Effects of silencing green peach aphid (Myzus persicae) genes via RNA interference.

This thesis is presented for the degree of

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by

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DECLARATION

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

Signature: VBilgi  Date: July 14, 2016
ABSTRACT

The green peach aphid (GPA), *Myzus persicae* (Hemiptera: Aphidae), is a polyphagous insect that feeds on a broad range of more than forty different plant families and also vectors over one hundred plant viruses. Feeding damage, and viral diseases that they transmit, contribute to yield losses in many economically important crops worldwide. The current measures used to control GPAs include genetic resistance and the application of insecticides. Although chemical insecticides provide control and are widely used for managing aphid infestations, they are expensive and some are not environmentally safe. Moreover, insecticides are not always effective because GPAs readily develop resistance. An alternate approach is to exploit the naturally occurring eukaryotic phenomenon of RNA interference (RNAi) to silence vital genes of aphids. RNAi is the sequence-specific degradation of homologous RNA molecules guided by small RNAs and can be triggered by the introduction of double-stranded RNA (dsRNA). This approach is environmentally-friendly, appears to be an effective strategy to silence vital genes of plant pathogens and pests such as nematodes and insects, and could be a competitive alternative to current methods of pest control. However, to develop transgenic plants expressing GPA genes, first suitable gene targets have to be identified and tested for their effects on aphid survival and reproduction through RNAi. Hence, the aims of this research were to identify essential genes of GPAs and to investigate the effects of gene silencing by artificial feeding of dsRNA and delivery of dsRNA through transgenic plants.

Comparative bioinformatics was undertaken for *in silico* identification of candidate genes of GPA. Six putative functional classes: embryogenesis, osmoregulation, moulting, cuticularisation, feeding, and locomotion were identified based on the life-cycle of GPA. All available expressed sequence tags (ESTs) of GPA were compared to the genomic resources of the free-living nematode, *Caenorhabditis elegans* to identify genes belonging to the putative functional class and also having lethal RNAi phenotypes. In this way, GPA orthologues of *C. elegans* genes with an RNAi phenotype belonging to the six putative functional classes were identified and also compared to nucleotide sequences of other insects to identify genes with known or predicted functions. Fifty target genes were identified *in silico* of which ten were then used for molecular characterisation and RNAi studies. The ten genes chosen for detailed study were named with a prefix *Mp* representing *Myzus persicae* and were: *MpVha-8* (vacuolar

A study to optimise *in vitro* RNAi conditions for GPAs was done which involved the assessment of eleven dyes for their suitability in tracing uptake of artificial diet containing sucrose and dsRNA after 24 hr feeding in an artificial feeding chamber. The dyes tested were: fluorescein isothiocyanate, fluorescein diacetate, phloxine B, methylene blue, acid fuschin, fast green, congo red, neutral red, acridine orange, and two food colours, red and yellow. The visibility of dyes inside the aphid body, effects of ingested dye on aphid survival, and effects on the purity of dsRNA in the presence of dye were studied. The optimal concentrations of two vital dyes, neutral red (0.02%) and acridine orange (0.0025%) when mixed with 30% sucrose were easily visualised in the aphid body upon ingestion, did not significantly (*p > 0.05*) affect aphid survival after 24 hr feeding compared to sucrose-only controls, and also did not affect the quality and purity of dsGFP after 24 hours.

To demonstrate that adding vital dyes to trace uptake of dsRNA allowed effective assessment of target gene silencing, *MpVha-8* was used to study the effects of *in vitro* RNAi. The lowest concentration of neutral red mixed with 30% sucrose and 2 *µg/µL* of ds*MpVha-8* was provided to GPA in an artificial feeding chamber in which aphids were allowed to feed *ad libitum*. After 24 hr feeding, aphid survival and transcript abundance of the target gene were studied in only those aphids which had fed and showed the presence of dye. A reduction of 21.59% and 29.17% was observed in survival of aphids that fed on ds*MpVha-8* with dye and without dye respectively. For both the treatments, there were also long-term effects on aphid survival in comparison to dsGFP-fed aphids when transferred to tobacco; mortality was recorded by day 6 and day 5 in aphids that fed on ds*MpVha-8* with dye and without dye respectively. Transcript abundance of the target gene in aphids that fed on ds*MpVha-8* mixed in sucrose and dye was compared to that of aphids fed on ds*MpVha-8* mixed in sucrose alone and were pooled together. There was a pronounced knockdown of target gene in aphids that had taken up dsRNA with dye. This study demonstrated that the addition of dyes to feed containing dsRNA
enabled the assessment of only those aphids that had ingested dsRNA, thus providing a more accurate measure of transcript abundance than when assessing aphids pooled after 24 hr feeding.

In vitro RNAi studies using the optimised conditions were undertaken to study the effects of silencing nine target genes. These were: MpEat-6, MpCct-6, MpPod-2, MpPdi-2, MpLev-11, MpSox-2, MpTnc-2, MpCars-1, and MpAqp-4. When dsRNA of target genes was delivered to GPA nymphs in a feeding chamber, differences in behaviour, reduction in target transcript levels and survival were found compared to controls. After 24 hr feeding, survival of nymphs fed on all nine dsRNA was between 48-75%, and was significantly lower than treatments of dsGFP and no-dsRNA control. The most obvious behavioural changes were found in aphids fed on dsRNA for genes involved in movement and locomotion. There was paralysis and limited movement for aphids fed on dsMpLev-11, dsMpSox-2 and dsMpTnc-2, and this was accompanied by a reduction in target transcript abundance. When aphids that had fed for 24 hours on dsMpEat-6, dsMpPod-2, dsMpLev-11, dsMpSox-2 and dsMpTnc-2 were transferred to tobacco to study the effects on long-term survival, it was found that aphids survived only until days eight, four, one, one, and three respectively. Conversely, for aphids that fed on dsMpPdi-2, dsMpCars-1, dsMpCct-6 and dsMpAqp-4, even though there was a gradual decrease in the percentage of aphid survival, complete mortality was not observed over 12 days on tobacco.

In planta RNAi studies were undertaken to study the effects on survival and reproduction of GPA reared on transgenic tobacco plants expressing target genes. For this, tobacco leaf disc transformation using recombinant Agrobacterium tumefaciens harbouring the hairpin expression cassette was done to generate primary transformants (T0). Transgenic T0 plants showing resistance to kanamycin and T1 plants that tested positive for the presence of the neomycin phosphotransferase gene were challenged with nymphs. Amongst the T0 plants tested, dsMpLev-11-expressing tobacco had significantly lower aphid survival as compared to empty vector- and dsGFP-expressing plants. Of all the T0 transgenic plants, dsMpLev-11-expressing tobacco had the most events, a total of nine, which had no aphids surviving by day 16; survival was only until day five or six. The mean number of aphids alive on the T1 events of target dsRNA-expressing tobacco at each time-point (beyond day zero) were found to be significantly lower as compared to the control group of untransformed wild type, GFP and null
events. At day 16, the T1 events for each gene that showed the lowest aphid number were: *MpSox-2 E8*, *MpAqp-4 E10*, *MpLev-11 E12*, *MpCars-1 E8*, *MpTnc-2 E8*, *MpPod-2 E6*, *MpCct-6 E6*, *MpEat-6 E8*, *MpPdi-2 E9*, and *MpVha-8 E10*.

The use of *in vitro* RNAi studies as a preliminary screen to determine the potential of a candidate gene before progressing into *in planta* RNAi studies was found to be useful. This was because the effects on survival after feeding on dsRNA through artificial feeding and on transgenic tobacco plants were somewhat similar. For example, aphid survival after feeding on ds*MpSox-2* and ds*MpLev-11* for 24 hours was 58.3% and 50% respectively and was accompanied by impaired locomotion, reduction in target gene expression, and also affected long-term survival when transferred to tobacco. There was also a reduction in aphid survival on the T1 events *MpSox-2 E8* and *MpLev-11 E12* as compared to controls. Results of *in vitro* RNAi for five genes, *MpEat-6*, *MpLev-11*, *MpSox-2*, *MpTnc-2* and *MpVha-8* complemented the results of *in planta* RNAi demonstrating the practicality of using an *in vitro* RNAi screen for selecting genes prior to advancing into host-mediated RNAi.

Except for V-ATPase, which has been tested previously for RNAi effects on aphids including *M. persicae*, none of the nine target GPA genes studied have ever been tested in GPA and are novel targets. Based on the behavioural changes and effects on aphid survival after *in vitro* RNAi, and the survival on transgenic T1 events of tobacco, genes were ranked for their efficiency. In an ascending order of their performance these were: *MpSox-2*, *MpLev-11*, *MpTnc-2*, *MpPod-2*, *MpEat-6*, *MpAqp-4*, *MpCct-6*, *MpPdi-2*, *MpCars-1* and *MpVha-8*. 
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<tr>
<td>35S</td>
<td>35S RNA transcriptional promoter of CaMV</td>
</tr>
<tr>
<td>AO</td>
<td>acridine orange</td>
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<tr>
<td>AF</td>
<td>acid fuschin</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
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<tr>
<td>bp</td>
<td>base pair</td>
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<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CR</td>
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<td>cultivar</td>
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<tr>
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<td>deoxyribonucleic acid</td>
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<td>nucleotide mix</td>
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<td>dsRNA</td>
<td>double-stranded DNA</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetra-acetate acid disodium salt</td>
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<tr>
<td>FDA</td>
<td>fluorescein diacetate</td>
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<td>FG</td>
<td>fast green</td>
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<td>green fluorescent protein</td>
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<tr>
<td>kb</td>
<td>kilobase</td>
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<td>LB broth</td>
<td>Luria Bertani broth</td>
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<td>MB</td>
<td>methylene blue</td>
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<td>tris-acetate-EDTA</td>
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<td>TBLASTX</td>
<td>search translated nucleotide database using a translated nucleotide query</td>
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<tr>
<td>YFC</td>
<td>yellow food colour</td>
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DEDICATION

To

my best friend,

soul-mate and husband,

Sameer.
CHAPTER 01

General Introduction and Literature Review
1.0 General Introduction

Aphids (Hemiptera: Aphidae) are a major group of insect pests that predominantly feed on plant phloem sap, a feeding behaviour that severely affects the growth of their hosts resulting in stunting, loss of vigour, reduction in photosynthesis and eventual reduction in crop yield (Smith and Boyko, 2006). As successful plant virus vectors, several species of aphids are able to transmit more than one virus. They feed on many economically important crops e.g. canola, corn, cotton, potato, rice, and wheat causing physical damage and extensive economic losses worth hundreds of millions of dollars worldwide (Blackman and Eastop, 2000; Ramsey et al., 2007). This makes aphids important pests posing a severe threat to global agriculture.

The peach-potato aphid or green peach aphid (GPA), *Myzus persicae* (Sulzer) is a polyphagous insect with a very broad host range (Blackman and Eastop, 2000; Moran et al., 2002). It is also a successful vector that can transmit many viruses including Potato Leafroll Virus (PLRV), Tobacco Mosaic Virus (TMV), Cucumber Mosaic Virus (CMV), Beet Yellows Virus (BYV), Beet Western Yellows Virus (BWYV), Beet Mosaic Virus (BtMV) and Lettuce Mosaic Virus (LMV) either non-persistently, semi-persistently or persistently (Blackman and Eastop, 2000). GPA has been recorded as an agriculturally important pest that accounts for severe yield losses in economically important crop commodities such as canola, cabbage, lupins, potato, wheat, etc. (Blackman and Eastop, 2000; Dixon, 1998).

Strategies to control damage caused by aphids include the use of virus-free certified seeds, planting resistant varieties of crops, and the application of insecticides. Although chemical insecticides may provide considerable control, they are expensive and mostly not environmentally-friendly. Crop protection through classical breeding for resistant varieties has been well-established but has its limitations. The advent of agricultural biotechnology has provided genetically-engineered plants expressing different bacterial proteins (*Bt* proteins) that are now widely deployed, particularly from crops like cotton and maize, but are only effective against chewing insects and not against sucking pests such as aphids. A robust method of crop protection that exploits the use of a well-studied naturally occurring phenomenon of RNA interference (RNAi) has gained considerable attention worldwide. There is now tremendous scope for research to use the highly conserved cellular process of RNAi to silence specific genes in sucking insects such as aphids, before the mRNA is translated. RNAi can be triggered when
double-stranded RNA (dsRNA) is introduced into a cell where a series of cellular processes lead to the unwinding of the RNA and binding to a complex which degrades a corresponding target mRNA resulting in loss-of-function of its protein.

In experimental work, RNAi has been shown to be an effective strategy for crop protection against several different plant pathogens and pests including nematodes and some insects (Baum et al., 2007; Fairbairn et al., 2007). Double-stranded (dsRNA) sprays have been effective in controlling certain viral diseases of tobacco. Although initially this mode of delivery was not economically viable (Tenllado et al., 2004), further work may deliver commercial products, for example, BioDirect™ (Monsanto, USA). RNAi has recently been demonstrated in some insect pests of the orders Lepidoptera and Coleoptera such as the western corn rootworm, Diabrotica virgifera virgifera and cotton bollworm, Helicoverpa armigera through injection of dsRNA, feeding artificial diets containing dsRNA as well as transgenic plants expressing dsRNA (Baum et al., 2007; Mao et al., 2007; Zha et al., 2011). Plant-mediated RNAi might be recognised as broad-spectrum control strategy for economic insect pests, which are not effectively controlled through Bt toxins, but it’s beauty is the narrow-spectrum that is possible with it. The application of such a technology could be more economical and potentially avoid the environmental effects of chemical insecticides.

However, there is still much to be learned about the mechanism of gene silencing in general and dsRNA processing in insects; this includes knowledge of the type, length, composition and conformation of dsRNA that elicit the effective down-regulation, the best target genes for RNAi-mediated control and the possible additive effects of knocking down multiple genes involved in one or more biochemical pathways. An aim of the research presented in this thesis was to identify potential essential genes of GPA through comparative bioinformatics and functional genomics, and to use such genes to provide insights into the mechanism of RNAi in insect pests. The research includes the use of dsRNA to elicit gene silencing, generate constructs that produce an RNAi response, and effects on GPA via in vitro feeding and through transgenic plants. It was expected that the results would provide target genes which through RNAi would effectively disrupt development, reproduction and survival of GPA, and by extension many of the world’s other damaging aphid and insect pests.
1.1 Green peach aphid as a crop pest

Of the 4,700 aphid species described to date, 250 are known to be pests and more than 190 are known to transmit plant viruses (Blackman and Eastop, 2000; Nault 1997). About 95% of aphid species are monophagous while 5% are polyphagous (Blackman and Eastop, 2000). *M. persicae* (Sulzer), first described by the Swiss entomologist Johann Heinrich Sulzer in 1776, is found throughout the world and is a polyphagous crop pest known to transmit plant viruses. The earliest reports of GPA in Australia date back to 1910 in New South Wales (NSW) however, the Australian National Insect Collection records its presence since 1893 from south-east Victoria (VIC) (Wilson *et al.*, 2002).

GPA has a very broad host range in over 40 different plant families (Moran *et al.*, 2002). It is the parthenogenetic stage of this sucking pest that causes most damage to the crops. The primary host of GPA is the stone fruit peach, (*Prunus persicae*), and in VIC, NSW, Tasmania (TAS) and also in certain regions of South Australia (SA), Queensland (QLD) and Western Australia (WA) GPA populations have been found on both peach and nectarine trees (Wilson *et al.*, 2002). In regions where peach is not found GPA infests several secondary hosts (Blackman and Eastop, 2007). The commonly found secondary hosts include herbaceous crop species belonging to the families, Chenopodiaceae, Compositae, Cruciferae, Cucurbitaceae, Fabaceae, and Solanacea. Some examples of economically important secondary hosts are barley (*Hordeum vulgare*), cabbage (*Brassica oleracea*), canola (*B. napus*), potato (*Solanum tuberosum*), tomato (*S. lycopersicum*), tobacco (*Nicotiana* species), wheat (*Triticum aestivum*), or herbaceous weed such as *Chenopodium amaranticolour* or *C. album*. It can transmit more than 100 different viruses such as potato leafroll virus (PLRV), tobacco mosaic virus (TMV), cucumber mosaic virus (CMV), etc. either non-persistently, semi-persistently or persistently causing severe yield losses (Blackman and Eastop, 2000). Its characteristics such as polyphagous nature and its capacity as a viral vector together with its cyclical parthenogenesis, telescoping of generations and recent records of resistance to many insecticide chemistries make GPA an agriculturally important pest in many parts of the world (Klein and Waterhouse, 2000; Blackman and Eastop, 2000; Dixon, 1998).
1.1.1 Taxonomy

*M. persicae* is an insect with a soft cuticle and belongs to the tribe Macrosiphini, sub-family Aphidinae, family Aphididae, sub-order Sternorrhyncha, order Hemiptera, class Insecta, phylum Arthropoda and kingdom Animalia. Members of the Macrosiphini tribe include agriculturally important crop pests such as the pea aphid (*Acyrthosiphon pisum*) and the Russian wheat aphid (*Diuraphis noxia*) (Martinez-Torres *et al.*, 2001; Ortiz-Rivas *et al.*, 2004). Members of the sub-order Sternorrhyncha include whiteflies, scale insects and aphids. They are characterised by the position of their mouthparts with respect to their head. The species belonging to the order Hemiptera possesses mouthparts such as maxillae and mandible that are sheathed within a labium. The maxillae interdigitate to form two needle-like channels called the stylet which is used for sucking sap during feeding (Kaloshian and Walling, 2005). Hemiptera is the largest order with insect species that have a hemimetabolous life-cycle *i.e.* the absence of metamorphosis between a pupal and an adult stage instead several nymphal stages prelude an adult stage (Dixon, 1998; Williams and Dixon, 2007).

1.1.2 Life-cycle

GPA typically leads a heteroecious life-cycle alternating on the hosts it colonises. It survives on a primary host, a peach species during winter, migrates to an unrelated secondary host species during summer and finally returns back to the primary host in autumn. Whether heteroecious or monoecious, aphid species that undergo sexual reproduction and parthenogenesis are said to have a ‘complete’ life-cycle often referred to as holocyclic. However, under certain conditions, aphids can survive parthenogenetically throughout the year thereby living an ‘incomplete’ life-cycle termed as anholocyclic. This is especially true when the primary host such as the *Prunus* species is not available or when the temperature of the geographical region is most suited for parthenogenetic stage (Blackman and Eastop, 2007; Williams and Dixon, 2007). In that case, GPA survives continuously on the secondary hosts, which often include economically important commodity crop. For example, in the UK, where the primary host, *P. persicae* is rarely found, GPA lives an anholocyclic life style, constantly surviving through parthenogenesis on several secondary hosts (Williams and Dixon, 2007). In warmer states of the US such as Florida and also in regions of Australia where *Prunus* species cannot be cultivated GPA survives on secondary hosts. However, where the primary host is readily
available such as in the peach-growing states of the US, GPA overwinters in the egg stage on Prunus species (Williams and Dixon, 2007). In spring, the eggs hatch and the nymphs feed on the young foliage, stems and flowers of Prunus. After several parthenogenetic generations, winged migrants from the Prunus species give birth to nymphs on secondary hosts such as potato, tomato, etc. The cycle continues and eventually adults return to Prunus species in autumn where after sexual reproduction eggs are deposited (Figure 1.1). A study done by Vorburger et al. (2003) that used microsatellite analysis to characterise clonal populations of GPA in Victoria revealed that the number of days with temperatures below 0°C and the regional availability of the primary host (peach) limits cyclical parthenogenesis, and asexual lineages seemed predominant. Interestingly, it was reported that obligate and cyclical parthenogens form mixed populations and coexisted in southeastern (Blackman, 1974).

Eggs are produced as a result of sexual reproduction in autumn on the primary host. These eggs are usually 0.6 mm long and 0.3 mm wide with a green or yellow colour that eventually turns black (Dixon, 1998; Williams and Dixon, 2007). The eggs are able to overwinter and survive adverse weather conditions. In spring, eggs hatch to form two highly fecund wingless (apterous) forms; the fundatrix, also known as the stem mother and the fundatrigenia. These wingless morphs, usually 1.0 mm to 2.1 mm in length, give rise to winged morphs, the spring migrants that are usually 1.5 mm to 2.5 mm in length. These colonise secondary host and during summer give birth through viviparity to live nymphs (Williams and Dixon, 2007). There are four nymphal instar stages and the average period of each instar is 2.0, 2.1, 2.3, and 2.0 days respectively (Horsfall, 1942). The wingless parthenogenetic female carries within herself embryos that begin to develop immediately after ovulation. The prospective progeny also carries embryos making them virtually pregnant even before birth. This phenomenon is called telescoping of generations which aids in the rapid development of aphid populations (Kindlmann and Dixon, 1989).

Generally, aphids begin to reproduce between seven and 10 days after their birth with an average age of 10.8 days at the first nymph produced. The reproductive period usually averages about 14.8 days but varies largely (Dixon, 1998). The life-cycle of an aphid usually lasts for about 23 days and in this period parthenogenetic females can give birth to about 80 young ones in a period of 10 days (Le Trionnaire, et al., 2008). After several parthenogenetic
generations, winged (alatae) gynoparae (a stage preluding sexual females) and sexual males are produced which return to the primary host. The gynoparae produces several sexual females which then undergo maturation to mate with the sexual males before they can oviposit (Dixon, 1998). The females then lay between 4 to 13 eggs in the crevices near the buds on the Prunus trees. The cycle then continues once the eggs hatch in spring (Figure 1.1).

![Figure 1.1. A generalised life-cycle of a heteroecious aphid. Source: Life-cycles and Polymorphism from Williams and Dixon (2007).](image)

1.1.3 Economic importance of GPA

GPA is the primary aphid found on Brassica species and is responsible for extensive crop damage, and yield reduction due to viral diseases caused by barley western yellows virus (BWYV), cauliflower mosaic virus (CaMV) and turnip mosaic virus (TuMV). For example, in WA alone, yield losses in canola due to BWYV transmitted by GPA are up to 50% (Berlandier et al., 2010). In another WA study, yield losses of up to 33% in canola were recorded from feeding damage caused by three different aphid species that included GPA (Berlandier et al., 2010). In lupin species, GPA actively transmits CMV and BYMV and is responsible for yield losses up to 60% in the Mediterranean climatic regions of South Australia (SA) and the ‘grain-belt’ region of south-west Australia (Thackray et al., 2004). In WA, yield losses of up to 50% in susceptible
lupin species due to CMV transmitted by GPA have been reported (Berlandier, 1999). In susceptible narrow-leafed as well as yellow lupin varieties, yield losses up to 90% have been recorded in the absence of virus infection and up to 30% in intermediate resistant lupin varieties (Berlandier, 1999). In NSW and SA, crop losses in beans, chickpeas, peas, lentils, and lupins due to GPA transmitted viruses such as CMV, BWYV and pea seed-borne mosaic virus (PSbMV) have also been reported (Jenkins et al., 2011). In addition to attacking economically important crops, GPA also attacks volunteer crops, weeds and ornamental plants thereby allowing its survival in regions with unsuitable weather.

1.1.4 Current strategies of aphid control and their limitations
Strategies to control damage caused by aphids include the use of virus-free certified seeds, cultural practices, planting resistant varieties of crops, biological control, and the application of insecticides (Dewar, 2007; Powell and Pell, 2007; van Emden, 2007; Wratten et al., 2007). Planting virus-free seeds, seedlings, transplants, cuttings and tubers is the first strategy of controlling viral diseases that can be transmitted by aphids. Healthy virus-free seeds or seedlings ensure that there are no viruses at a very early stage of crop growth and do not provide a reservoir of viruses that can be further spread by aphids. Some states in Australia such as WA, NSW and VIC have developed seed certification schemes especially for vegetative crops like potatoes to help reduce viral diseases and this has contributed to the safety of the Australian potato industry.

Many breeding programs have been designed and efforts are being made to develop insect resistant crop cultivars in Australian cereals, legumes, crucifers, and sorghum supported by the Grains Research Development Corporation (GRDC), Commonwealth Scientific and Industrial Organisation (CSIRO), Department of Agriculture, Fisheries and Forestry (DAFF), and Department of Agriculture and Food, WA (DAFWA) using the principle of host plant resistance (HPR). HPR has been studied in great details in order to develop resistant crop cultivars and many breeding programs make use of antibiosis, antixenosis, and tolerance as mechanisms for crop protection (van Emden, 2007). For example, in WA, lupin breeders at DAFWA have developed narrow-leafed lupin cultivars with a high level of resistance to aphids as well as antibiosis based resistance in legumes (Perry et al., 2010). Currently, in peach cultivars,
‘Rubira’ is the only known cultivar that confers strong avoidance resistance causing aphids to leave the plant within a few days (Sauge et al., 2002). Resistance in ‘Rubira’ is a major dominant gene and is utilised in many breeding programs around the world (Pascal et al., 2002; Sauge et al., 2011).

Biological control through the use of the aphid parasitoids belonging to the families Braconidae and Aphelinidae have been found to impact the growth of aphid populations (Mackauer, 1968; Takada, 2002). *Aphelinus abdominalis* and *A. colemani* are two parasitoid wasps that are commercially available in Australia specifically for the control of GPA (http://www.sardi.sa.gov.au/). The applications are recommended as a preventive measure in order to keep aphid populations in check and avoid the build-up of large populations (Powell and Pell, 2007). Cultural practices include the use of polyethylene mulches to repel aphids to reduce the incidence of aphid colonisation, altering planting dates, high planting densities, intercropping, and the use of trap crops are some other cultural practices (Wratten et al., 2007). However, none of these control strategies can be deployed singularly and are they are also not completely reliable.

For controlling heavy infestations of aphid populations, the use of chemicals still remains the most reliable method of control. Although chemical insecticides may provide considerable control, they are expensive and mostly not environmentally-friendly. The primary choice of insecticide classes are carbamates, organophosphates, pyrethroids, neonicotinoids, pyridine carboxamide, azomethine, and pyrazole (Dewar, 2007). These insecticides are systemic, contact or used as a fumigant. They act on the central nervous system or the peripheral nervous system by inhibiting the enzyme acetylcholinesterase, disrupting the voltage-gated sodium channel or post-synaptic nicotinic acetylcholine-receptors, obliterating nerves that control the salivary pump, or inhibiting ATPase affecting respiration (Dewar, 2007). Neonicotinoids, for example, imidacloprid, a systemic insecticide is a growing class of insecticide which is also fast selling and is particularly efficient in controlling GPA in crops such as cabbage. The Australian Pesticides and Veterinary Medicines Authority currently recognises the registered use of insecticides belonging to the categories: carbamate, organophosphate, tetronic and tetramic acid derivatives, diamides, pyrethroids, neonicotinoid, sulfoxaflor, and
pymetrozine specifically for the control of GPA crops including capsicum, eggplant, potato, stone fruits, tomato, etc. (Umina et al., 2014a; www.apvma.gov.au).

When chemicals are used to control insect pests the potential for resistant phenotypes to develop is well-documented. An interesting yet an alarming fact is that GPA has the ability to adapt very rapidly to insecticide chemistries that results in resistance development to more active ingredients in comparison to any known insect (Georghiou, 1991). GPA is found to have developed resistance to many of the widely used chemicals such as carbamates, organophosphates, and pyrethroids (Foster et al., 2007).

In the United States and Chile, for example, resistance of GPA against carbamates, organophosphates, cyclodienes or organochlorines, and pyrethroid insecticides has been reported (Georghiou, 1963; Silva et al., 2012; Sudderuddin, 1973; Unruh et al., 1996). More recently, in 2010, the first GPA resistance to pirimicarb, a selective carbamate, was recorded in two different locations that are about 350 km apart, namely Kojonup and Moora, WA. Until then, this insecticide was the most reliable registered chemical against GPA (Cesar Pest Facts, 2010). Carbamate resistance in GPA is becoming more common in WA, SA and QLD, and is generally not recommended to use (Umina et al., 2014b). Carbamate and organophosphate resistant field populations have been reported in several vegetable and stone fruit growing regions of Australia (Hamilton et al., 1981). Also, neonicotinoid resistance in GPA is also emerging in some regions of Australia (Umina et al., 2014b). With growing number of reports of GPA resistance to insecticides, alternative strategies to control GPA will become important.

1.1.5 Genetic engineering as a strategy of crop protection

The ever increasing global population as well as the rise of food prices makes meeting future food demands a challenge. On the other hand, plant diseases, global rise in atmospheric temperatures, deforestation, and rapid urbanisation has had a tremendous effect on agriculture worldwide. The application of agricultural biotechnology through genetically modifying crops can provide some solutions to many of the world’s agricultural problems. Example of areas in which genetically modified (GM) crops may contribute are in enhanced agronomic traits and nutrition, weed control, higher yields, pest and disease resistance, and
particularly insect resistance. Traits such as herbicide, disease or insect resistance allow the grower to use far less chemicals. The use of chemicals is expensive, environmentally-unfriendly and in various instances have had health impacts on consumers. Although the use of chemicals is efficient in controlling certain diseases or pest attacks, the adverse effect on non-targets as well as selection pressure on aphid populations to develop resistant phenotypes has been reported (Georghiou, 1963; Griffitts and Aroian, 2005; Silva et al., 2012; Sudderuddin, 1973; Unruh et al., 1996).

Classical genetics as an approach for crop breeding to develop disease resistant or insect resistant crops is time-consuming. Loss of gene pools during domestication and crop breeding has limited the availability of genetic resources. Also, certain naturally occurring traits that may be beneficial in one plant tissue may be deleterious in other tissues (Wani et al., 2010). With the advent of new molecular tools and techniques, genetically engineering crops for human benefits is being applied widely and increasingly becoming acceptable worldwide. A striking example of a successful GM crop designed for the benefit of humankind and provided a long-term solution to fight against vitamin-A deficiency induced diseases in many countries is ‘golden rice’. With a gene for plant phytoene synthase (psy) and bacterial phytoene desaturase (crt I), ‘golden rice’ is able to produce an enhanced level of beta-carotene, a precursor of vitamin-A, in the grain where it is usually not expressed: its implementation would reduce death, blindness and related health problems cause by vitamin-A deficiency by rural poor in some regions of SE Asia (Paine et al., 2005).

GM crops with insect resistance have been developed and introduced in many parts of the world. Currently, these crops include cotton, corn, eggplant, potato, rice, soybean, and tomato. In Australia, insect resistant transgenic cotton, corn, potato, and soybean have been registered either for growth or importation as foodstuffs (www.isaaa.org/gmapprovaldatabase/default.asp). Some of the transgenic events have stacked traits, in particular, herbicide tolerance and resistance against insects belonging to the order Lepidoptera and Coleoptera, which are primarily chewing insects such as corn borer, corn rootworm, cotton bollworm, and the Colorado potato beetle (www.isaaa.org/gmapprovaldatabase/default.asp). These GM crops have been developed to express the bacterial Cry toxins from the soil bacterium Bacillus thuringiensis (Bt) that confer
resistance to the plant by selectively damaging the mid-gut lining, preventing the insect from feeding, eventually leading to the death of the insect due to starvation. The Bt expressing GM crops however, do not protect crops from sucking pests belonging to the order Hemiptera, for example, GPA.

Some of the methods that have been used so far to develop transgenic crops include *Agrobacterium*-mediated plant transformations, microparticle bombardment, electroporation, chemical-mediated introduction into protoplast, pollen-tube pathway and cross hybridisation with transgenic donor(s) (www.isaaa.org/gmapprovaldatabase/traitintrolist/default.asp). The innovative approach for developing transgenic crops through RNA interference (RNAi) based control of insect pests is a fairly recent development, although discovery of the phenomenon took place nearly 20 years ago. RNAi based research is now being undertaken worldwide. A RNAi-based strategy for crop protection exploits the highly conserved, naturally occurring cellular process of silencing or down-regulating genes before their mRNA is translated. The rapid advances made in the use of RNAi-based research to understand gene functions in model insects such as *Drosophila melanogaster* and non-model insect pests, both in vitro and *in planta*, show a promising future in developing an environmentally-friendly and an economical strategy for crop protection. Application of RNAi-based technology to control insect pests, particularly insects belonging to the order Hemiptera, has only just begun recently. Identifying a reliable dsRNA delivery system and a suitable target gene which upon silencing will result in significant control of the insect, without harmful off-target effects, is challenging and needs detailed research.

1.2. RNA interference (RNAi)

RNAi is a naturally occurring, highly conserved cellular mechanism for regulating the transcript abundance of gene(s) through a sequence-specific process of RNA degradation. Previously described as post-transcriptional gene silencing (PTGS) in plants (Hamilton *et al.*, 1999) and quelling in fungi (Romano and Macino, 1992), RNAi is now known to modulate gene expression and development of many organisms such as nematodes, insects, fish, and crustaceans. The mechanism is well-studied and has been used as a powerful tool in functional genomics to study gene function via loss-of-function analyses in many organisms such as *Arabidopsis*
thaliana (Xie et al., 2004), D. melanogaster (Elbashir et al., 2001) and Caenarhabditis elegans (Fire et al., 1998).

1.2.1 History of RNAi

RNA molecules for long were believed to serve only as messengers that bear genetic information from DNA. It was in the early 1980’s that it was discovered that small RNA molecules can bind to a complementary sequence of mRNA to inhibit the process of translation in Escherichia coli (Nordstrom and Wagner, 1994). The initial discovery of RNAi was in the year 1990, when Rich Jorgensen and his research group observed a perplexing phenomenon in Petunia flowers. They introduced extra copies of the gene, chalcone synthase (CHS-A), an enzyme that plays a role in anthocyanin pigment production, with the aim to achieve enhanced purple flowers. However, the result was far from expected. The purple colour disappeared completely in some of the progeny and resulted in variegated and white Petunias. This phenomenon of degradation of the homologous transcripts of the endogene as well as the transgene was termed as co-suppression (Napoli et al., 1990).

In 1995, Guo and Kemphues reported a confusing observation in C. elegans in an experiment to silence the expression of par-1 gene using antisense RNA. They noted that injecting antisense RNA did reduce the function of par-1, however, injecting sense RNA too resulted in the blocking of the expression of par-1, suggesting that the sense RNA could base pair with the endogenous target mRNA thereby inhibiting translation (Guo and Kemphues, 1995).

It was in 1998 that the paradox of the observations made by Guo and Kemphues was solved and an explanation was provided by Fire et al. while studying the gene function several locomotion and muscle regulatory genes such as unc-22 in C. elegans (Fire et al., 1998). They reported that injecting both single stranded (sense and antisense) dsRNA molecules were able to disrupt the expression of the target endogenous mRNA with only a few molecules of dsRNA required to have a greater impact on gene silencing. They drew four conclusions; the phenomenon of interference was not triggered by dsRNA corresponding to the intron sequences, injecting dsRNA resulted in significant silencing of the endogenous gene, the effect of interference could spread systemically from the gut to the rest of the body as well as
through the germ-line to many progenies, and finally, the process of interference occurred after transcription in the cytoplasm.

Since then, much research has been performed to study the mechanisms and effects of RNAi in various organisms and to evaluate its possibility as a strategy for crop protection. For example, effects of RNAi have been studied in several plant pathogenic fungi such as *Cladosporium fulvum*, *Magnaporthe oryzae*, plant parasitic nematodes such as *Meloidogyne* and *Heterodera* species, and insect pests such as *H. armigera*, *D. virgifera virgifera*, etc. (Bakhetia, *et al.*, 2005; Baum *et al.*, 2007; Hamada and Spanu, 1998; Kadotani, N. *et al.*, 2003; Mao *et al.*, 2007, 2011; Urwin, *et al.*, 2002).

### 1.2.2 Molecular mechanism of RNAi

RNAi is the sequence-specific degradation of homologous RNA molecules that is guided by small RNAs. Based on the structure of their precursors small RNAs have been classified as short interfering RNAs (siRNAs), micro RNAs (miRNAs) and piwi-interacting RNAs (piRNAs). SiRNA and miRNA are generally ~21 to 25 nt long while piRNA are ~24 to 31 nt long however, piRNAs of *C. elegans* called 21U-RNAs are 21 nt long (Xue *et al.*, 2012). Although different small RNAs have different production pathways, the steps involving processing and effect have a common mode in vertebrates, insects, nematodes, fungi and plants (Xue *et al.*, 2012; Tang, 2005; Tomari and Zamore, 2005). A diagrammatic representation of the small RNA pathways in *Drosophila* is given in Figure 1.2 (Vodovar and Saleh, 2012).

The mechanism of RNAi can be explained in two stages; initiation and effector (Das, *et al.*, 2011; Hannon 2002). The first step involves the generation of miRNA and siRNA. MiRNAs are synthesised from short hairpin precursor miRNA (pre-miRNA) while siRNAs are synthesised from an exogenous long dsRNA which may be introduced artificially. The cleavage of long dsRNA and pre-miRNA molecules into short RNA duplexes ranging between 21-25 nt is carried out by the endonuclease enzymes called Dicers which are a large group of multidomain RNase III (Bartel, 2004).
Fig 1.2. Small RNA pathways in Drosophila. (A) miRNA pathway initiated by the transcription of miRNA genes, (B) siRNA pathway initiated by a dsRNA molecule, and (C) piRNA pathway initiated by maternally deposited piRNAs. Source: Vodovar, and Saleh (2012).

Four classes of dicers have been identified in plants (A. thaliana), one in nematodes (C. elegans), mouse and humans, and two in the model insect Drosophila, which have been named dcr-1 and dcr-2 (Bernstein et al., 2001). Although dcr-1 and dcr-2 show a structural similarity, their requirements for ATP and substrate are different (Jiang et al. 2005).

Within the nucleus, the RNase III, Drosha together with the dsRNA-binding protein (dsRBD) Pasha processes the primary miRNA (pri-miRNA) transcript ~70 nt hairpin pre-miRNA (Lee et al., 2003). The pre-miRNA is exported into the cytoplasm by Exportin 5/Ran GTP which is then cleaved by dcr-1 into 21-22 nt mature miRNA (Yi et al., 2003; Bohnsack et al., 2004; Lund et al., 2004). Dcr-1 is an ATP-independent enzyme which together with its co-factor, the dsRBD protein, Loquacious (loqs) or R3D1-L (long) preferentially cleaves near the loop of the hairpin pre-miRNA (Jiang et al., 2005; Lee et al., 2004). The effector step involves the incorporation of the guide strand into the RNA inducing silencing complex (RISC). The RISC comprises of a core
protein, argonaute (ago) which has multiple members with several functions such as RNA cleavage. In Drosophila, five ago proteins have been identified of which ago-1 and ago-2 are directly involved in the RISC (Rehwinkle, et al., 2006). In the miRNA pathway, the mature miRNA is then delivered to ago-1 containing RISC. The miRNA is eliminated and the guide miRNA is responsible for the cleavage of the homologous mRNA (Jaubert-Possamai et al., 2010; Vodavar and Saleh, 2012).

In cases where exogenous or viral dsRNA is introduced it is cleaved by the endonucleases dcr-2 (Vodavar and Saleh, 2012). Dcr-2 functions together with loqs to generate ~21 nt siRNA in an ATP-dependent catalytic step. Subsequently, the dsRBD protein, R2D2 along with dcr-2 forms a complex which deliver the duplex siRNA to ago-2 containing RISC (Vodavar and Saleh, 2012; Xue, et al., 2012). The ago-2 is responsible for converting the pre-RISC to holo-RISC by the removal of the passenger strand. The pre-RISC contains siRNA duplex while the holo-RISC contains only the guide strand of the siRNA after the removal of the passenger strand (Das et al., 2011). The 5’-end of the siRNA is responsible for the target recognition guide for the mechanism of RNAi (Matranga, et al., 2005). The passenger strand is eliminated and the guide siRNA directs the degradation of the target RNA (Das et al., 2011; Hannon, 2002; Jaubert-Possamai et al., 2010; Jiang et al., 2005; Vodavar and Saleh, 2012; Xue, et al., 2012).

The biogenesis of piRNAs is the least understood among all the small RNAs. Independent of dicer enzymes, it requires piwi proteins and seems to be processed from single-stranded primary transcripts transcribed from defined genomic regions rather than dsRNA (Vadovar and Saleh, 2012). PiRNAs have been found to be involved in the silencing of transposable elements specifically in animal gonads. Ago-3, aubergine and piwi proteins are involved in the processing of piRNA and are thought to play a role in chromatin modification in the nucleus (Aravin et al., 2003, 2007; Brennecke et al., 2007; Olivieri, et al., 2010; Vagin et al., 2006).

In summary, drosha, pasha, dcr-1, loqs, and ago-1 play a role in the miRNA pathway while dcr-2, R2D2, and ago-2 play a role in the siRNA pathway (Jaubert-Possamai et al., 2010; Jiang et al., 2005; Xue, et al., 2012). The closest insect relative of GPA for which a genome is available, the pea aphid, A. pisum, has four copies of pasha, one copy of drosha and exportin-5, and two copies each of dcr-1, loqs and ago-1 which are involved in the miRNA pathway. One copy each
of dcr-2, R2D2, and ago-2 which are involved in the siRNA pathway have also been found in the genome of pea aphid (Jaubert-Possamai et al., 2010).

### 1.2.3 Natural functions of RNAi

RNAi is known to play an important role in several biological processes such as development, antiviral defence mechanism, heterochromatin formation, and maintenance of genomic stability (Bartel, 2004; Hannon, 2002). For example, in A. thaliana, mutations in ago-1 resulted in improper development of leaves and floral organs as well as axillary meristem (Bohmert et al., 1998). In C. elegans, it has been shown to play a role in proper germ-line development; mutations in dcr-1 and ego-1 results in complete sterility (Knight and Bass, 2001). The role of RNAi in antiviral defence mechanism was first identified in plants and in the nematode, C. elegans (Ratcliff et al., 1997; Wilkins et al., 2005). One of the natural functions of RNAi is to protect the genome from viruses and transposable elements (Hannon, 2002; Meister and Tuschl, 2004). For example, in C. elegans, members of the RNA deficient (rde) mutant argonaute family, such as rde-1 and rde-4 have been found to exhibit mobilisation of transposable elements in the germline suggesting that transposon silencing may be a one of the natural roles of RNAi (Sijen and Plasterk, 2003). Similarly, a mutator gene, mut-7, has been found to play an essential role in germline silencing of the transposon Tc1 which is found abundantly in all natural isolates of C. elegans and through RNAi (Ketting et al., 1999).

In Drosophila dcr-2 mutants, four viruses namely, flock house virus (FHV), drosophila C virus (DCV), cricket paralysis virus (CrPV) and Sindbis virus are found to be more pathogenic with increased viral replication than in the wild type, suggesting the role of RNAi in antiviral immunity (Galiana-Arnoux et. al., 2006; Wang et. al., 2006; Zambon et al., 2006). Although dcr-2 protects against the four viruses, replication of drosophila X virus (DVX) is not affected by the presence of dcr-2, instead it is affected by ago-2, R2D2 and piwi (Pal-Bhadra et al., 2002).

In the animal kingdom, Drosophila ago-2 was the first RNAi component that was identified to play a role in antiviral RNAi silencing pathway (Li et al., 2002). Li et al. reported that reducing the ago-2 mRNA significantly increased the accumulation of FHV RNAs (Li et al., 2002). Drosophila dcr-1 is required for stem cell maintenance as well as oogenesis (Jin and Xie, 2007). Similarly, in cockroach, Blatella germanica, dcr-1 of the miRNA pathway is an important
enzyme in miRNA biogenesis and is involved in proper development of oogenesis (Tanaka and Piulachs et al., 2012).

At least three piwi class ago family proteins namely, ago-3, piwi, and aubergine, have been identified in Drosophila that play a major role in regulating transposable elements (TEs) in germline cells (Aravin et al., 2007; Hartig et al., 2007) suggesting the role of piRNA pathway in protecting against TEs by degrading transcripts and by heterochromatin formation (Obbard et al., 2008). Although the natural role of RNAi in innate immunity as well as growth and development has been extensively studied in plants, vertebrates, nematodes and insects particularly, Drosophila, there is very little information in insects of the order Hemiptera.

1.2.4 Inhibitors of RNAi

Both plant and animal viruses are able to prevent host immune response. Certain viruses, such as tombusvirus, possess dsRNA binding proteins that interrupt the host’s RNAi machinery. The p19 suppressor protein of the tombusvirus has been co-crystallised with a duplex of 21 nt siRNAs (Vargason, et al., 2003; Ye et al., 2003). In plants, inhibitors of RNAi target RISC or interrupt the spread of systemic silencing from infected cells (Silhavy et al., 2002; Vargason, et al., 2003; Ye et al., 2003). In Drosophila S2 cells, Li et al. (2002) demonstrated that infection with virions of FHV triggered a strong RNA silencing signal and that FHV has a silencing suppressor gene, B2, which plays a key role in suppressing RNA silencing and successfully establishing infection.

Two examples of genes that negatively regulate RNAi in C. elegans are exonuclease-domain-containing eri-1 and rrf-3. Eri-1 expressed specifically in neurons of C. elegans preferentially utilises siRNA as a substrate (Kennedy et al., 2004). Among the RNA-dependent RNA polymerase (RdRP) family proteins namely, rrf-1, ego-1 and rrf-3, the apparently inactive rrf-3 is presumed to compete for templates or primers of RNA amplification, inhibiting the production of additional short RNAs (Meister and Tuschl, 2004; Simmer et al., 2002). Found in plants and C. elegans, RdRPs amplify the silencing effect of dsRNA synthesis of the target mRNAs or its cleavage products and are essential in systemic silencing (Sijen et al., 2001; Vaistij
et al., 2002; Voinnet et al., 1998). However, orthologues of RdRPs involved in inhibition of RNAi have not been reported so far since insects appear to lack RdRPs (Huvenne and Smagghe, 2010).

1.3 RNAi in insects

Three types of RNAi responses have been described: cell-autonomous, environmental, and systemic; the latter two account for non-cell autonomous (Whangbo and Hunter, 2008). Systemic RNAi in C. elegans, has been studied extensively and it was found that RdRP activity is extremely important in systemic and transitive effects of RNAi. RdRPs are considered to amplify the silencing effect by synthesising dsRNA of the target mRNA or its cleaved products (Cogoni and Macino, 1999; Dalmay et al., 2000; Mourrain et al., 2000; Smardon et al., 2000).

However, in insects, RdRPs are absent and dsRNA is quite likely not amplified suggesting that another mechanism may exist (Burand and Hunter, 2013; Huvenne and Smagghe, 2010; Tomoyasu et al. 2008). To achieve efficient control of insects through RNAi, non-cell-autonomous RNAi is the most suitable and for that, the insect must take up the dsRNA through feeding (Burand and Hunter, 2013; Huvenne and Smagghe, 2010). The dsRNA must be taken up from the gut lumen into the gut cells demonstrating environmental RNAi. If the targeted gene is outside the gut, then the silencing signal must spread through cells and tissues for systemic RNAi to occur (Huvenne and Smagghe, 2010). Interestingly, environmental RNAi has also been shown to be achieved in Apis mellifera through nebulised nanoparticle-siRNA complex that was introduced via the spiracles (Li-Byarlay et al., 2013).

1.3.1 Mechanisms and uptake of small RNAs

Two mechanisms involved in the uptake of dsRNA have been identified: transmembrane channel-mediated uptake and endocytosis-mediated uptake (Huvenne and Smagghe, 2010; Katoch et al., 2013). In C. elegans, sid-1 and sid-2 (systemic RNAi defective mutant), multispanspan transmembrane proteins are responsible for the transport of dsRNA into cells and are essential for systemic RNAi in both somatic and germ cells (Feinberg and Hunter, 2003; Winston et al., 2002).
Interestingly, the model insect, *D. melanogaster*, neither has a robust systemic RNAi mechanism nor a *sid-1* orthologue but cell-autonomous RNAi is present (Rognant et al., 2008; Gordon and Waterhouse, 2007; Miller et al., 2008). When S2 cells of *D. melanogaster* were soaked in a medium containing dsRNA, the effect of RNAi was reported; this suggested that an alternative dsRNA uptake mechanism exists (Feinberg and Hunter, 2003; Saleh et al., 2006). Several insect taxa seem to have conserved the *sid-1* homologue and many species of aphids also show the presence of *sid-1* suggesting the occurrence of systemic RNAi (Gordon and Waterhouse, 2007; Tomoyasu et al., 2008; Xu and Han, 2008). For example, the red flour beetle, *Tribolium castaneum*, which has a well characterised genome is found to be capable of systemic RNAi and also has three homologues of *sid-1* (Tomoyasu et al., 2004, 2008). Homologues of *sid-1* gene have been identified in other insects such as *Bombyx mori*, *Spodoptera exigua*, *A. mellifera*, *Schistocerca americana*, and *A. glycines* (Terenius et al., 2011; Tomoyasu et al., 2008; Xu and Han 2008). *Sid-1* genes of several insects and vertebrates share similarities with the *tag-I30* genes in *C. elegans* that are not involved in systemic RNAi (Tomoyasu et al., 2008). This suggests that in certain insects *sid-1* may not be involved in systemic RNAi or in dsRNA uptake and that an alternative dsRNA uptake mechanism might exist (Tomoyasu et al., 2008). *Sid-1*-like (*sil*) genes have been reported in the cotton aphid, *A. gossypii*, *T. castaneum*, *A. mellifera*, *S. americana*, and *Sitobion avenae* (Tomoyasu et al., 2008; Xu and Han, 2008). Genes involved in systemic RNAi in *C. elegans* do not seem to be in conserved in *T. castaneum*, contradicting the earlier understanding that *sil* genes may be sufficient for systemic RNAi (Tomoyasu et al., 2008). Systemic RNAi has been reported in *A. pisum* and also in mosquito even though *sid-1* and *sil* genes are absent thus suggesting an alternative dsRNA uptake mechanism (Jaubert-Possamai et al., 2007; Mutti et al., 2006, 2008; Xu and Han, 2008).

In *C. elegans*, three other genes, *rsd-2*, *rsd-3*, and *rsd-6* play a role in systemic RNAi in the germ-line but not in the somatic cells (Grishok, 2005; Tijsterman et al., 2004). The *C. elegans* orthologues of *rsd-2* and *rsd-6* have not been found in many insects suggesting that these genes are either not conserved in insects or have evolved rapidly to be detected in the evolutionary pathway (Tomoyasu et al., 2008). In *C. elegans*, *rsd-3* encodes for a protein containing an epsin amino-terminal homology (ENTH domain) and is responsible in vesicle trafficking suggesting the possible involvement of endocytosis in systemic RNAi (Tijsterman et
Interestingly, orthologues of *rsd-3* have been found not only in *Tribolium* but also in *Drosophila*, which lacks systemic RNAi, suggesting that these proteins may not be involved in determining the presence or absence of RNAi (Tomoyasu *et al*., 2008).

Endocytosis-mediated uptake may be possible through the involvement of *clathrin-heavy chain*, vacuolar H+ ATPase (*vha*), scavenger proteins (SR), and *rsd-3* (Huvenne and Smagghe, 2010). In *Drosophila* S2 cells, more than 20 genes required for dsRNA uptake have been identified (Ulvila *et al*., 2006; Saleh *et al*., 2006). Many of these genes have been found to be involved in endocytosis suggesting that this process is involved in dsRNA uptake also in other cells (Ulvila *et al*., 2006; Saleh *et al*., 2006). In *Drosophila*, *vha* may be responsible in breaking the normal endocytosis pathway to induce RNAi in the S2 cells (Saleh *et al*., 2006). Several other genes such as *clathrin-heavy chain*, *eater*, SRs have been identified in both *Tribolium* and *Drosophila* that play a role in dsRNA uptake and have a biological function in endocytosis, phagocytosis and innate immunity (Tomoyasu *et al*., 2008; Ulvila *et al*., 2006). Specifically, SR-CI and *eater* in *Drosophila*, are responsible for more than 90% of dsRNA uptake by the S2 cells (Ulvila *et al*., 2006).

### 1.3.2 Double-stranded RNA delivery systems

To achieve crop protection via RNAi, the biggest challenge is to develop a reliable and suitable dsRNA delivery system. Microinjection, feeding, soaking (insect cell-lines), and spraying have been found to be effective in nematodes and have been utilised in insects as well. Of these delivery systems, oral delivery of host-derived dsRNA has been demonstrated in GPA so far. Some of the successful work to develop delivery systems, with special reference to insects of the order Hemiptera are discussed below.

Microinjection of dsRNA has been performed in several insect orders. Specifically, in the order Hemiptera, microinjection of dsRNA has been administered to insects such as *Lygus lineolaris*, *Oncopeltus fasciatus*, *Riptortus pedestris*, *Nilaparvata lugens*, *Bactericerca cockerelli*, and *A. pisum* (Walker and Allen, 2010, 2011; Turchyn *et al*., Ikeno *et al*., 2011a, b; Liu *et al*., 2010; Wuriyanghan *et al*., 2011; Mutti *et al*., 2006; Jaubert-Possamai *et al*., 2007). It is accurate, the dose of dsRNA administered is known, it can be manipulated easily and has been
demonstrated successfully in nymphs of insects such as *A. pisum*, *Bemisia tabaci*, *N. lugens* (Ghanim et al., 2007; Jaubert-Possamai et al., 2007; Liu et al., 2010; Mutti et al., 2006; Shakesby et al., 2009; Upadhyay et al., 2011). However, microinjection is also laborious, requires a substantial level of skill to reduce undue stress injury or even death of the aphid and also requires choice of appropriate size of microinjection needles as well as empirical injection volumes (Jaubert-Possamai et al., 2007). In addition, it is not suitable for large scale screening of target sequences and it may be difficult to inject small nymphs of some insects such as GPA (Katoch et al., 2013; Li et al., 2012; Whangbo and Hunter, 2008; Yu et al., 2012; Zhang et al., 2013a).

Tenllado et al. (2004) demonstrated that spraying bacterial lysates of *E. coli* expressing dsRNA against plant viral sequences was successful in reducing viral infection and this application may be a potential strategy in crop protection. To successfully apply this strategy large scale fermenters would have to be developed for the commercial production of bacteria expressing dsRNA and more work towards this end is required (Tenllado et al., 2004). Delivery of dsRNA formulations through sprays has been shown to be possible in controlling the Asian corn borer, *Ostrinia furnanalis*, and has demonstrated larval lethality and developmental disorders (Wang et al., 2011). In this research, 10 larval stage specific genes were selected for spraying dsRNA along with an artificial diet of which nine dsRNA sprays resulted in stunting and significant mortality. The study showed that it was possible to deliver dsRNA (in forms of sprays) into the haemolymph of the insect larva through its integument. The approach of spraying dsRNA formulations may be viable, provided the mass production of dsRNA is relatively inexpensive, accompanied by technologies to make the dsRNA more stable under field conditions.

Oral delivery of dsRNA either through artificial diet or transgenic plants has been undertaken for several insects. It is successful, non-invasive and also convenient for functional genomic studies in which large numbers of target genes or sequences are screened (Zhang et al., 2013a). Oral delivery of dsRNA and siRNA through artificial feeding systems has since been applied as a method to feed insects such as *R. pedetris*, *N. lugens*, *B. tabaci*, *S. avenae*, and *A. pisum* (Araujo et al., 2006; Chen et al., 2011; Li et al., 2011a; Shakesby et al., 2009; Upadhyay et al., 2011; Zha et al., 2011; Zhang et al., 2013b). While feeding dsRNA to GPA through an artificial feeding system has not been reported yet, the success of doing so may be significant.
considering generations of GPA have been kept alive on a complex artificial diet for about 30 years (Douglas and van Emden, 2007). Besides, artificial diets have been successfully used to deliver dsRNA in *A. pisum* (Shakesby *et al*., 2009).

A typical feeding chamber includes a clear bottle or a small petridish made of glass or plastic in which the aphids are collected. The artificial feed sandwiched between a thinly stretched parafilm is placed over the mouth of the container and the aphids are allowed to feed *ad libitum* (Mitler and Dadd, 1963 a, b; Mitler and Dadd, 1964). The first attempt of using parafilm in aphid studies was reported by Bradley in 1956 for studying the loss of infectivity of viruliferous GPA on insertion of their stylets into an inert substrate (Bradley, 1956). There are several examples of early studies on aphids in which artificial feeding chambers were used to study the uptake of fluids such as water, sucrose solutions, dyes and radioactive solutions in many aphid species including GPA (Day and Iezykiewicz, 1953; Maltais, 1952; Mitler and Dadd, 1962, 1963 a, b). However, in such a system it is not possible to confirm and measure the uptake unless the feed is coloured and can be visualised in the aphid body.

Transgenic *Arabidopsis* and tobacco expressing dsRNA for the genes cytochrome *P450* and glutathione-S-transferase of the cotton bollworm, *H. armigera* (order: Lepidoptera) have been developed successfully (Mao *et al*., 2007, 2011). Transgenic corn expressing dsRNA for the gene V-type proton ATPase (*V*-ATPase) of the western corn rootworm, *D. virgifera virgifera* (order: Coleoptera), has also been developed successfully (Baum *et al*., 2007). Transgenic plants expressing the *Bt* cry toxins against insect pests belonging to Lepidoptera and Coleoptera have been developed and are successful however, these toxins do not work against insect pests belonging to the order Hemiptera. Transgenic rice expressing dsRNA for three *N. lugens* genes namely, *Nlht1*-a hexose transporter, *Nlcar*-a carboxypeptidase gene, and *Nltry*-a trpsin like serine protease gene, was developed successfully however, although there was a reduction in the transcript abundance there were no lethal phenotypic effects (Zha *et al*., 2011). So far, there are only four reports of RNAi in GPA and all these are primarily host-delivered siRNA of target genes. Hence, there is considerable scope for research to identify suitable target genes of GPA, testing them *in vitro*, and assessing their effectiveness *in planta*.
1.3.3 Factors impacting efficacy of RNAi

The success of achieving efficient RNAi of target genes relies considerably on the mode of dsRNA delivery, its concentration, its length, the sequence itself, the persistence of the silencing effect, and finally the life stage of the insect pest (Huvenne and Smagghe, 2010; Li, et al., 2012; Yu et al., 2012; Zhang et al., 2013a).

The effect of the target gene silencing varies greatly based on the method used for dsRNA delivery. Microinjecting dsRNA has certainly proved to be effective in several insect species including insects of the order Hemiptera. For example, in the tarnished plant bug, *L. lineolaris*, delivering dsRNA for the inhibitor of an apoptosis gene, through microinjection resulted in significant reduction in the lifespan as compared to delivering dsRNA through an artificial diet (Walker and Allen, 2010, 2011). In the triatomine bug, *Rhodnius prolixus*, similar success was achieved through microinjection of dsRNA for the salivary gland nitrophorin rather than feeding dsRNA (Araujo et al., 2006). However, in contrast to the above, lethality was observed in *A. pisum* through feeding of dsRNA for the V-ATPase in an artificial diet (Whyard et al., 2009). These data suggested that different insect species differ in their RNAi responses.

Although some research on *A. pisum* and *N. lugens* demonstrated positive correlation between the concentrations of dsRNA and the effect of RNAi (Mutti et al., 2006; Liu et al., 2010) it is not always found to be true in other insects. A directly proportional relationship between concentrations of dsRNA and the effect of RNAi were not observed in *L. lineolaris* or in the beet armyworm, *Laphygma exigua* (Walker and Allen, 2010; Zhang et al., 2013a). Different concentrations of dsRNA have shown to be required to initiate different RNAi efficiencies in the light brown apple moth, *Epiphyas postvittana* (Turner et al., 2006). Optimisation of the dsRNA concentration used has been shown to be an important part in achieving desired RNAi effects in *T. castaneum* (Tomoyasu and Denell, 2004). These data suggest that the optimal concentration for efficient RNAi also varies for different insect species.

The length of the dsRNA sequence used plays a role in its uptake, for example, in the S2 cells of *Drosophila*, the minimum length of dsRNA is 211 bp and can range between 300 to 520 bp (Saleh et al., 2006). In contrast, Whyard and colleagues demonstrated that feeding dsRNA, less than 40 bp in length, designed on a variable region such as the 3’-UTR of the gamma-
tubulin gene, could selectively kill four species of *Drosophila* (Whyard et al., 2009). The length of dsRNA also determines the silencing efficiency in intact organisms (Mao et al., 2007; Saleh et al., 2006). A study on *H. armigera* by Wang et al. suggested that dsRNA shorter than 50 bp is insufficient to trigger an RNAi response (Wang et al., 2013b).

DsRNA and siRNA both have been found to be effective in inducing RNAi effects in different insects. For example, Wang et al. tested two genes in *T. castaneum* namely, ultrabithorax (*ubx*) and achaete-scute-homologue (*ash*) responsible for wing and sensory structure formation respectively, and studied the effect of RNAi through injection of dsRNA and siRNA designed on different regions of these genes. They demonstrated that dsRNA was very effective in silencing the target genes while siRNA only caused transient effects which did not lead to any apparent phenotype as feeding dsRNA (Wang et al., 2013b). In contrast to the results obtained by Wang et al., (2013b) Kumar and colleagues reported that feeding artificial diet containing siRNA designed on the conserved region of the two homologous acetylcholinesterase gene in *H. armigera* resulted in mortality, growth inhibition of larvae, reduction in the pupal weight, malformation and significant reduction in fecundity as compared to control larvae (Kumar et al., 2009). Similarly, in *B. mori* embryos, injecting siRNA resulted in greater reduction in target transcript levels as well as obvious phenotypic differences in larvae compared with injecting long dsRNA (Yamaguchi et al., 2011).

Some research suggests that the silencing effect persists in the organism only transiently, for example, the effect of silencing an aquaporin gene in *A. pisum* lasted for only about five days after which the effect was reduced (Shakesby et al., 2009). Finally, the life stage at which dsRNA is delivered also determines the efficiency of RNAi. The older the organism, the easier it can resist RNAi, however, younger organisms often show greater RNAi effects (Huvenne and Smagghe, 2010; Li et al., 2012; Zhang et al., 2013a).

The nucleotide sequence of the dsRNA for a particular species may also have the ability to downregulate its orthologue in a closely related species if there is enough sequence similarity. For example, dsRNA for the *V-ATPase* gene in the Colorado potato beetle, *Leptinotarsa decemlineata*, also downregulated its orthologue in *D. virgifera virgifera* when higher concentrations of dsRNA were used (Baum et al., 2007). Feeding dsRNA designed on a variable
region of the 3′-UTR of the gamma-tubulin gene could selectively kill four species of *Drosophila* (Whyard *et al.*, 2009). Species-specific dsRNA against *V-ATPase* gene fed to *D. melanogaster*, *T. castaneum*, *A. pisum*, and *Manduca sexta* resulted in mortality (Whyard *et al.*, 2009). More recent examples of successful species-specific RNAi also demonstrate that feeding dsRNA against specific genes can result in stunted growth or insect death (Gong *et al.*, 2011; Porta *et al.*, 2011; Upadhyay *et al.*, 2011). The ease of finding a species-specific target gene will increase as more insect genomes are sequenced, and targeting such candidate genes will reduce off-target effects. Some of the questions that still remain to be answered for GPA include identifying suitable target genes, the effect of their silencing, whether it is long term or transient, what are the optimal concentrations of dsRNA required for efficient RNAi?

### 1.4 Successful accounts of RNAi in insects belonging to the order Hemiptera

There are many examples of successful RNAi in insects belonging to the order Hemiptera such as the tarnished plant bug (*L. lineolaris*) and milkweed bug (*O. fasciatus*) and some of these are discussed in this section with special reference to GPA (Bhatia *et al.*, 2012; Guo *et al.*, 2013; Mao and Zeng, 2013; Pitino *et al.*, 2011; Walker and Allen, 2010, 2011; Turchyn *et al.*, 2011). The dsRNA delivery systems in these studies include microinjection into adult or nymphal morphs and oral administration through transgenic plants. Table 1 provides a summary of some species belonging to Hemiptera in which various target genes were silenced by different dsRNA delivery system so far.

The first example of RNAi in GPA was demonstrated using transgenic *Arabidopsis* and *agro-*infiltrated tobacco expressing dsRNA for the genes, *MpRack-1* and *MpCOO2*, expressed predominantly in the aphid gut and salivary gland respectively (Pitino, *et al.*, 2011). They showed a significant reduction in transcript levels as well as reduced fecundity. The second example of host-delivered RNAi was demonstrated in transgenic *Arabidopsis* expressing the endopeptidases serine proteases, *MySP* which resulted in attenuation of target transcripts, aphid fecundity and also gut protease activity (Bhatia *et al.*, 2012). Mao and Zeng in 2013 demonstrated similar effects of reduced fecundity and transcript levels of the gap gene hunchback, *Mphb* in GPA fed on transgenic tobacco. More recently, transgenic tobacco expressing artificial hairpin-RNA (hpRNA) and micro-RNA (amiRNA) vectors were studied and it
was shown that whether the small RNAs were processed from hpRNA or pre-amiRNA it was possible to induce RNAi effects in GPA and that selecting a suitable target gene was important (Guo et al., 2014). In the same study, nine candidate genes including V-type proton ATPase subunit E (V-ATPase E), tubulin folding cofactor D (TBCB) were used to develop tobacco expressing hpRNA vectors. A comparison of the performance of transgenic tobacco expressing two amiRNA vectors versus hpRNA vector containing the acetylcholinesterase (MpAchE2) gene was also carried out. Insect challenge experiments suggested that transgenics with amiRNA vectors expressing MpAchE2 provided higher levels of aphid resistance and significant reduction in transcript abundance than hpRNA vectors expressing the same gene. The authors also reported that silencing of V-ATPase-E and TBCD resulted in significant reduction of fecundity and were suitable targets for RNAi in GPA.
Table 1. Examples of RNAi in insect species of Hemiptera, their target genes and the dsRNA delivery system used.

<table>
<thead>
<tr>
<th>Insect species</th>
<th>Target gene</th>
<th>dsRNA/ siRNA delivery system</th>
<th>Concentrations</th>
<th>Size (bp)</th>
<th>Location</th>
<th>Life stage</th>
<th>Silencing of mRNA</th>
<th>Gene expression evaluation method</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Acyrthosiphon pisum</em></td>
<td>COO2</td>
<td>Injection</td>
<td>50 ng</td>
<td>21-23</td>
<td>Salivary glands</td>
<td>7-day old 3rd instar</td>
<td>-</td>
<td>Mortality</td>
<td>Mutti <em>et al.</em>, 2006</td>
</tr>
<tr>
<td></td>
<td>Calreticulin</td>
<td>Injection</td>
<td>276 ng</td>
<td>434</td>
<td>Whole body</td>
<td>3rd instar</td>
<td>40%</td>
<td>-</td>
<td>Jaubert-Possamai <em>et al.</em>, 2007</td>
</tr>
<tr>
<td></td>
<td>Cathepsin-L</td>
<td>Injection</td>
<td>276 ng</td>
<td>353</td>
<td>Gut</td>
<td>1st instar</td>
<td>40%</td>
<td>-</td>
<td>Whyard <em>et al.</em>, 2009</td>
</tr>
<tr>
<td></td>
<td>V-ATPase</td>
<td>Feeding</td>
<td>3 ug/µL</td>
<td>185</td>
<td>Gut</td>
<td>6-day old</td>
<td>-</td>
<td>Mortality</td>
<td>Whyard <em>et al.</em>, 2009</td>
</tr>
<tr>
<td></td>
<td>Aquaporin</td>
<td>Feeding</td>
<td>1 ug/µL</td>
<td>-</td>
<td>Gut</td>
<td>-</td>
<td>Elevated osmotic pressure of haemolymph</td>
<td>Shakesby <em>et al.</em>, 2009</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hunchback</td>
<td>Feeding</td>
<td>0.75 mg/mL</td>
<td>528 &amp; 564</td>
<td>Central nervous system</td>
<td>2-day old</td>
<td>54%</td>
<td>Mortality</td>
<td>Mao &amp; Feng, 2012</td>
</tr>
<tr>
<td><em>Bactericerca cockerelli</em></td>
<td>BC-Actin, ATPase, Hsp-70, CLIC</td>
<td>Injection &amp; feeding</td>
<td>100 ng/mL</td>
<td>21-23</td>
<td>Whole body</td>
<td>Nymph &amp; adult</td>
<td>30-50%</td>
<td>Mortality</td>
<td>Wuriyghan <em>et al.</em>, 2011</td>
</tr>
<tr>
<td><em>Bemisia tabaci</em></td>
<td>BtCG585</td>
<td>Injection</td>
<td>0.1-0.5 ug</td>
<td>370</td>
<td>Midgut</td>
<td>Newly emerged females</td>
<td>70%</td>
<td>-</td>
<td>Ghanin <em>et al.</em>, 2007</td>
</tr>
<tr>
<td></td>
<td>BtGATAd</td>
<td>Injection</td>
<td>0.1-0.5 ug</td>
<td>233</td>
<td>Salivary glands</td>
<td>Newly emerged Newly emerged</td>
<td>70% &amp; 60%</td>
<td>-</td>
<td>Ghanin <em>et al.</em>, 2007</td>
</tr>
<tr>
<td></td>
<td>BtSnap</td>
<td>Injection</td>
<td>0.1-0.5 ug</td>
<td>593</td>
<td>Gut &amp; salivary glands</td>
<td>Newly emerged Newly emerged</td>
<td>70% &amp; 60%</td>
<td>-</td>
<td>Ghanin <em>et al.</em>, 2007</td>
</tr>
<tr>
<td></td>
<td>Chickadee</td>
<td>Injection</td>
<td>0.1-0.5 ug</td>
<td>401</td>
<td>Ovaries</td>
<td>2-day old old 2-day old</td>
<td>70%</td>
<td>-</td>
<td>Ghanin <em>et al.</em>, 2007</td>
</tr>
<tr>
<td></td>
<td>Actin; ADT/ATP translocase; α- tubulin; RPL9; V-ATPase s.u.A</td>
<td>Injection</td>
<td>1, 5, 10, 20 &amp; 40 ug/mL (dsRNA siRNA)</td>
<td>293 &amp; 244 &amp; 189 &amp; 283 &amp; 184 &amp; 189</td>
<td>Whole body</td>
<td>-</td>
<td>Low to significant mortality</td>
<td>Upadhyay <em>et al.</em>, 2011</td>
<td></td>
</tr>
<tr>
<td></td>
<td>V-ATPase s.u. A</td>
<td>Transgenic</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>62%</td>
<td>Reduced fecundity</td>
<td></td>
<td>Thakur <em>et al.</em>, 2014</td>
</tr>
<tr>
<td>Lygus lineolaris</td>
<td>Inhibitor of apoptosis gene (LiAP)</td>
<td>Injection</td>
<td>250-500 ng</td>
<td>-</td>
<td>Whole body</td>
<td>4th instar &amp; adult</td>
<td>Significant decrease</td>
<td>Mortality; reduced lifespan</td>
<td>Walker &amp; Allen, 2011</td>
</tr>
<tr>
<td>------------------</td>
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</tr>
<tr>
<td>Polygalacturonase (PG)</td>
<td>Injection</td>
<td>300-400 ng</td>
<td>-</td>
<td>Salivary glands</td>
<td>4th-5th instar</td>
<td>77-81%</td>
<td>-</td>
<td></td>
<td>Walker &amp; Allen, 2010</td>
</tr>
<tr>
<td><strong>Myzus persicae</strong></td>
<td>MpCOO2 and Rack-1 Serine protease MySP</td>
<td>Transgenic</td>
<td>-</td>
<td>Salivary gland &amp; Gut</td>
<td>1st instar</td>
<td>30-60%</td>
<td>Reduced fecundity</td>
<td>Pitino <em>et al.</em>, 2011</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gap gene hunchback Mphb</td>
<td>Transgenic</td>
<td>-</td>
<td>239 Gut</td>
<td>ug/day</td>
<td>3-day old</td>
<td>27-74%</td>
<td>Reduced fecundity and gut protease activity</td>
<td>Bhatia <em>et al.</em>, 2012</td>
</tr>
<tr>
<td></td>
<td>Nine genes namely, V-ATPase-E, CPRR1, RPSS, SMARCD1, TBCD, delta-COP, RPS14, Med31, AchE2</td>
<td>Transgenic</td>
<td>-</td>
<td>Between 312 &amp; 499 Central nervous system</td>
<td>1st instar</td>
<td>12-32.4%</td>
<td>Reduced fecundity</td>
<td>Guo <em>et al.</em>, 2014</td>
<td></td>
</tr>
</tbody>
</table>

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<p>| Nilaparvata lugens | Hexose transporter; carboxypeptidase; trypsine-like serine protease | Feeding &amp; transgenic | - | - | Nymph | 30-60% | - | | Zha <em>et al.</em>, 2011 |
| V-ATPase s.u. E Calreticulin Cathepsin-B Nicotinic Ach receptor (NIB2) Trehalose phosphate synthase | Feeding | 0.05 ug/mL | 0.5 ug/mL | 250-500 ng | 0.5 ug/mL | - | Mortality | Li <em>et al.</em>, 2011 |
| | Injection | 276, 499 | - | 250 ng | 659 | Fat body, midgut &amp; ovary | 3rd instar | Mortality | Liu <em>et al.</em>, 2010 |
| | Injection | 675 | - | 250 ng | 585 | Gut | 3rd instar | - | Liu <em>et al.</em>, 2010 |
| | Injection | 574 | - | 250-500 ng | 574 | Gut | 3rd instar | - | Liu <em>et al.</em>, 2010 |</p>
<table>
<thead>
<tr>
<th>Species</th>
<th>Gene(s)</th>
<th>Method</th>
<th>Concentration</th>
<th>Subcellular Location</th>
<th>Stage</th>
<th>Effect</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Oncopeltus fasciatus</em></td>
<td>Nubbin Hox genes</td>
<td>Injection</td>
<td>2.5 ug/μL</td>
<td>-</td>
<td>Adult</td>
<td>Freshly laid eggs; Gnathal appendage</td>
<td>Turchyn et al., 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
<td>Reveal novel roles</td>
<td>Hughes &amp; Kaufman, 2000</td>
</tr>
<tr>
<td><em>Rhodnius prolixus</em></td>
<td>Nitrophorin 2</td>
<td>Feeding</td>
<td>13 ug</td>
<td>502</td>
<td>2&lt;sup&gt;nd&lt;/sup&gt; instar</td>
<td>Salivary glands</td>
<td>Araujo et al., 2006</td>
</tr>
<tr>
<td></td>
<td>Nitrophorins</td>
<td>Injection</td>
<td>15 ug</td>
<td>502</td>
<td>4&lt;sup&gt;th&lt;/sup&gt; instar</td>
<td>Salivary glands</td>
<td>Lower ingestion rate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Injection</td>
<td>10.5 ug</td>
<td>502</td>
<td>4&lt;sup&gt;th&lt;/sup&gt; instar</td>
<td>Salivary glands</td>
<td>Araujo et al., 2009</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>502</td>
<td>4&lt;sup&gt;th&lt;/sup&gt; instar</td>
<td>Salivary glands</td>
<td></td>
</tr>
<tr>
<td><em>Riptortus pedestris</em></td>
<td>Circadian clock gene period; cycle; mammalian-type cryptochrome; nitrophorin 2 (NP2)</td>
<td>Injection &amp; feeding</td>
<td>1 ug</td>
<td>-</td>
<td>Adult</td>
<td>-</td>
<td>Ikeno et al., 2011a,b</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>
While some target genes when silenced resulted in mortality of the insect, their orthologues in closely related species resulted in only a significant reduction in the transcript abundance. For example, siRNA microinjection of the salivary gland transcript, \textit{COO2} in seven day-old pea aphid resulted in mortality and significant reduction on transcript abundance by day three after microinjection (Mutti \textit{et al.}, 2006). However, when new-born GPA nymphs were fed on transgenic F3 homozygous \textit{Arabidopsis} lines expressing \textit{MpCOO2} for 16 days, the target gene expression was reduced by 60% and resulted in reduced fecundity but not survival (Pitino \textit{et al.}, 2011).

Another example that illustrates differences in RNAi effects for the same gene is the \textit{hunchback} which was silenced in the pea aphid and GPA. When second instar pea aphid was fed with the gap gene \textit{hunchback, Aphb}, continuously in an artificial feeding system, by day seven there was 36.7% and 54% mortality and reduction in transcript abundance respectively (Mao and Feng, 2012). But when first instar nymphs were fed on transgenic T2 homozygous tobacco expressing \textit{Mphb}, there was a 17.6% and 32.4% reduction in fecundity and transcript abundance respectively by day 14 (Mao and Feng, 2013). In both of the above examples, although the target genes are orthologous in the two insect species, the dsRNA or siRNA delivery systems were different and it is possible that the amount of dsRNA expressed by the transgenic plants may not have been sufficient or the amount of siRNA taken up by GPA may have been inadequate to trigger RNAi effects equivalent or greater than the ones observed in pea aphid.

Application of RNAi as a strategy for crop protection against the GPA requires selection of suitable target genes to achieve significant silencing results. The preliminary genome sequence data as pre-publication data-access are now available for GPA clones ‘O’ and ‘G006’ with an estimated size of 398 Mb (www.aphidbase.org). However, gene annotation and global analysis of the whole genome is yet to be made publicly available, and this will shed light on knowledge of the functions of genes in GPA. The completely sequenced and annotated pea aphid genome until then remains the closest insect genome which will be useful in identifying orthologues of target genes in GPA (IAGC, 2010). So far, there are few reports of RNAi in GPA and there is tremendous scope for research on identifying potential target genes, and their amenability to \textit{in vitro} and \textit{in planta} RNAi.
### 1.5 RNAi as a strategy for insect pest control

Robust RNAi delivery systems have been deployed and successful results in several *in vitro* studies have been achieved. RNAi technology undoubtedly offers a new gateway for developing insect-proof crops (Baum *et al.*, 2007; Gordon & Waterhouse, 2007; Mao *et al.*, 2007). In addition, model plants such as *Arabidopsis* and tobacco and crops like, cotton, corn, and rice have also been engineered to express dsRNA against target insect pests of the order Coleoptera, Lepidoptera, and Hemiptera. To combat several challenges of agriculture, such as increased insecticide resistance and limited genetic resistance, RNAi technology-based crop protection is certainly a promising strategy to safeguard crops against insect pests.

#### 1.5.1 Limitations of RNAi technology for insect pest control

There are numerous challenges and questions that need to be addressed before RNAi technology-based crop protection is commercially deployed. For a transgenic crop expressing dsRNA to reach the status of wide acceptance such as the *Bt* crop it will require extensive research to deliver effective results. Resistance, biosafety and ecological concerns will also have to be addressed. Some of the important challenges in developing dsRNA transgenic crops are: whether the plants express dsRNA continuously in all the parts or expression is restricted to a particular tissue, and what concentrations of dsRNA need to be expressed for efficient RNAi. If RNAi-based technology is proposed as an alternative bio-insecticide, then additional questions that need to be addressed including: the stability of the dsRNA, the cost effectiveness, the ecological impact of dsRNA, and mechanism of delivery of dsRNA perhaps by special coating that protects the dsRNA from degradation when used as a spray. Finally, more research is required to understand the effects of employing different sizes, sequences, concentrations and delivery systems for dsRNA to silence specific target insect genes (Huvenne and Smagghe, 2010; Yu *et al.*, 2012; Zhang *et al.*, 2013a).

#### 1.5.2 Future prospects for RNAi-based technology in insect pest control

The feasibility of RNAi-based technology as a strategy for crop protection against different species of insect pests has been demonstrated in principle by many different research
groups. Although RNAi-based technology may not be an immediate alternative solution of crop protection, once certain questions are answered and limitations or challenges are overcome, it has the potential to be applied successfully in the field. The success of RNAi technology could be enhanced by targeting multiple essential genes of insect pests and also through cross-species protection provided there are no off-target effects caused to beneficial organisms (Katoch et al., 2013; Price and Gatehouse, 2008). An alternative to targeting genes that result in mortality of the insect pests when silenced, is to target the insects’ pheromone receptors, resulting in the disruption of the mating behaviour or communication systems, thereby negatively impacting on insect populations (Trivedi et al., 2010; Turner et al., 2006; Zhao et al., 2011). Similarly, targeting genes associated with insecticide resistance may result in increased sensitivity to insecticide chemistries thereby improving their efficiency and reducing the amount required for use. This kind of approach has also been suggested as an important crop protection strategy (Bautista et al., 2009). Some of the essential steps in assessing the feasibility of RNAi technology require further research to identify and efficiently screen potential candidate genes through insect bioassays, to optimise dsRNA or siRNA concentrations, to investigate the effects of in vitro RNAi, and correlate these effects with in planta RNAi.

1.6 Aims and objectives of this research
The overall aims of this research were to investigate effects of silencing essential genes of GPA and to evaluate the potential of the RNAi technology as an effective strategy to control GPAs. This included identifying target genes and evaluating their effectiveness through in vitro and in planta delivery of dsRNA. This could then provide information critical for the generation of effective constructs for producing transgenic plants for aphid pest control. Specific objectives of this research were as follows:

1. To undertake a comparative bioinformatics study to identify candidate target genes for RNAi. This work was to make use of the expressed sequence tags (ESTs) available for GPA and compare them in silico to the available genomic resources of all other insects including the close insect relative of GPA, the pea aphid, A. pisum. In addition to this, genomic database of the most highly annotated multicellular organism to date, the free-living
nematode, C. elegans were to be used as a reference for identifying lethal RNAi phenotypes to select target genes in GPA.

2. To amplify and sequence candidate genes using molecular techniques, clone them in to transcription vectors suitable for the synthesis of dsRNA for in vitro RNAi experiments, and to make binary vector constructs expressing hairpin cassette for in planta RNAi.

3. To establish conditions for artificial feeding of dsRNA for candidate genes. For this, an in vitro RNAi protocol was to be established for oral delivery of dsRNA using an artificial feeding system. The approach was to investigate which dyes were suitable for tracing the uptake of dsRNA, effects on aphid survival, and stability of dsRNA. Following optimisation of concentrations of dye and dsRNA for target genes, GPAs were fed with target dsRNA to determine which ones affected GPA growth, behaviour and reproduction. Transcript levels of target genes in fed GPAs were to be assessed via semi-quantitative PCRs and long-term effects of gene silencing on growth and reproduction were to be studied.

4. To generate transgenic plants expressing dsRNA corresponding to candidate genes showing promise as efficient targets in the in vitro feeding experiments. In planta RNAi work would involve generating binary vector constructs for Agrobacterium tumefaciens mediated plant transformations, transforming tobacco using leaf disc transformation, characterisation of transgenic plants, and assessing the fecundity of GPA feeding on those plants.
CHAPTER 02

General Materials and Methods
2.0 Insect rearing

The GPA lineage used was obtained from the Plant Pathology Section of the Department of Agriculture and Food, Western Australia (DAFWA). The insects were reared on *Nicotiana tabacum* cv. Wisconsin-98 kept in custom-built insect proof mesh cages (42 cm L X 32.5 cm W X 62 cm H) and/or acrylic cages (101.6 cm L X 51.8 cm W X 17.8 cm H) at 23° to 24°C under a 16:8 hour light:dark cycle. For *in vitro* RNAi studies, nymphs fed for 24 hr on double-stranded RNA (dsRNA) were transferred to two-leaf stage tobacco seedlings grown in 620 mL clear plastic disposable cups filled with soil. Clear plastic lids were placed tightly on the cups immediately after the aphids were transferred. The lids had a small window of 1.5 cm x 1.5 cm covered with white cloth mesh (2 x 2 cm) for air exchange. For *in planta* RNAi studies, aphid challenge experiments were carried out on two-leaf stage transgenic tobacco seedlings that were planted in steam-sterilised Murdoch soil mixture (composted pine bark: coarse river sand: ‘Coco’ peat in a 2:2:1 ratio) supplemented with calcium carbonate (15 g), dolomite (20 g), ‘Grower’s Blue’ (60 g) and Osmocote (60 g) per 40 L of soil. Plants were maintained in a temperature controlled Physical Containment Level 2 (PC2) glasshouse (21 ± 5°C). These plants were covered with inverted 620 mL clear plastic disposable cups that were placed tightly into the soil and had been modified to have a small window of 2.5 cm x 2.5 cm at the base covered with stretched Parafilm M (Pechiney Plastic Packaging, IL, USA). Plants were fertilised with an all-purpose soluble fertiliser, Thrive (N:P:K ratio of 25:5:8.8; Yates, Australia) when required.

2.1 Extraction of total RNA and cDNA synthesis from aphids and tobacco

An aphid population containing mixed life-stages was used for extracting RNA used for synthesis of cDNA. RNA was extracted using the TRizol Reagent (Life Technologies Corporation, Australia) followed by ethanol precipitation. About 150-200 aphids were first macerated with liquid nitrogen in 1.5 mL RNAse-free centrifuge tubes after which 600 μL TRizol diluted with 200 μL RNAse-free water was added. The mixture was vortexed for 5 min and RNA was extracted with 200 μL of chloroform. The upper aqueous phase was added to 1/20 volume of 5 M sodium acetate (pH 5.2) and 2 volumes of ice cold 100% ethanol and incubated overnight at -20°C. The solution was precipitated for 30 min at 12,000 g and resuspended in RNAse-free water followed by RNase-free DNase (Qiagen, Australia)
treatment as per manufacturer’s protocol. To extract RNA from dsRNA-fed aphids, Arcturus PicoPure RNA Isolation Kit (Life Technologies Corporation, Australia) was used following manufacturer’s protocol and then treated with RNase-free DNase (Qiagen, Australia). For transgenic plant analyses, 1-2 g fresh leaf tissue was macerated in liquid nitrogen using a sterile mortar and pestle treated with RNaseZap (Life Technologies Corporation, Australia). RNA was extracted as described above and stored at -80°C until further use.

Single-stranded cDNA from 100 nanogram (ng) of total RNA extracted from unfed and/or dsRNA-fed aphids was synthesised using MultiScribe reverse transcriptase (High Capacity Transcription Kit, Applied Biosystems, Australia). The 20 µL reaction mixture consisted of 1 X RT buffer, 1 X deoxynucleotide triphosphates (dNTPs), 1 X Random Primers, 2.5 U/ µL MultiScribe reverse transcriptase and RNase Inhibitor. Reactions were subjected to PCR thermal cycler conditions of 25°C for 10 min, 37°C for 120 min, 85°C for 5 min followed by 4°C. The cDNA was stored at -20°C until further use. Both DNA and RNA were quantified on a NanoDrop (Thermo Fisher Scientific, Australia) and the purity of the samples were checked based on 260/280 ratios.

2.2 Extraction of genomic DNA from tobacco

For transgenic plant analyses, tobacco leaves were harvested and flash-frozen in liquid nitrogen. A rapid method of genomic DNA extraction was adapted from Henry (1997). Approximately 1-2 mg leaf tissue was placed in a 1.5 mL centrifuge tube and macerated in 200 µL extraction buffer (100 mM Tris HCl, pH 9.5, 1 M KCl and 10 mM EDTA). Tubes were heated at 95°C for 10 min on a heat block, briefly centrifuged, and 1 µL of the supernatant was used in a 20 µL PCR.

2.3 Primer design

Gene specific primers (GSPs) were designed to amplify selected regions of GPA ESTs identified after comparison with several characterised genes of C. elegans and other insects. The restriction enzymes (REs) in the regions of the ESTs selected for amplification were checked for compatibility with those in cloning vectors. This was done using NEB cutter V2.0
GSPs were designed manually and the Extended Nucleic Acid Sequence Massager program was used for reverse complementation (http://www.cmbn.no/tonjum/seqMassager-saf.htm). For cloning into a transcription vector, RE sites for KpnI and XhoI were appended to the primers. A list of primers containing the GPA EST identity number, orthologous *C. elegans* gene, primer names, sequences, total length of EST and position of the primers are shown in Table 2.1. These primers were used for initial amplification of sequences from GPA cDNA as well as for semi-quantitative PCRs to study transcript abundance of target gene in GPA.

Several other primers were also used depending upon the purpose and the plasmid vector used for cloning. These primers were used for colony PCRs and DNA sequencing and their use has been described in the relevant sections. The primers designed were; M13-F (TAA AAC GAC GGC CAG T) and M13-R (CAG GAA ACA GCT ATG AC) for pDoubler, SIntron (TCA TCA TCA TAG ACA CAG GA), S35S (GAT TGA TGT GAC ATC TCC ACT GA), ASNosT (ATT GAG ACG CAA TCC ACA CGC T), ASIntron (TCG TGT GTC TAT GAT GAT GAT GA) and 35SART (GTC TGGATG AGA CCT GCT GCG TA) for pCleaver-NosT, SP6 (ATT TAG GTG ACA CTA TAG AAT), sNpt II-F (AAT ATC ACG GGT AGC CAA CG) and sNpt II-R (AGC ACG TAC TCG GAT GGA AG) for pART27. All primers were synthesised by Integrated DNA Technologies, USA.
Table 2.1. List of primers used for RT-PCRs, semi-quantitative PCRs, colony PCRs and DNA sequencing.

<table>
<thead>
<tr>
<th>GPA EST</th>
<th>Orthologous <em>C. elegans</em> gene</th>
<th>Total length of EST; Position of primers</th>
<th>Mp-GSP</th>
<th>Primer sequence</th>
</tr>
</thead>
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<td>EE261986</td>
<td>Pod-2</td>
<td>819; 182-809</td>
<td>Pod-2 X F</td>
<td>TCA CTCGAG</td>
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<td></td>
<td></td>
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<td>Pod-2 K R</td>
<td>GAGTCAGGATTCTTTTGATAAAGG</td>
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<td>TCA GTTACC</td>
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<td>Eat-6</td>
<td>631; 9-628</td>
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<td>Actin</td>
<td>886; 381-716</td>
<td>Actin X F</td>
<td>TCA CTCGAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ACAGGTCACTCACATCGGAACGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TCA GGTACC</td>
</tr>
</tbody>
</table>

Restriction enzyme sites for *XhoI* (CTCGAG) and *KpnI* (GGTACC) were introduced on forward and reverse primers respectively. Asterisks denote GSP pair used specifically for semi-quantitative PCRs.
2.4  Polymerase Chain Reaction (PCR)

2.4.1  Routine PCRs

PCRs to amplify target genes from aphid cDNA were performed using MyTaq (Bioline, Australia), DreamTaq and Pfu (Fermentas, Australia). The total volume in a PCR was 20 µL and the concentrations of the components were as follows; 1 X buffer, 0.2 mM dNTPs, 1 U polymerases, 2 mM MgSO₄, 10 pm/µL primers, 300 ng of cDNA or 100 ng of plasmid DNA. For most PCRs the thermal cycle was set at 94°C or 96°C for 3 min for the initial denaturation stage, 25 to 45 cycles of denaturation at 94°C or 96°C for 15 to 30 sec, primer annealing at 55°C or 60°C for 15 to 30 sec, extension at 72°C for 30 sec to 1 min and a final extension at 72°C for 7 to 10 min. For amplifying several genes using gene specific primers with restriction enzyme sites, touch-down PCRs with varying annealing temperatures were used where the annealing temperature was decreased in steps of 1°C beginning at 65°C. These PCRs enhanced the specificity of the initial primer-template duplex formation. PCRs to amplify a short fragment (356 bp) within the sequence of the selectable marker gene, neomycin phosphotransferase II (npt II) from genomic DNA of transgenic plants were performed using MyTaq Red Polymerase (Bioline, Australia) and sNpt II primer pair as described in section 2.3. Similarly, PCRs on cDNA synthesised from total RNA of selected transgenic tobacco plants were carried out using gene specific primers listed in Table 2.1 to confirm the expression of the hairpin cassette. The total volume in a PCR was 20 µL and the concentrations of the components were as follows; 1 X buffer containing 0.2 mM dNTPs and 2 mM MgCl₂, 1 U DNA Polymerase, 1:10 dilution of cDNA and clean water to make up the volume. Semi-quantitative PCRs were carried out in triplicate for each biological replicate. PCR conditions were as follows, 96°C for

2.4.2  Semi-quantitative PCRs

Semi-quantitative PCRs were performed to determine expression of target genes in aphids after 24 hr feeding on dsRNA. For reverse transcription, 100 ng of RNA was used throughout the study and first strand of cDNA was synthesised as described in section 2.1. The total volume of PCR was 20 µL and the concentrations of the components were as follows; 1 X reaction buffer comprised of 0.2 mM dNTPs and 2 mM MgCl₂, 1 U DNA Polymerases, 1:10 dilution of cDNA and clean water to make up the volume. Semi-quantitative PCRs were carried out in triplicate for each biological replicate. PCR conditions were as follows, 96°C for
3 min for the initial denaturation stage, 30, 33 and 35 cycles of denaturation at 96°C for 30 sec, primer annealing at 60°C for 30 sec, extension at 72°C for 30 sec and a final extension at 72°C for 7 to 10 min.

2.5 Agarose gel electrophoresis and DNA markers

All gel electrophoreses were carried out in submerged horizontal electrophoresis system (Bio-Rad, Australia). Depending on the expected amplicon sizes, 1.5 to 2 % agarose gels were prepared in 1 X TAE buffer (40 mM Tris acetate, 1 mM EDTA) (Sambrook et al., 1989) with SYBR Safe DNA stain (Life Technologies, Australia) for visualisation of DNA. Electrophoresis procedures was performed at 70 to 80 V. The expected sizes of amplicons were compared to 100 bp or 1 kb DNA ladders (Axygen Biosciences, Fisher Biotech, Australia or New England Biolabs, Genesearch, Australia). Gels were observed with a transilluminator attached to a camera system (Fisher Biotech, Australia) and analysed with BioVision software (www.vilber.com).

2.6 DNA purification from agarose gel

DNA from agarose gel was purified using the Wizard SV Gel and PCR Clean-up System (Promega, Australia). Desired DNA bands were excised, placed in pre-weighed 1.5 mL centrifuge tubes and membrane binding solution (4.5 M guanidine isothiocyanate, 0.5 M potassium acetate pH 5.0) at a ratio of 10 µL per 10 mg agarose gel was added. Gel was melted at 50 to 65°C on a heat block and tubes were vortexed intermittently. The dissolved gel mixture was transferred to a SV minicolumn assembly and centrifuged for 1 min at 16,000 g. Bound DNA was washed twice, first with 700 µL and then 500 µL column wash solution (after addition of ethanol: 10 mM potassium acetate pH 5.0, 80% ethanol, 16.7 µM EDTA pH 8.0) previously diluted with 95% ethanol. Spin column assembly was centrifuged at 16,000 g first for 1 min and then for 5 min. Finally, DNA was eluted in 20 to 50 µL clean RNase-free water and stored at -20°C until further use. All centrifugation steps were performed on a benchtop centrifuge at room temperature.
2.7 Vectors used for cloning

Three plasmid vectors were used in this research. The vectors pDoubler and pCleaver-NosT developed and kindly provided by Dr. John Fosu-Nyarko (formerly Nemgenix Pty. Ltd.) were used for in vitro transcription and cloning of a hairpin cassette respectively while pART27 (Gleave, 1992) was used for plant transformations. GPA target sequences were cloned into an in vitro transcription vector, pDoubler that was also used for dsRNA synthesis (Figure 2.1). For this, purified PCR products and the plasmid vector, pDoubler, were digested with restriction enzymes, XhoI and KpnI to yield complementary ends used for ligation. To generate sense (S) and antisense (AS) fragments, restriction enzyme pairs, XhoI/KpnI and BamHI/XbaI were used respectively to digest out the fragments from pDoubler. Subsequently, both fragments were sub-cloned into the plasmid vector, pCleaver-NosT also cleaved with the same restriction enzyme pairs. Plasmid pCleaver-NosT was designed to have a CaMV35S promoter, bean catalase intron and Nos terminator as shown in Figure 2.2. The RNAi hairpin cassette was finally cleaved from pCleaver-NosT using NotI and ligated to the binary vector, pART27 also cleaved with NotI. A schematic representation of the steps involved in cloning of the RNAi hairpin cassette is given in Figure 2.3.

Figure 2.1. In vitro transcription of vector pDoubler used for cloning of GPA EST sequences and dsRNA synthesis.
Figure 2.2. Vector pCleaver-NosT used for cloning sense and antisense fragments of target GPA sequences.

Figure 2.3. A schematic representation of cloning of a RNAi hairpin cassette in pART27 (not to scale).
2.8 Ligation

Purified PCR products were ligated to pDoubler, sense and antisense fragments were ligated to pCleaver-NosT, and final hairpin RNAi expression cassettes were ligated to a dephosphorylated pART27 (described below) using a 3:1 insert:vector molar ratio following the formula: ng of insert=(concentration of vector in ng) X (size of insert in kilobase (kb))/size of vector in kb X 3/1. All ligation reactions were carried out in a total reaction volume of 10 or 20 µL overnight at 16°C. Reactions comprised of 1 X T4 DNA Ligation Buffer (50 mM Tris-HCl, 10 mM MgCl₂, 1 mM ATP, 10 mM DTT), 1 U T4 DNA Ligase (NEB, Genesearch, Australia). Heat inactivation of ligase was carried out in a thermal cycler at 65°C and reactions were stored at -20°C until further use.

2.9 Dephosphorylation of pART27

Before ligating the hairpin RNAi cassette that was cleaved from pCleaver-NosT using NotI into pART27, the binary vector was dephosphorylated using Antarctic Phosphatase (AP) (NEB, Genesearch, Australia). The enzyme catalyses the removal of 5’ phosphate from DNA thus preventing self-ligation of the vector. A 20 µL dephosphorylation reaction containing 1 X AP Reaction Buffer, 0.5 U AP, 1 µg NotI digested pART27 and RNase-free water was incubated at 37°C for 30 min followed by heat inactivation at 65°C for 5 min.

2.10 Bacterial transformation and DNA purification

All plasmid vectors were transformed into chemically competent Escherichia coli JM109 cells and recombinant plasmid DNA was isolated and purified from cell culture. The final recombinant pART27 vector was transformed into competent Agrobacterium tumefaciens GV3101 cells and used for plant transformations.

2.10.1 Preparation and transformation of competent E. coli JM109 cells

Commercial stock of E. coli JM109 (Promega, Australia) was used to prepare chemically competent cells using a modified rubidium chloride method (Promega’s Protocol and Application Guide, 1996). Glycerol stock was first streaked on Luria Bertani (LB) (10 g
tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar) medium plates and incubated overnight at 37°C. A single bacterial colony was picked with a sterile pipette tip and cultured in 10 mL of LB (prepared as above but without agar) broth in McCartney bottles at 37°C on a shaker (225 strokes per min) overnight. One tenth volume of the overnight culture was used to inoculate 100 mL of LB in 2 L conical flasks and the culture incubated at 37°C on a shaker (225 strokes per min) until the OD600 reached 0.4 to 0.6. The culture was centrifuged at 4,500 g for 5 min in a pre-cooled Beckman Avanti™ J-251 High Performance Centrifuge at 4°C. The resulting pellet was resuspended with 40 mL of filter-sterilised titration buffer-I (100 mM RbCl2, 10 mM CaCl2, 50 mM MnCl2.4H2O, 30 mM KOAc and 15% glycerol adjusted to pH 5.8 with 0.2 M glacial acetic acid). After 5 min of incubation on ice the solution was centrifuged as before and the pellet gently resuspended in 4 mL of ice-cold filter-sterilised titration buffer-II (10 mM MOPS, 75 mM CaCl2, 10 mM RbCl2, and 15% glycerol adjusted to pH 6.5 with 1 M KOH) and later incubated on ice for 10 min after which 100 µL cell suspension were aliquoted into 1.5 mL centrifuge tubes. Tubes were flash-frozen in liquid nitrogen immediately and stored at -80°C until further use.

The heat-shock transformation method was used to transform chemically competent E. coli JM109 cells. Aliquots of competent cells stored at -80°C were first thawed and 5 to 10 µL of the ligation reaction was added to the 25 to 50 µL cells in 1.5 µL centrifuge tubes. Cell-ligation mixture was mixed by gently flicking the tubes two to four times before incubating on ice for 20 minutes. The cells were then heat-shocked at 42°C in a water bath for 45 to 60 sec and then placed on ice for 5 to 10 min. About 500 to 800 µL LB medium without any antibiotics was added and this culture was incubated at 37°C on a shaker at 225 rpm. After 2 to 3 hours, 200 to 400 µL of culture was streaked on LB agar plates containing appropriate antibiotics depending on the plasmid vector used. For pDoubler and pCleaver-NosT, 25 mg/L kanamycin was used while for pART27 100 mg/L spectinomycin was used. Plates were incubated at 37°C overnight.

2.10.2 Preparation and transformation of competent A. tumefaciens GV3101 cells

Glycerol stock of A. tumefaciens GV3101 was used to prepare competent cells using CaCl2. Glycerol stock was first thawed on ice and culture was streaked on LB medium plates with 50
mg/L gentamicin and 25 mg/L rifampicin. Plates were incubated at 28°C overnight. A single colony was used to inoculate 10 mL of LB broth with antibiotics in McCartney bottles and placed at 28°C on a shaker (225 strokes per min) overnight. One tenth volume of the overnight culture was used to inoculate 100 mL of LB with antibiotics in 2 L conical flasks and incubated at 28°C on a shaker (225 strokes per min) until the OD$_{600}$ reached 0.8 to 1.0. The cell culture was transferred into 50 mL Falcon tubes and centrifuged at 3,000 g for 30 min at 4°C in a pre-cooled Beckman Coulter Allegra® X-15R benchtop centrifuge. The pellet was resuspended in 1 mL of 20 mM CaCl$_2$ at 4°C and 50 µL cell suspension was aliquoted in 0.5 mL centrifuge tubes. Tubes were flash-frozen in liquid nitrogen immediately and stored at -80°C until further use.

A freeze-thaw transformation procedure was used to transform competent A. tumefaciens GV3101 cells with recombinant plasmid vectors. Ten microlitre aliquots were thawed on ice and approximately 350 ng plasmid vector was added. Tubes were gently flicked for a few times to mix the cells and frozen immediately by immersing into liquid nitrogen for 5 min. Tubes were then removed and allowed to thaw for 5 min at room temperature followed by incubation at 37°C for 5 min in a pre-warmed water bath. About 500 to 800 µL LB medium without any antibiotics was added and this culture was incubated at 28°C for 4-5 hr. Cell cultures were centrifuged for 2 min at room temperature 12,000 g in a benchtop centrifuge. Pellet was resuspended in 1 mL of LB broth with 100 mg/L spectinomycin and 25 mg/L rifampicin, and 250 µL of this suspension was plated on LB agar plates with antibiotics. Plates were incubated at 28°C for 2 to 3 days.

2.10.3 Plasmid DNA isolation

Plasmid minipreps were prepared using Wizard Plus SV Minipreps DNA Purification System (Promega, Australia) kit. Cell cultures were aliquoted in 2 mL centrifuge tubes, centrifuged for 5 min at 10,000 g and the pellet was resuspended in 250 µL of cell resuspension buffer (50 mM Tris-HCl pH 7.5, 10 mM EDTA, 100 µg /mL RNase A) by inverting the centrifuge tubes. Two hundred and fifty µL of cell lysis solution (0.2 M NaOH, 1% SDS) was added, mixed by inverting four times and incubated for 5 min until the suspension cleared. Ten µL of alkaline protease solution was added, mixed by inverting four times and incubated for another 5 min
after which 350 µL neutralisation solution (4.09 M guanidine hydrochloride, 0.759 M potassium acetate, 2.12 M glacial acetic acid, final pH 4.2) was added immediately, mixed by inverting four times and the bacterial lysate was centrifuged at maximum speed for 10 min. The cleared lysate was decanted onto a spin column assembly without transferring the white precipitate and centrifuged at maximum speed for 1 min. Bound DNA was washed twice, first with 750 µL and then 250 µL column wash solution (162.8 mM potassium acetate, 22.6 mM Tris-HCl pH 7.5, 0.109 mM EDTA pH 8.0) previously diluted with 95% ethanol. Spin column assembly was centrifuged at maximum speed for 1 min both times. Finally, plasmid DNA was eluted in 50 to 75 µL clean RNase-free water and stored at -20°C until further use. All centrifugation steps were performed on a benchtop centrifuge at room temperature.

2.11 Analyses of transformants
Appropriate antibiotic selection based on the vectors used for cloning, PCRs, and restriction digestion were carried out to analyse transformants and are described below.

2.11.1 Selectable markers for analyses of transformants
A kanamycin resistance gene was used as a selectable marker for screening of recombinant pDoubler and pCleaver-NosT, and spectinomycin resistance was used to identify recombinant pART27. Additionally, blue/white colony selection based on LacZ gene expressing beta galactosidase was used for screening recombinant pART27 vectors.

2.11.2 PCR analyses of transformants
To identify positive transformants of pDoubler clones, M13-F and M13-R or gene specific primer pairs were used. In colonies transformed with the inserts, the amplicon size included insert plus 361 bp of the vector. To confirm ligation of sense fragments to pCleaver-NosT, primer pair Sintron and S35S was used whereas ligation of antisense fragment was confirmed with the primer pair ASNosT and ASIntron. In colonies transformed with sense and antisense fragments, the amplicon size included insert plus 311 bp and 159 bp respectively. Presence and orientation of a hairpin cassette in pART27 were assessed by PCRs using SP6 (designed
on the SP6 primer binding site on pART27) and 35SART (designed on CaMV35S promoter on pCleaver-NosT) primers. Transformed colonies yielded an amplicon size of 760 bp. For these PCRs, single colonies were picked with sterile pipette tips and resuspended in 20 µL of clean injection water of which 5 µL of the culture was used for PCRs. Routine PCRs with 1 X buffer containing 0.2 mM dNTPs and 3 mM MgCl₂, 0.5 to 1U Taq polymerases (MyTaq, Bioline, Australia), and 10 pm/µL primers was performed. The thermal cycle was set at an initial denaturation at 96°C for 3 min followed by 25 cycles of denaturing at 96°C for 30 sec, annealing at 55°C for 30 sec, extension at 72 for 30 sec, and a final extension at 72°C for 7-10 min.

2.11.3 Restriction digest analyses of transformants
Based on the results of antibiotic selection and PCR analyses, 5 to 10 µL of culture (prepared by suspending single colonies in clean injection water) from positive transformants was used to inoculate 10 mL of LB broth with appropriate antibiotics in McCartney bottles and were incubated at 37°C on a shaker (225 rpm) overnight. Plasmid DNA was isolated from bacterial cell cultures and was digested with appropriate restriction enzyme(s) to confirm the presence, size, direction of inserts in a vector. For recombinant pDoubler, XhoI/KpnI were used whereas XhoI/KpnI and XbaI/BamHI were used to confirm the presence of sense and antisense fragments in pCleaver-NosT. Finally, XhoI/XbaI were used to confirm the presence and direction of a hairpin cassette in pART27. All restriction enzymes were obtained from NEB, Genesearch, Australia, and reactions were set up as per manufacturer's protocol. Based on the enzyme(s) used in single or double digests, appropriate concentrations of DNA and enzymes, buffers, incubation temperatures for digestion and enzyme inactivation were used with or without BSA. Generally, reactions were incubated at 37°C and heat inactivated at 65°C in a thermal cycler when required.

2.12 DNA sequencing and analyses
All recombinant vector clones were sequenced at the WA State Agricultural Biotechnology Centre (SABC) Sanger sequencing facility using the dye terminator (version 3.1) chemistry based ABI 3730 Sanger Sequencer (Applied Biosystems, Life Technologies, Australia). For
sequencing of pDoubler clones, M13 forward or gene specific primers were used. To confirm the identities of the sense and antisense fragments cloned in pART27, either primer SIntron or ASIntron was used.

A modified version of the Big Dye terminator v3.1 Cycle Sequencing protocol from Applied Biosystems was used. Template preparation for sequencing was done with 1/16th dilution of the recommendation reaction mixture. This was performed using 3.2 pm/µL M13 forward primer, 1 X reaction buffer, Big Dye terminator (dye T), and 150-300 ng plasmid DNA in a total volume of 10 µL. The thermal cycle was set at a hold temperature of 96°C for 2 min followed by 25 cycles of denaturation at 96°C for 10 sec, annealing at 55°C, and an extension at 60°C for 4 min. After PCRs, the reactions were purified to remove any salts or unincorporated dye terminator through ethanol precipitation. This involved the addition of 1 µL of 125 mM EDTA, 1 µL of 3M sodium acetate pH 5.2, and 30 µL of 100% ethanol in the same order without making a master mix for samples. This mixture was pipetted well and incubated at room temperature for 20 min, followed by centrifuging at maximum speed for 30 min at room temperature. The supernatant was discarded and the tubes were air-dried after which 125 µL of 70% ethanol was added and the tubes were centrifuged again for 5 min at room temperature at 12,000 g. The tubes were finally air-dried in the dark for 20 min and submitted to the SABC Sequencing facility. Sequencing was carried out by Frances Brigg, SABC, Murdoch University. Raw sequencing result was viewed and edited with FinchTV (www.geospiza.com/products/finchtv.html) Resulting nucleotide sequence was checked against the ESTs of GPA using TBLASTX program on NCBI.

2.13 In vitro RNAi

For in vitro RNAi studies, dsRNA was synthesised and delivered to GPA through an artificial diet consisting of 30% sucrose and/or vital dyes. Synthesis of dsRNA, artificial feeding system and post-feeding set-up that was used in this research is described below.
2.13.1 DsRNA synthesis

The vector pDoubler was designed to contain T7 promoter regions on either sides of the multiple cloning site with two NotI and EcoRI sites flanking the T7 promoter sites. Either NotI or EcoRI was used to digest the cloned fragments after which the digestion reactions were electrophoresed on agarose gel. DNA bands of the expected size were excised excluding the vector backbone, cleaned-up using Wizard SV Gel and PCR Clean-up kit, quantified on a NanoDrop spectrophotometer and used as a template for in vitro transcription reactions. The Hi-Scribe T7 in vitro transcription kit (NEB, Genesearch, Australia) was used to generate dsRNA for templates greater than 300 bp as per the protocol supplied by the manufacturer. Between 1 to 2 µg template, 1 X reaction buffer, 10 mM of ATP, GTP, CTP and UTP, 2 µL T7 RNA polymerase mix, and appropriate volume of RNase-free water was used in a 20 µL reaction. Tubes were incubated at 37°C for 16 hr in a thermal cycler. To remove template DNA, reactions were treated with 2.5 µL RNase-free DNase, 10 µL RDD buffer (Qiagen, Australia), and appropriate volume of RNase-free water was added to make the volume to 200 µL. Tubes were kept at 20 to 25°C for 10 min after which 1 µL of the DNase I treated solution was aliquoted for gel electrophoresis to examine the quality of dsRNA. RNA was precipitated out from the remaining solution using chloroform followed by sodium acetate and ethanol precipitation described in section 2.1. RNA pellet was washed with 70% ethanol, dissolved in 15 to 20 µL RNase-free water, quantified on a NanoDrop spectrophotometer and stored at -80°C until further use.

2.13.2 Artificial feeding of dsRNA

To study the effects of feeding dsRNA of target genes to GPA, in vitro RNAi experiments were carried out that made use of an artificial feeding system. DsRNA with or without dyes were fed to GPA via feeding sachets. For this, 2nd or 3rd instar nymphs established from a single parthenogenetic adult were carefully collected from tobacco plants and placed in 5 mL clear yellow cap bottles (Sarstedt, Australia) using a fine paint brush. These bottles were put in a 24-well plate to stay in place while dsRNA solution was loaded. Parafilm M (Pechiney Plastic Packaging, IL, USA) pieces of 2 cm x 2 cm were cut and cleaned with Ambion RNaseZap (Life Technologies, Australia) prior to use. A thin layer of parafilm was stretched over the bottles immediately to prevent the escape of aphids. A 40 µL of artificial diet containing dsRNA
suspended in filter-sterilised 30% sucrose solution prepared using RNase-free water was placed on the first layer of parafilm. Another layer of parafilm was stretched to cover the droplet taking precautions to avoid any air bubble formation or spillage. For experiments carried out to trace uptake of dsRNA (Chapter 4), various dyes were first tested without dsRNA and the vital dyes, neutral red and acridine orange were optimised for their use with dsRNA. The number of aphids and feeding chambers used varied depending upon the experiment and are described in the relevant chapters. The feeding system was kept undisturbed on the lab bench for 24 hr at a temperature of 21 to 23°C allowing the aphids to feed on the dsRNA solution *ad libitum* after which dye uptake (presence or absence of dye), aphid survival and phenotypic observations were recorded.

### 2.13.3 Post-feeding set-up

After 24 hr feeding on dsRNA, five surviving aphids were collected in a centrifuge tube, frozen immediately in liquid nitrogen and stored at -80°C for studying gene expression. The remaining dsRNA-fed live aphids were transferred to tobacco and the numbers recorded daily for 12 days to assess long term effects of feeding on aphids. In experiments that made use of vital dyes, live aphids showing the presence of dyes were transferred.

At least five GPA with dye were transferred onto two-leaf stage tobacco plants, one aphid per plant, representing five replicates for each dsRNA treatment. These plants were growing in 285 mL clear plastic party cups and were filled to half full with pasteurised Murdoch soil mix. Clear plastic lids were placed tightly on the cups immediately after the aphids were transferred. These lids had a small window of 1.5 x 1.5 cm for air exchange which was covered with mesh (2 x 2 cm) that was stuck on the lid to prevent escape of aphids. Plants were put in a tray and the set-up was placed in a plant growth chamber under controlled conditions of a 16:8 hr light:dark cycle at 23.5°C. The number of aphids surviving was recorded each day for a period of 12 days.
CHAPTER 03

In silico identification of target genes of *Myzus persicae* for RNAi studies.
3.0 Introduction

Several factors impact the efficiency of RNAi including composition and length of nucleotide sequence, concentration of dsRNA administered and the function of the gene (Huvenne and Smagghe, 2010; Li, et al., 2012; Zhang et al., 2013). In the application of RNAi as a strategy of crop protection against insect pests it is important to achieve significant RNAi effects. Hence it is important to target suitable genes that play roles in important biological processes and which when silenced will disrupt the life-cycle of the organism. Several important GPA genes that encode salivary gland proteins, odorant-binding and chemosensory proteins, peptide hormones and detoxification enzymes have been identified in silico and have been functionally characterised (Bos et al., 2010; Christie, 2008; Ramsey et al., 2010; Xu et al., 2009).

Identification of vital genes that are expressed during various life stages is now possible because genomes and transcriptomes of many invertebrates including insects and nematodes have been sequenced. For example, genome sequencing of 5000 arthropod species was initiated in 2012 by the i5K (arthropod genomes initiative) Consortium (i5K Consortium, 2013) and this will lead to the availability of significantly more genomic information which will be useful in studying gene function. Currently, the International Aphid Genomics Consortium (IAGC) has promoted access to the pre-publication genome assembly of the GPA clone O (GPA_Ov1), the genome size of which is estimated to be 398 Mb, and there is also access to the genome of the GPA clone (G006) (www.aphidbase.org), however, it is not annotated. There is abundant information available from cDNA libraries and ESTs (expressed sequence tags) generated from the transcriptome of GPA (Ramsey et al., 2010). In that study, 16 GPA cDNA libraries made from sexual and asexual whole aphids, gut, head and salivary glands were sequenced using the Sanger sequencing method. This generated 26,669 ESTs, and 10,525 unique genes were identified by Agilent eArray platform (Ramsey et al., 2007).

Comparative bioinformatics is a powerful tool and can be used to identify novel GPA genes in silico. In the current research, to identify potential target genes and test their amenability to RNAi, ESTs of GPA were compared with genes of C. elegans and other insects. All available ESTs of GPA downloaded (December 12, 2012) were used to identify target genes which
were then categorised into six major putative functional classes each representing an important biological process based on the life-cycle of GPA. The non-redundant nucleotide collections of *C. elegans* and insects were mined using BLAST (Basic Local Alignment Tool) suites to identify candidate GPA genes. The databases of *C. elegans* and WormBase, were used as a reference to search for genes with RNAi phenotypes. This provides information that can be used to select candidate target genes for *in vitro* RNAi studies and also to develop transgenic model plants expressing dsRNA for the selected targets.

Based on the life-cycle of GPA, putative functional classes of genes that may be involved at specific stages of the life-cycle were developed. These were embryogenesis, osmoregulation, moulting, cuticularisation, feeding, and locomotion (Figure 3.1). Since both reproductive and phenotypic plasticity is observed in the life-cycle of GPA, the two forms (plasticity) involve distinct embryonic developments and rates, and hence targeting genes involved in embryogenesis can serve as potential targets for RNAi. Genes involved in embryogenesis also included those required for overall growth and development of the embryos, nymph and adults.

Throughout their life-span aphids feed on the plant phloem which has an osmotic pressure four to five times higher than the body fluids of the insect thus making water-cycling or osmoregulation an imperative process to maintain an osmotic balance. Moulting is a hallmark of insects and nematodes including *C. elegans* and hence was a choice of functional class. To survive and reproduce, the aphid must feed and successful feeding involves puncturing the plant tissues, probing, salivation into the sieve elements and ingesting phloem sap. Since feeding occurs in all the life stages of GPA, feeding was also chosen as one of the functional classes to identify target genes. Locomotion was also included as a functional category because it includes genes responsible not only for physical movement of aphids, but also genes required for normal body muscle development in both phenotypic and reproductive morphs. This functional class includes several genes that play a role in muscle contraction of body-wall such as the smooth muscle fibres, actin and myosin. It includes genes that play a role in muscle movement during feeding, development of reproductive organs, and are extremely important in normal functioning throughout the life-cycle. *C. elegans* has well developed body-wall muscle and many of the locomotion genes have been
characterised, and there is abundant information that would be useful in the current study. Thus, it was hypothesised that targeting genes involved in any of the six functional categories would result in the disruption of gene functions that are extremely important in the completion of the GPA life-cycle.

Figure 3.1. Important biological functions (in text boxes) that may be involved at specific periods in the life-cycle of GPA. Pea aphid life-cycle adapted from Le Trionnaire, et al. 2008.

3.1 Aims and objectives of Chapter 03
The overall aim of this chapter was to identify essential genes of GPA as potential targets for its control through comparative bioinformatics. The objectives were to perform in silico comparison of all available 27,721 ESTs of GPA with the non-redundant nucleotide (nr/nt) collection of *C. elegans* and insects curated by the National Center for Biotechnology
Information (NCBI) and to identify GPA ESTs homologous to *C. elegans* genes with RNAi phenotypes and also to find those that shared similar functions in insects.

3.2 Materials and methods

3.2.1 Bioinformatic analyses of ESTs of GPA

All available GPA ESTs, a total of 27,721 curated by the NCBI ESTs database were downloaded on December 12, 2012. To search for sequence similarities, TBLASTX 2.2.26+ (Altschul *et al*., 1997) was performed using CLC Genomics Workbench 7.0.3 (www.clcbio.com). BLAST searches were done with an expected value (e-value) cut-off of 1.0E-05 against the *C. elegans* and insect (excluding GPA) database of nr/nt collection curated by the NCBI. The resulting alignments were retrieved with e-values and bit scores as an XML file used for analyses.

3.2.2 Selection of target genes

Based on the life-cycle of GPA six functional classes of genes were created: embryogenesis, osmoregulation, moulting, feeding, cuticularisation and locomotion. Wormbase was then used to search for *C. elegans* genes involved in each functional class and which also had RNAi phenotypes such as embryonic and larval lethality, sterility, paralysed, moult and growth defective, short life-span, and reduced pharyngeal pumping, development and reproduction. GPA orthologues to *C. elegans* genes with RNAi phenotypes were then identified in the results of TBLASTX that was done to find sequence similarities between GPA ESTs and *C. elegans*. The resulting subset of ESTs was also compared with translated nucleotide sequences of other insects excluding GPA to identify genes with known or predicted functions.

3.3 Results

3.3.1 *In silico* comparison of GPA ESTs with *C. elegans* and other insects.

An important objective of the comparative bioinformatics was to identify target genes of GPA that shared similarities with *C. elegans* genes having lethal RNAi phenotypes, and insect genes with known or predicted functions. Out of the 27,721 GPA ESTs, a total of 13,099 and
25,962 ESTs shared sequence homologies to those of *C. elegans* and insects respectively on the NCBI database. Their distribution based on e-values and maximum scores is represented in Figures 3.2 and 3.3 respectively. There were 2,371 GPA ESTs that shared the highest number of matches to *C. elegans* and had e-values between 1E-11 and 1E-20. On the other hand, 2,382 GPA ESTs shared a maximum number of similarities with other insects and had e-values between 1E-91 and 1E-100. Among the ESTs that matched to insects, a total of 25,393 shared similarities with *A. pisum*.

**Figure 3.2.** Distribution of GPA ESTs by E-values as a result of TBLASTX against the non-redundant nucleotide databases of *C. elegans* (orange) and insects (blue).
3.3.2 Identification of target GPA genes based on functional classes

The search for *C. elegans* genes involved in each putative functional class resulted in a total of 1858 genes from Wormbase (Table 3.1). Of these, a total of 284 GPA orthologues to *C. elegans* genes with RNAi phenotypes were then identified in the collection of 13,099 ESTs of GPA that shared sequence homologies to those of *C. elegans*. The resulting orthologues were then also checked in the collection of 25,962 GPA ESTs that shared similarities with insect genes. Subsequently, a total of 50 GPA ESTs based on the RNAi phenotypes recorded in *C. elegans* and with an e-value cut-off of 1E-05 in TBLASTX searches against *C. elegans* and insect genes were identified (Appendix 1). All 50 GPA ESTs shared similarities with genes of *A. pisum*. Forty-three of the *A. pisum* genes had similar functions, either known or predicted, as those of *C. elegans*. For further confirmation of the choice of the 50 GPA ESTs being identified, the resultant EST queried against the non-redundant nucleotide collection of *C. elegans* in TBLASTX with an e-value cut-off of 1E-05. This resulted in the same *C. elegans* genes that were identified as orthologous to GPA. Appendix 1 provides a detailed description of the GPA ESTs homologous *C. elegans* and *A. pisum* genes that have been sorted by the best e-value represented in a putative functional class. A subset of 10 GPA target genes were selected for molecular characterisation and RNAi studies. These genes were: *MpVha-8* (vacuolar H ATPase), *MpEat-6* (EATing: abnormal pharyngeal pumping), *MpCct-6* (Chaperonin
Containing TCP-1), MpLev-11 (LEVamisole resistant; tmy-1), MpSox-2 (alternative splicing transcription factors), MpTnc-2 (Troponin C), MpAqp-4 (aquaporin or aquaglyceroporin related), MpPdi-2 (protein disulphide isomerase), MpPod-2 (polarity and osmotic sensitivity defect), and MpCars-1 (cysteinyl amino-acyl tRNA synthetase) (Table 3.2). The prioritisation of these gene in terms for advancing them into in vitro RNAi studies was based on a number of parameters: (i) smaller e-values and/or higher maximum scores (Hsp bit) of the selected genes that resulted after a TBLASTX against C. elegans and insect nr/nt nucleotide collection, (ii) assessment that if a gene was cross-listed with one or more putative functional class, it would be selected, (iii) similarly, priority was also given to genes for which there was research data on RNAi studies in C. elegans and/or insects, and if there were lethal effects or phenotypic changes reported.

Table 3.1. Functional classification of GPA ESTs homologous to C. elegans with RNAi phenotypes based on comparative bioinformatics and a subset of GPA ESTs shortlisted for in vitro RNAi.

<table>
<thead>
<tr>
<th>Functional class of genes</th>
<th>No. of C. elegans genes in Wormbase for a specified functional class</th>
<th>No. of GPA ESTs out of 13,099 homologous to C. elegans genes with RNAi phenotype</th>
<th>No. of GPA ESTs shortlisted for in vitro RNAi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryogenesis</td>
<td>293</td>
<td>59</td>
<td>11</td>
</tr>
<tr>
<td>Osmoregulation</td>
<td>22</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Moulting</td>
<td>78</td>
<td>19</td>
<td>10</td>
</tr>
<tr>
<td>Feeding</td>
<td>52</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>Cuticularisation</td>
<td>137</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Locomotion</td>
<td>1276</td>
<td>177</td>
<td>12</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>1858</strong></td>
<td><strong>284</strong></td>
<td><em><em>50 (46</em>)</em>*</td>
</tr>
</tbody>
</table>

The asterisk represents the actual total of GPA ESTs which was less than the sum since three of the C. elegans genes listed in cuticularisation were also cross-listed with moulting genes and one locomotion gene was cross-listed with osmoregulation gene.
Table 3.2. Subset of 10 GPA ESTs representing functional classes, major RNAi phenotypes in Wormbase and results of TBLASTX against non-redundant nucleotide collection of *C. elegans* and insects selected for RNAi studies.

<table>
<thead>
<tr>
<th>Putative functional class</th>
<th>Gene</th>
<th>GPA EST</th>
<th>C. elegans nr/nt database</th>
<th>Accession no.</th>
<th>E-value</th>
<th>Maximum score</th>
<th>Percent sequence identity</th>
<th>Major RNAi phenotypes in C. elegans (Wormbase)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Embryogenesis</strong></td>
<td><em>MpVha-8</em></td>
<td>EC387265</td>
<td>NM_068639</td>
<td></td>
<td>4.72</td>
<td>142</td>
<td>75</td>
<td>Embryonic lethal, maternal sterile, endocytic transport, ovulation, moult and growth defect.</td>
</tr>
<tr>
<td><strong>Feeding</strong></td>
<td><em>MpEat-6</em></td>
<td>ES224834</td>
<td>NM_073868</td>
<td></td>
<td>1.85</td>
<td>338</td>
<td>99</td>
<td>Growth defect, protein degradation &amp; mitochondria morphology variant, sick, embryonic lethal, abnormal pharyngeal pumping.</td>
</tr>
<tr>
<td><strong>Locomotion / Osmoregulation</strong></td>
<td><em>Mpcct-6</em></td>
<td>EE263803</td>
<td>NM_171135</td>
<td></td>
<td>7.70</td>
<td>147</td>
<td>83</td>
<td>Apoptosis, locomotion, life span, protein aggregation &amp; germcell variant; embryonic lethal; maternal sterile, sick.</td>
</tr>
<tr>
<td><strong>Locomotion</strong></td>
<td><em>Mplev-11</em> (tmy-1)</td>
<td>EE264045</td>
<td>D38540</td>
<td></td>
<td>6.32</td>
<td>284</td>
<td>82</td>
<td>Actin cytoskeleton filament morphology, ovulation &amp; body muscle variant; embryonic lethal, paralysed, sick, sluggish, sterile.</td>
</tr>
<tr>
<td><strong>Locomotion</strong></td>
<td><em>Mpsox-2</em></td>
<td>EC389404</td>
<td>NM_171725</td>
<td></td>
<td>2.79</td>
<td>189</td>
<td>30</td>
<td>Embryonic &amp; larval lethal, locomotion &amp; morphology variant, sick, slow growth, sterile.</td>
</tr>
<tr>
<td><strong>Locomotion</strong></td>
<td><em>Mptnc-2</em></td>
<td>ES224908</td>
<td>NM_063850</td>
<td></td>
<td>1.93</td>
<td>133</td>
<td>71</td>
<td>Larval arrest, maternal sterile, reduced brood size</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Insects nr/nt database</th>
<th>Accession no.</th>
<th>E-value</th>
<th>Maximum score</th>
<th>Percent sequence identity</th>
<th>Gene function in A. plasman</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NM00116278</td>
<td>2.00E-130</td>
<td>387</td>
<td>99</td>
<td>V-type proton ATPase s.u.E-like</td>
</tr>
<tr>
<td></td>
<td>XM000818455</td>
<td>6.00E-142</td>
<td>506</td>
<td>99</td>
<td>sodium/potassium-transporting ATPase subunit alpha-like</td>
</tr>
<tr>
<td></td>
<td>XM001946937</td>
<td>1.00E-121</td>
<td>438</td>
<td>95</td>
<td>PREDICTED: t-complex protein 1 subunit zeta-like, transcript variant 1</td>
</tr>
<tr>
<td></td>
<td>XM001947785</td>
<td>3.00E-154</td>
<td>548</td>
<td>96</td>
<td>PREDICTED: tropomyosin-2-like, transcript variant 1</td>
</tr>
<tr>
<td></td>
<td>XM003242400</td>
<td>1.3E-138</td>
<td>304</td>
<td>91</td>
<td>PREDICTED: hypothetical protein</td>
</tr>
<tr>
<td></td>
<td>NM_01162240</td>
<td>1.00E-131</td>
<td>425</td>
<td>98</td>
<td>troponin C-like mRNA</td>
</tr>
<tr>
<td>Role</td>
<td>Accession</td>
<td>Refseq</td>
<td>Fold</td>
<td>Overlap</td>
<td>Description</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>-----------</td>
<td>---------</td>
<td>------</td>
<td>---------</td>
<td>-------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Osmoregulation</td>
<td>MqAqp-4</td>
<td>EE570677</td>
<td>1.81</td>
<td>E-20</td>
<td>59 43 No obvious defects but it is required in conjunction with Aqp-2,3 and 8 to recover from hypotonic stress.</td>
</tr>
<tr>
<td>Moulting / Cuticularisation</td>
<td>MqPdi-2</td>
<td>ES225480</td>
<td>1.04</td>
<td>E-67</td>
<td>238 93 Embryonic lethal, larval arrest, locomotion, development, morphology, population fitness &amp; protein aggregation variant, sterile, sluggish.</td>
</tr>
<tr>
<td>Moulting</td>
<td>MqPod-2</td>
<td>EE261986</td>
<td>1.41</td>
<td>E-68</td>
<td>260 76 Embryonic lethal, locomotion, larval growth, protein aggregation variant, sick, shortened lifespan</td>
</tr>
<tr>
<td>Moulting</td>
<td>MqCars-1</td>
<td>EC388786</td>
<td>1.73</td>
<td>E-90</td>
<td>324 88 Increased apoptosis, embryonic lethal, germ cell morphology variant, larval arrest &amp; lethal, moult defect, slow growth, sterile</td>
</tr>
</tbody>
</table>
In *C. elegans*, some of these genes are involved in more than one function and are required for maintaining normal germline, embryonic and larval viability, development, growth and fertility, pharyngeal pumping, muscle development and contraction, and osmoregulation. For example, *Vha-8* encodes subunit E of the cytoplasmic domain (V1) of the vacuolar proton-translocation ATPase, plays multiple roles in *C. elegans* and insects and is phylogenetically conserved. In *C. elegans*, it plays a role in receptor-mediated endocytosis, pH homeostasis, and is required for embryonic and larval viability, and ovulation.

### 3.4 Discussion

The main objectives of the research in this Chapter were to identify potential target genes of GPA amenable to RNAi using *C. elegans* as a reference genome, and to search for genes with known functions and lethal RNAi phenotypes. Comparison between GPA ESTs and insect genes was also done to study sequence similarities and gene functions. This study led to the identification of 50 potential target GPA genes similar to those required for embryogenesis, feeding, osmoregulation, moulting, cuticularisation, and locomotion in *C. elegans*, and silencing them may disrupt the life-cycle of GPA.

While, it would have been exciting to undertake *in vitro* RNAi studies for all of these, it was not feasible in terms of resources and time. Hence, it was decided that 10 of them would be advanced further. Priority was given based on smaller e-values and/or higher maximum (Hsp bit) scores of the selected genes that resulted after a TBLASTX against *C. elegans* and insect nr/nt collection. Another criterion was that if a gene was cross-listed with one or more putative functional class, then it would be of interest, as it would mean that successful silencing would impact more than one biological process. Similarly, priority was given to genes that had more research data such as any RNAi studies in *C. elegans* and/or insects, and if there were lethal effects or phenotypic changes reported for a particular gene reported in Wormbase. The selection process of one of the genes is explained: In the functional class embryogenesis, GPA EST EC387265 shared high homologies with *C. elegans* Vha-8 with an e-value of 4.72E-61 and Hsp of 142.20 but was second best after EST DW013146 which shared homologies with *C. elegans* gene Let-92 with an e-value of 2.1E-113 and Hsp of 407.04. However, GPA EST EC387265 was selected over EST DW013146 because it not only shared
homologies *C. elegans* but also with a known gene function in pea aphid, and had RNAi phenotypes such as lethal, sterile, moult defective recorded in Wormbase. Additionally, for EST DW013146, the TBLASTX against nr/nt collection of insects did not result in a known or predicted gene further strengthening the choice of EST EC387265 (*MpVha-8*) over EST DW013146 among the final 10 genes. Besides, Vha-8 has been a popular RNAi target in many other insects and has been tested in GPA through plant-mediated RNAi but not *via in vitro* RNAi (Guo *et al.*, 2014). Another example of the selection process that considered cross-listing between putative functional classes, e-values and maximum scores resulting from TBLASTX against *C. elegans* and insect nr/nt collection, and RNAi studies in *C. elegans* was the gene designated *MpPdi-2*. Representing the putative functional class, cuticularisation, *MpPdi-2* had the smallest e-value of 1.04E-67 and the highest maximum score of 238.23, was cross-listed with moulting, and was reported to have several RNAi phenotypes including embryonic and larval lethal, locomotion and development variant, etc. in *C. elegans*.

One of the factors for successful RNAi is the selection of target nucleotide sequence (Huvenne and Smagghe, 2010; Li, *et al.*, 2013; Yu *et al.*, 2012; Zhang *et al.*, 2013a). Identification of target genes for RNAi in insects has been approached in many different ways (Baum *et al.*, 2007; Mao *et al.*, 2007; Wang 2011). The approach most used to select such genes is based on previously reported successful targets for RNAi in other insects (Mao and Zeng, 2014; Pitino *et al.*, 2011; Zhao *et al.*, 2011; Zhu *et al.*, 2011). In this study, *C. elegans* genes with RNAi phenotypes were used as a reference point to identify GPA genes. *C. elegans* was chosen as a reference genome because it is currently the best studied eukaryotic model organism with a completely sequenced and annotated genome, and with the use of RNAi as a tool for reverse genetics, many important genes, their functions and phenotypes are known. The use of WormBase to search for *C. elegans* genes with known RNAi phenotypes allowed the identification of orthologues in GPA EST collection (Table 3.2). Out of the 27,721 GPA ESTs, a total of 13,099 ESTs were found to have similarities with *C. elegans*. On the other hand, more GPA ESTs, 25,962, shared similarities with other insects, most of which were shared with *A. pisum*. The high similarities that were found between GPA and pea aphid sequences were not surprising since there is extensive genomic data available for the pea aphid (IAGC, 2012) and also the two insects belong to the same tribe.
Macrosiphini (sub family: Aphidinae) and are phylogenetically related (von Dohlen et al., 2006).

In GPA, some genes have been investigated for their silencing effects through transgenic plant-mediated RNAi. The first report of successful RNAi in GPA was demonstrated by silencing the gut-specific Rack-1 and the salivary gland-specific COO2 (Pitino et al., 2011). So far, eleven more genes, including the hunchback gap gene, V-type proton ATPase subunit E, RR1 cuticle protein-1, 40S ribosomal protein S5-like isoform-1, and serine proteases have been studied for their gene knockdown effects via plant-mediated RNAi (Bhatia et al., 2012; Guo et al., 2014; Mao and Zeng, 2013). In all these studies, orthologues of A. pisum with known functions were selected and tested via RNAi.

The 10 genes, eat-6, cct-6, pod-2, pdi-2, lev-11, sox-2, tmy-2, cars-1, vha-8 and aqp-4 selected for RNAi in GPA have previously been silenced through RNAi in nematodes (Amin et al., 2007; Culetto & Sattelle, 2000; Doi and Iwasaki, 2008; Frand et al., 2005; Greene et al., 2011; Hamilton et al., 2005; Huang et al., 2007; Jones et al., 2005; Kamath et al., 2003; Mariol et al., 2007; Ono, 2014; Ono and Ono, 2004; Ruksana et al., 2005; Shima et al., 1998; Simmer et al., 2003; Terami et al., 1999). However, except for the genes, vha-8, aqp-4 and lev-11 the target genes selected for RNAi studies in GPA are novel candidates that have not been tested previously in any insect species. V-ATPase is a target that has been studied for the effects of RNAi-mediated silencing in many insect species including M. persicae (Baum et al., 2007; Guo et al., 2014; Upadhyay et al., 2011; Whyard et al., 2009). Aquaporin and tropomyosin have also been investigated for the effects of in vitro RNAi in A. pisum and H. vitripennis respectively (Rosa et al., 2012; Shakesby et al., 2009). Results of the comparative bioinformatics analyses from this study suggested that the 10 genes chosen play essential roles in various biological functions and were suitable targets for further investigation of the effects of down-regulation using RNAi in GPA.

3.5 Conclusions

In this study, through the use of comparative bioinformatics, GPA ESTs were classified into putative functional classes and 10 potential target genes were identified and advanced for
RNAi studies. In addition, this study has provided valuable information on the 10 genes chosen as targets and on additional essential genes of GPA which may also serve as suitable targets for RNAi-mediated crop protection.
CHAPTER 04

The use of vital dyes to trace uptake of dsRNA for effective assessment of target gene knockdown via in vitro RNAi.
4.0 Introduction

Several approaches have been used to deliver dsRNA into insects such as soaking cell-lines, microinjection, and oral feeding through artificial diets and transgenic plants (Baum et al., 2007; Chen et al., 2008; Clemens et al., 2000; Eaton et al., 2002; Mao et al., 2007; Tian et al., 2009). In many cases for in vitro RNAi studies in insects, dsRNA is delivered through microinjection or orally where insects are allowed to feed ad libitum (Burand and Hunter, 2013; Gu and Knipple, 2013; Huvenne and Smagghe, 2010; Li et al., 2013, Zhang et al., 2013a). Microinjection of dsRNA or siRNA into the abdomen or thorax involves the use of very fine glass needles to administer a known amount of dsRNA into various nymphal stages of insects such as A. pisum, B. tabaci, and N. lugens (Ghanim et al., 2007; Jaubert-Possamai et al., 2007; Liu et al., 2010; Mutti et al., 2006). This method requires substantial skill to reduce undue stress, injury or death, involves the use of appropriate size of needle as well as empirical injection volumes and doses of dsRNA (Jaubert-Possamai et al., 2007). Microinjection can be laborious and may not be suitable for large-scale functional analyses of target genes for small nymphs of some insects microinjection may cause injury (Li et al., 2013; Whangbo and Hunter, 2008; Yu et al., 2012; Zhang et al., 2013a).

Oral delivery of dsRNA to insects (also called artificial feeding) is convenient, non-invasive, allows the use of delicate nymphs, and ad libitum feeding which is close to the natural feeding behaviour of aphids (Li et al., 2013; Yu et al., 2012; Zhang et al., 2013a). Artificial feeding is also well suited for large-scale functional analyses and has been used successfully for example, to screen 290 dsRNA targets in D. virgifera virgifera (Baum et al., 2007). However, one of the limitations of oral delivery is that it is difficult to determine the amount of dsRNA ingested by the insect or if any solution is taken up (Surakasi et al., 2011). Most published research on RNAi that uses artificial feeding of insects do not use markers to trace uptake of solutions. In such experiments it is assumed that all insects have fed, and the measure of gene silencing is averaged (Chen et al., 2010; Li et al., 2011; Mao and Zeng, 2012; Shakesby et al, 2009; Upadhyay et al., 2011). This could result in underestimation of gene knockdown if in fact not all insects have fed. However, if uptake of solution could be traced and confirmed, those insects that have taken up feed containing dsRNA can be identified and only those used to deliver a more accurate analysis of gene silencing. Fluorescent-labelled Cy-3 has been used to investigate uptake of dsRNA in Hemipterans such as the glassy-winged
sharpshooter, *Homalodisca vitripennis*, the grain aphid, *Sitobion avenae*, and the potato/tomato psyllid *B. cockerelli* (Rosa et al., 2012; Zhang et al., 2013b; Wuriyanghan et al., 2011). But Cy-3 labelling is expensive when replicated experiments for testing many different target dsRNAs is done. For large-scale screening of target dsRNAs by *in vitro* RNAi the use of inexpensive dyes to trace uptake of dsRNA would allow effective identification of those aphids that have fed.

One of the factors that determine the efficiency of *in vitro* RNAi is the concentration of dsRNA administered. Although a positive correlation between dsRNA concentrations and the effects of RNAi have been found in *A. pisum* and *N. lugens*, this was not the case for the tarnished plant bug, *L. lineolaris* or the beet armyworm, *Laphygma exigua* (Liu et al., 2010; Mutti et al., 2006; Walker and Allen, 2010; Zhang et al., 2013a). Differences in the extent of silencing have been found when varying concentrations of dsRNA for *in vitro* RNAi experiments were used in studies on the light brown apple moth, *E. postvittana* and *T. castaneum* (Turner et al., 2006; Tomoyasu and Denell, 2004). These studies indicate the importance of the need to optimise dsRNA concentrations depending upon the target gene and the method of dsRNA delivery used in order to achieve efficient RNAi.

### 4.1 Aims and objectives of Chapter 04

The aim of the work undertaken in this chapter was to identify dyes that were non-toxic to GPA, and which did not affect the stability of dsRNA, could be visualised easily inside the aphid body, and would enable selection of dsRNA-fed GPA for studying the effects of *in vitro* RNAi. For this, the suitability of 11 inexpensive dyes was assessed to trace the uptake of feed, their effects on dsRNA quality and the effects of ingesting dsRNA at an optimal concentration with and without dye(s). Optimal concentrations of the most suitable dye(s) and dsRNA were subsequently chosen and the effectiveness was assessed by studying the effects of silencing the vacuolar (H+)-ATPase subunit E-like (*Vha-8*) gene. This chapter consisted of three parts:

Part 1: Preliminary studies on dsRNA concentrations and limitations of the study.

Part 2: Identification and optimisation of suitable dyes to trace dsRNA uptake.
Part 3: *In vitro* RNAi using the effective concentrations of dsRNA and vital dye to study knockdown of a target gene.

4.2 Materials and methods

4.2.1 *In vitro* feeding of dsRNA and post-feeding set-up

DsRNA was added to a solution of 30% sucrose with or without dyes, nymphs were allowed to feed on this solution *via* feeding sachets (Figure 4.1). The *in vitro* feeding system was set-up as described in section 2.13.2. In the preliminary studies undertaken to identify a suitable concentration of dsRNA for feeding, one feeding chamber containing 12 aphids was used and aphids were fed with 1 µg/µL and 1.5 µg/µL of *dsMpVha-8*, *dsGFP*, and 30% sucrose as a no-dsRNA control. After 24 hr feeding, 10 aphids were transferred onto tobacco plants, five per plant, and aphid survival was recorded for 12 days to study the long-term effects of *in vitro* RNAi. The remaining live aphids were used to study transcript abundance of target genes by semi-quantitative PCR.

For *in vitro* RNAi using optimal concentration of vital dye and dsRNA to study knockdown of a target gene, 2 µg/µL of dsRNA with and without dye was used. *DsMpVha-8*, *dsGFP*, and 30% sucrose were mixed with and without 0.002% neutral red (NR) and provided to nymphs for feeding trials. Each treatment had two replicates of feeding chambers each containing 12 aphids. After 24 hr feeding the presence of dyes in the aphid body were observed, and their survival and any changes in behaviour were recorded. The number of aphids active/alive and inactive/dead with and/or without the presence of dyes was recorded and expressed as a percentage of the total number of aphids used. To assess long-term effects of feeding, five aphids that had fed on dsRNA with (dye visible) and without dye were transferred on tobacco plants and the numbers of aphids were recorded daily for 12 days.
Figure 4.1. Artificial feeding of dsRNA and post-feeding set-up. (4.1A, 4.1B and 4.1C) A 40 uL droplet of dsRNA was mixed with 30% sucrose sandwiched between two layers of stretched parafilm fastened onto a 5 mL bottle containing GPA nymphs. (4.1D) Post-feeding set-up consisting of tobacco seedlings planted in clear plastic cups and covered with lids with mesh windows.

4.2.2 Dyes

Eleven dyes, these included two fluorescent, six vital, one non-vital and two food colours were artificially fed to GPA nymphs. The fluorescent dyes were fluorescein isothiocyanate (FITC), fluorescein diacetate (FDA), the vital dyes were NR, phloxine B (PB), congo red (CR), methylene blue (MB), acridine orange (AO) and fast green (FG), the non-vital dye was acid fuchsin (AF), and the two food colours were red (RFC) and yellow (YFC) (Queen Fine Foods, Australia).

To identify a dye which could be ingested and observable in the aphid body without any detrimental effects on growth and development, three concentrations each of the fluorescein dyes FITC at 0.5, 1, and 2 mg/mL, FDA at 0.01, 0.001 and 0.0001% w/v, and one concentration of PB at 0.75% v/v, NR (0.5%), CR (0.5%), AF (0.25%), MB (0.5%), FG (0.25%), AO (0.05%), RFC (1:10) and YFC (1:10) were provided in sachets to 20 nymphs to enable them
to feed for 24 hr. To identify concentrations of NR and AO that was sufficient for visualisation and also did not affect aphid survival, solutions with 0.02%, 0.05%, 0.1%, 0.3% and 0.5% of NR and 0.0025%, 0.005%, 0.01%, 0.03% and 0.05% of AO were tested. For each concentration of dye, three replicates of feeding chambers each containing 10 nymphs were used. Finally, lowest concentrations of NR (0.02%) and AO (0.0025%) were further assessed for their effects on the quality, stability and purity of dsGFP. All dyes were first mixed with 30% sucrose and prepared in RNase-free water. After 24 hr feeding, aphids were examined using a fluorescence microscope or compound microscope for the presence of dyes. Aphids allowed to feed on FITC, FDA and AO were examined with an Olympus BX-51 microscope using an FITC filter or UV filter (400-500 nm for fluorochrome dye, AO) at 4x, 10x, and 20x magnifications.

4.2.3 Identification of *MpVha-8* EST

An EST of the vacuolar (H+)-ATPase subunit E-like protein, designated *MpVha-8* was used as target gene to assess the effects of feeding dsRNA (suspended in 30% sucrose) with or without dye. The sequence was identified after a TBLASTX search of the non-redundant nucleotide insect database of the NCBI using *C. elegans* Vha-8: this resulted in 24 ESTs of which the best match was the *M. persicae* EST, EC387265 (771 nucleotide (nt), 75% coverage, total score of 142, and an e-value of 4.72E-61). A schematic representation of EC387265 in comparison to sequences of *A. pisum, D. melanogaster, M. sexta*, and *T. castaneum*, for which RNAi of the Vha-8 gene is reported to have detrimental effects on development (Whyard *et al.*, 2009) is shown in Figure 4.2.
Figure 4.2. Schematic comparison of the mRNA sequences of vacuolar (H+)-ATPase subunit E-like protein (V-ATPase subunit E) depicting the target regions used for dsRNA synthesis in five insect species. Blue arrows indicate 185 bp-long target sequence amplified in *D. melanogaster*, *M. sexta*, *T. castaneum*, *A. pisum* (Whyard *et. al.*, 2009) and red arrows indicate the 477 bp-long target sequence from *M. persicae* used in the current study.

### 4.2.4 Amplification of *MpVha-8* EST

RNA was extracted from a mixed stage aphid population using TRIzol Reagent (Life Technologies Corporation, Australia) and cDNA was synthesised using the High Capacity Transcription kit (Applied Biosystems, Australia) as described in section 2.1. Primers (*MpVha-8*-F: 5’-TCACTCGAGCGTCTGGTCCACAC-3’ and *MpVha-8*-R: 5’-TCAGGTACCTCAGTCCGATTCCGTCACAC-3’) were designed based on the GPA EST, EC387265 to amplify a 477 bp fragment from cDNA. PCRs were performed using MyTaq DNA Polymerase (Bioline, Australia) following the manufacturer’s protocol in a Veriti 96-Well Thermal Cycler (Applied Biosystems) at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 30 sec, primer annealing at 60°C for 30 sec, extension at 72°C for 30 sec and a final extension at 72°C for 7 min. Amplified fragments were excised from 1% agarose gels, purified as in section 2.6 using Wizard® SV Gel and PCR Clean-Up System and stored at -20°C until further use.


4.2.5 Cloning and synthesis of dsRNA for MpVha-8 and GFP

Both, GFP and MpVha-8 DNA fragments were cloned into the *in vitro* transcription vector, pDoubler developed by Dr. John Fosu-Nyarko (formerly Nemgenix Pty. Ltd.) as described in section 2.7. Competent *E. coli* JM109 cells were transformed and transformants were screened through antibiotic selection, colony PCRs and restriction digests as in section 2.11. Clones were sequenced using an ABI 3730 96 capillary machine to confirm their identities as per section 2.12.

Double-stranded RNA corresponding to the 477 bp of *MpVha-8* was synthesised and used to assess the effectiveness of *in vitro* gene silencing. A 524 bp fragment of the green fluorescent protein (GFP) of *Aequorea victoria*, representing base 47 to 571 of the mGFP (M62653) was used as a control in the dsRNA feeding experiments. Templates used for dsRNA synthesis were prepared by cleaving the recombinant pDoubler with *EcoRI* or *NotI* to yield the cloned fragment having opposing T7 polymerase promoter regions. The Hi-Scribe T7 *in vitro* transcription kit (NEB) was used to synthesis dsRNA and was done as in section 2.13.1.

4.2.6 Analysis of the quality of dsRNA in dyes

To assess the stability and quality of dsRNA suspended in dye and/or sucrose solution for 24 hours, an artificial feeding system was set-up as described in section 2.13.2, except that no aphids were included. DsGFP at a concentration of 500 ng/µL was mixed with NR and AO at 0.02% and 0.0025% respectively and was suspended in 30% sucrose in a feeding sachet and allowed to ‘rest’ for 24 hours. The integrity of RNA samples was checked based on 260/280 and 260/230 ratios using a NanoDrop spectrophotometer. Three treatments were done; dsGFP in 30% sucrose; dsGFP and 0.02% NR in 30% sucrose; and dsGFP with 0.0025% AO in 30% sucrose. For all three treatments, the RNA concentration was quantified and its purity checked with a NanoDrop spectrophotometer at 0 hour (*i.e.* before preparing the sachet) and after 24 hr in dye solution. Dye stocks and sucrose solutions were freshly made with RNase-free water using RNase-free sterile filter tips. The quality of dsRNA was also checked through electrophoresis using 2% agarose gel in 1X TAE a 70V. The expected band size of 524 bp was confirmed after comparing it with a 100 bp ladder (Axygen, Australia).
4.2.7 Migration assay

To test if adding dyes to 30% sucrose would attract aphids, an experiment was conducted to study their migration towards the feeding mixture with and without NR (0.02%) and AO (0.0025%) over a period of three hours. To do this, one aphid per channel was placed at one end of a 12-channel isoelectric focusing tray (BioRad, Australia) (each channel was 15 cm long, 0.5 cm wide and 1 cm deep) with 100 µL of feeding mixture placed at the other end on a layer of stretched parafilm and the movement of aphids towards the mixture was measured at 15-minute interval for 3 hr (Figure 4.3). The feeding mixtures were 100 µL of NR (0.02%) + 30% sucrose, AO (0.0025%) + 30% sucrose, and 30% sucrose only, and for each mixture six aphids (replicates) were used.

![Migration assay setup](image)

Figure 4.3. Set-up used for migration assay. (4.3A and 4.3B) 12-channel isoelectric focusing tray containing aphid nymphs at one end and feed or no feed at the other end. (4.3C, 4.3D and 4.3E) Close-up images of single channels showing aphid migration (red arrows) towards the feed.
4.2.8 Semi-quantitative PCRs

Semi-quantitative PCRs were performed to determine the levels of expression of \textit{MpVha-8} in aphids 24 hours after feeding on dsRNA. RNA was extracted from up to five aphids using an Arcturus PicoPure RNA Isolation Kit (Life Technologies Corporation, Australia) followed by an on-column RNase-free DNaseI treatment according to the manufacturer’s protocol (Qiagen, Australia). Equal quantities of RNA extracted from live nymphs that showed the presence of dye after 24 hr (for those fed with NR) and all live nymphs treated without dye were used to generate cDNAs from which equal quantities were used for PCRs. Briefly, 100 ng of RNA was used to synthesise cDNA and subsequently a 1:10 dilution of cDNA was used for PCRs. \textit{MpVha-8} gene expression was normalised with the \textit{MpActin} gene. The primer pairs \textit{MpActin-F}: 5’-TCACTCGAGACAGGTCATCACCACATCGAAACGA-3’ and \textit{MpActin-R}: 5’-TCAGGTACCTCCACATCTGTTGGAGGTGGACA-3’ were designed based on an \textit{M. persicae} EST (EE261235) homologous to the known actin sequence NM_001142636 in \textit{A. pisum} to amplify a 335bp fragment. Semi-quantitative PCRs were carried out in triplicates: PCR conditions were as described in section 2.4.2 and for each biological replicate, \textit{MpVha-8} expression was assessed after 30, 33 and 35 cycles.

4.2.9 Statistical analysis of data

Data were analysed using a combination of descriptive and inferential statistics, and analyses were carried out using Excel and the R programming language (R Core Team 2016). For the analysis of aphid survival times after exposure to various types of feed, Kaplan-Meier survival curves and Cox proportional hazards models were used. Survival curves and Cox models were fit using the R “survival” package (Therneau and Grambsch 2000, Therneau 2015) and “coxphw” package (Heinze, Ploner, and Dunkler 2014), respectively. Additionally, linear regression was used to assess aphid population trajectories over time. For other analyses that considered aphid survival for a fixed period of time (specifically 24 hours), Fisher’s exact test was used to test for differences in survival rates based on feed type.
4.3 Results

4.3.1 Cloning and dsRNA synthesis of *MpVha-8*

A 477 bp-long *MpVha-8* fragment was amplified from cDNA of GPA, cloned into pDoubler and confirmatory PCRs on transformed *E. coli* colonies using M13-F/R and gene specific primer pairs generated the expected band sizes. PCRs using gene specific primer pairs and double digest with *XhoI/KpnI* on plasmid DNA isolated from transformants also confirmed the presence of *MpVha-8*. Sequence analyses indicated that the pDoubler *MpVha-8* clones were 99% and 96% identical to GPA EST EC387265 and *A. pisum* EST NM_001162178.2 respectively. The quality of dsRNA synthesised using the pDoubler clones containing the *GFP* and *MpVha-8* fragments was analysed through agarose gel electrophoresis (Figure 4.4).

![Figure 4.4](image)

**Figure 4.4.** Agarose gel electrophoresis results showing the quality of dsGFP and ds*MpVha-8* in lanes 2 and 3 respectively. Lane 1 contains 100 bp DNA ladder.

4.3.2 Preliminary studies with dsRNA concentrations

4.3.2.1 Expression of *MpVha-8* after 24 hr feeding on 1 μg/μL and 1.5 μg/μL of ds*MpVha-8*

Transcript abundance of *MpVha-8* was studied using semi-quantitative PCRs for aphids that were fed with 1 μg/μL and 1.5 μg/μL of ds*MpVha-8*, dsGFP, and 30% sucrose alone. The results showed that the expression of the house-keeping gene, *MpActin* was similar for aphids in all treatment (Figures 4.5A and 4.5B) and the expression of *MpVha-8* was not affected by dsGFP or sucrose control (Figures 4.5C and 4.5D) for aphids fed with either concentrations of target dsRNA. There was only a slight difference in transcript abundance of *MpVha-8* in aphids fed with 1 μg/μL and 1.5 μg/μL and this difference was seen in the intensities of DNA bands amplified at cycle 30 in both treatments. However, this observation
was not consistent at cycles 33 and 35, in fact, the intensities of DNA bands amplified at those cycles were greater in aphids allowed to feed on dsRNA at 1.5 µg/µL than 1 µg/µL.

Figure 4.5. Inverse images of agarose gel electrophoresis results of semi-quantitative RT-PCRs showing transcript abundance of *MpActin* (4.5A and 4.5B) and *MpVha-8* (4.5C and 4.5D) in aphids after 24 hr feeding. (4.5A) Expression of *MpActin* in aphids fed on sucrose only (lanes 2, 3, and 4 representing 30, 33 and 35 cycles respectively), 1 µg/µL dsGFP (lanes 5, 6, and 7 representing 30, 33 and 35 cycles respectively), and 1 µg/µL ds*MpVha-8* (lanes 8, 9, and 10 representing 30, 33 and 35 cycles respectively). (4.5B) Expression of *MpActin* in aphids fed on sucrose only (lanes 2, 3, and 4 representing 30, 33 and 35 cycles respectively), 1.5 µg/µL dsGFP (lanes 5, 6, and 7 representing 30, 33 and 35 cycles respectively), and 1.5 µg/µL ds*MpVha-8* (lanes 8, 9, and 10 representing 30, 33 and 35 cycles respectively). (4.5C) Expression of *MpVha-8* in aphids fed on sucrose only (lanes 2, 3, and 4 representing 30, 33 and 35 cycles respectively), 1 µg/µL dsGFP (lanes 5, 6, and 7 representing 30, 33 and 35 cycles respectively), 1.5 µg/µL dsGFP (lanes 5, 6, and 7 representing 30, 33 and 35 cycles respectively), 1 µg/µL ds*MpVha-8* (lanes 8, 9, and 10 representing 30, 33 and 35 cycles respectively).
cycles respectively), and 1 µg/µL dsMpVha-8 (lanes 8, 9, and 10 representing 30, 33 and 35 cycles respectively). (4.5D) Expression of MpVha in aphids fed on sucrose only (lanes 2, 3, and 4 representing 30, 33 and 35 cycles respectively), 1.5 µg/µL dsGFP (lanes 5, 6, and 7 representing 30, 33 and 35 cycles respectively), and 1.5 µg/µL dsMpVha-8 (lanes 8, 9, and 10 representing 30, 33 and 35 cycles respectively).

4.3.2.2 Effects on long-term survival of aphids in a post-feeding set-up

After 24 hr feeding on 30% sucrose containing dsRNA, two replicates of five aphids were transferred to tobacco plants and their survival was studied by recording the number of aphids alive (or reproduced) each day for 12 days. (Thus, the same aphids were followed over time, so as to have longitudinal observations.) This was done for each of four treatment groups consisting of aphids fed:
1. 30% sucrose containing 1 µg/µL of dsGFP,
2. 30% sucrose containing 1.5 µg/µL of dsGFP,
3. 30% sucrose containing 1 µg/µL of dsMpVha-8, and
4. 30% sucrose containing 1.5 µg/µL of dsMpVha-8.

A fifth treatment group consisting of aphids fed 30% sucrose only was also considered as a control, and this control group consisted of four replicates of five aphids per tobacco plant.

Two separate analyses were considered to understand the effects of the different feeds. First, a survival analysis was carried out for aphids in the five treatment groups for the first eight days after being fed the particular feed. The length of time selected was based on ability to distinguish the original aphids fed a particular feed-type from their progeny. For the survival analysis, all aphids assigned to a particular feed were treated as replicates, meaning that it was assumed that there was no effect of tobacco plant. (Sample sizes were not large enough to allow for a rigorous assessment of the validity of this assumption, although a log-rank test applied to the twenty aphids fed a diet of 30% sucrose only found no significant different in the survival curves for aphids assigned to each of the four replicates/tobacco plants [p-value = 0.0965], which is consistent with the assumption that there is no tobacco plant effect.)
Kaplan-Meier survival curves for aphids fed each of the various types of feed are shown in Figure 4.6, and they appeared to show a separation in the survival curves for those aphids fed sucrose only, those fed sucrose with dsGFP (regardless of concentration), and those fed sucrose with dsMpVha-8 (regardless of concentration). A Cox proportional hazards model considering the effect of feed type on hazard of death was fit, and the estimated coefficients and their level of significance are shown in Table 4.1. Coefficients provided a comparison of hazard of death for aphids in each of the treatment groups with those aphids fed a diet of 30% sucrose only, and it was noted that, although the p-values corresponding to the coefficients for the two groups fed different concentrations of dsGFP are small (0.063 and 0.058), these are not statistically significant. Coefficients corresponding to aphids fed both concentrations of dsMpVha-8 were highly statistically significant (p-values of 0.002 and 3.00e-05) and positive, indicative of significantly higher hazard of death for these aphids at any given time point as compared to aphids fed a diet of 30% sucrose only. In particular, the coefficients would suggest that aphids fed a diet of 30% sucrose with 1 μg/μL of dsMpVha-8 are roughly 12 times more likely to die at any given time point than aphids fed a diet of 30% sucrose only, and aphids fed a diet of 30% sucrose with 1.5 μg/μL of dsMpVha-8 are roughly 27 times more likely to die at any given time point than aphids fed a diet of 30% sucrose only.

To determine whether there are differences in the hazard of death between the other groups, all possible contrasts of treatment groups were considered, and the only significant differences in hazard were for those aphids fed a diet of 30% sucrose with 1.5 μg/μL of dsMpVha-8 and aphids fed the different concentrations of dsGFP (p-values of 0.0053 and 0.0062 for concentrations of 1 μg/μL of dsGFP and 1.5 μg/μL of dsGFP, respectively) with aphids fed the diet of 30% sucrose with 1.5 μg/μL of dsMpVha-8 being roughly five times more likely to die at any given time point than aphids fed either concentration of dsGFP. The hazard of death between aphids fed a diet of 30% sucrose with 1 μg/μL of dsMpVha-8 and 30% sucrose with 1.5 μg/μL of dsMpVha-8 was not statistically significant (p-value = 0.106), so there was insufficient evidence to suggest that the increased concentration of dsMpVha-8 was more effective in killing the aphids.
**Figure 4.6**: Kaplan-Meier survival curves for aphids fed each of five different feed treatments.

Survival curves are based on sample sizes of $n = 10$ per feed treatment except for the group fed sucrose only, which was based on a sample size of $n = 20$.

**Table 4.1**: Summary of model fit for a Cox proportional hazards model of aphid survival by feed type.

| dsRNA            | Coef. | Exponential function (Coef.) | Std. Err. | z-value | Pr(>|z|) |
|------------------|-------|------------------------------|-----------|---------|----------|
| 1 μg/μL of dsGFP | 1.609 | 4.998                        | 0.867     | 1.86    | 0.063    |
| 1.5 μg/μL of dsGFP | 1.643 | 5.170                        | 0.867     | 1.90    | 0.058    |
| 1 μg/μL of dsMpVha-8 | 2.491 | 12.073                       | 0.805     | 3.09    | 0.002**  |
| 1.5 μg/μL of dsMpVha-8 | 3.313 | 27.463                       | 0.794     | 4.17    | 3.00e-05*** |

Significance codes: ‘***’ 0.001, ‘**’ 0.01, ‘*’ 0.05.
In addition to the survival analysis, modelling of numbers of aphids over time for each of the treatment groups to get a sense of how the different feed types might affect population trajectories were considered. (For instance, dsMpVha-8 might influence aphid fecundity for those aphids that do survive, so it could be influential not only in killing aphids but also in retarding population growth for aphids that survive.) To model population growth over time, a linear regression of number of live aphids on time, a quadratic for time, treatment group, and a time-treatment interaction were considered. The introduction of a quadratic term for time allowed for non-linear trends, and a time-treatment interaction meant that different curves were fit for each treatment group. The number of live aphids for a given treatment group and point in time was normalised by the number of aphids originally assigned to that group, so the response in this linear model can be interpreted as the number of aphids alive as a proportion of the number of aphids at the start of the study. Output for this model is provided in Table 4.2. (It was noted that, whilst a more complex time series analysis that reflects the dependence in number of live aphids between successive days would be appropriate, the linear model considered fits population trajectories incredibly well [$R^2 = 0.99$], and residuals plots would suggest the introduction of a quadratic for time successively eliminated auto-correlation.)
Table 4.2: Summary of model fit for a linear regression of number of aphids alive on time, a quadratic for time, treatment group, and a time-treatment interaction. Number of aphids live have been normalised by the number of aphids alive at the beginning of the study (n = 10 for each treatment group except the group fed sucrose only, which had n = 20).

|                      | Coef. | Std. Err. | t-value | Pr(>|t|) |
|----------------------|-------|-----------|---------|----------|
| (Intercept)          | 0.910 | 0.087     | 10.40   | 4.20e-14*** |
| Time                 | 0.038 | 0.034     | 1.13    | 0.262    |
| 1 μg/μL of dsGFP     | 0.147 | 0.124     | 1.19    | 0.240    |
| 1.5 μg/μL of dsGFP   | 0.162 | 0.124     | 1.31    | 0.198    |
| 1 μg/μL of dsMpVha-8 | 0.189 | 0.124     | 1.53    | 0.133    |
| 1.5 μg/μL of dsMpVha-8 | 0.078 | 0.124     | 0.63    | 0.531    |
| Time^2               | 0.025 | 0.003     | 9.32    | 1.60e-12*** |
| Time:1 μg/μL of dsGFP | -0.160 | 0.048     | -3.35   | 0.002**  |
| Time:1.5 μg/μL of dsGFP | -0.213 | 0.048     | -4.44   | 4.90e-15*** |
| Time:1 μg/μL of dsMpVha-8 | -0.254 | 0.048     | -5.30   | 2.6e-6*** |
| Time:1.5 μg/μL of dsMpVha-8 | -0.287 | 0.048     | -5.99   | 2.3e-7*** |
| Time^2:1 μg/μL of dsGFP | 0.000 | 0.004     | -0.05   | 0.964    |
| Time^2:1.5 μg/μL of dsGFP | 0.005 | 0.004     | 1.32    | 0.194    |
| Time^2:1 μg/μL of dsMpVha-8 | -0.010 | 0.004     | -2.54   | 0.014*   |
| Time^2:1.5 μg/μL of dsMpVha-8 | -0.009 | 0.004     | -2.25   | 0.029*   |

Signif. codes: ‘***’ 0.001, ‘**’ 0.01, ‘*’ 0.05.

Residual standard error: 0.122 on 50 degrees of freedom

Multiple R-squared: 0.99, Adjusted R-squared: 0.988

F-statistic: 369 on 14 and 50 df, p-value: <2e-16

The highly significant time-treatment group interactions (all p-values < 0.002) and negative coefficients suggest that population trajectories are lower for aphids fed concentrations of dsGFP and dsMpVha-8 than aphids fed a diet of 30% sucrose only. When the interactions of treatment group with time and a quadratic for time are combined, the estimated population trajectories are highest for aphids on a sucrose only diet, next highest for aphids on diets with the two different dsGFP concentrations (which were not significantly different), next highest for aphids consuming 30% sucrose with 1 μg/μL of dsMpVha-8, and finally lowest for aphids consuming 30% sucrose with 1.5 μg/μL of dsMpVha-8. Figure 4.7 shows this clearly for the five treatment groups. In addition to mean trends, 95% confidence bounds are presented for these trends, demonstrating the clear divergence in population trends.
between groups fed sucrose only, sucrose with dsGFP and sucrose with dsMpVha-8. At the same time, a clear separation in the population trends for aphids fed sucrose with concentrations of 1 μg/μL and 1.5 μg/μL of dsMpVha-8 with the higher concentration corresponding to a lower population trajectory was seen. (This was not true for dsGFP.) This may indicate that higher concentrations of dsMpVha-8 were in fact more effective in managing aphid population sizes.

Figure 4.7: Normalised proportions of aphids alive by treatment group over a period of 12 days. Lines of best fit (along with 95% confidence bounds) for each group as determined by a linear regression incorporating time, a quadratic for time, treatment group, and a time-treatment interaction are overlaid. (95% confidence bounds corresponding to concentrations of 1.5 μg/μL are shaded in light grey.)
4.3.2.3 Limitation of the preliminary studies on dsRNA concentration

This initial study was undertaken to identify a suitable concentration of dsRNA to use for in vitro RNAi experiments. However, because the results of transcript abundance experiments on aphids fed on two concentrations of dsMpVha-8 were not consistent, an optimal dsRNA concentration for feeding was not identified. Additionally, no changes in behaviour or mortality were observed immediately in aphids that fed on target dsRNA after 24 hr feeding. Even though there were some long-term effects on survival of aphids that had fed on 1 µg/µL or 1.5 µg/µL dsMpVha-8, aphids began to reproduce by day 9. Perhaps, aphids may have overcome the effect of feeding on target dsRNA suggesting that the effect was transient or not all aphids studied for transcript abundance may have taken up the feed containing dsRNA.

4.3.3 Identification and optimisation of suitable dyes to trace dsRNA uptake

4.3.3.1 Identification of appropriate dyes to trace uptake in aphids

In order to identify those aphids which had taken up solution containing dsRNA, a series of experiments were undertaken to choose a suitable dye with which to monitor uptake of feeding solution. Eleven dyes were fed to GPA nymphs to identify the best one which when taken up by aphids would be visible in the body and would not have observable negative effects on movement and survival. Dyes were mixed with 30% sucrose and each fed to 20 nymphs for 24 hours after which the aphids were observed with Olympus BX-51 microscope under FITC filter or UV filter for FITC, FDA, AO and compound microscope for all other dyes. Dyes were visualised in six aphids fed with AO, five fed with NR and in only two and one respectively fed with CR and PB. In these aphids the dyes were observed in the stylets and guts and in some AO-fed aphids the fluorescence was also observed in the siphunculi (Figure 4.8C and 4.8D). In the only aphid that could be seen to have ingested PB, the dye was faintly visible in the moult (Figure 4.8F).
Figure 4.8. Visualisation of NR, CR, AO, PB, FITC, and FDA in the bodies of GPA after feeding for 24 hr. (4.8A) Presence of CR in the gut; (4.8B) Presence of NR in the stylet and gut; (4.8C and 4.8D) Presence of AO in the stylet, gut and siphunculi, (4.8E and 4.8F) Presence of PB in moult and in the gut; (4.8G, 4.8H and 4.8I) Autofluorescence in GPA fed with sucrose only, FITC and FDA.

Using an FITC filter at an excitation wavelength of 490 nm, autofluorescence was observed in aphids fed with 30% sucrose alone and the intensity could not be differentiated readily from fluorescence emitted by aphids fed with all concentrations of FITC and FDA (Table 4.3). The presence of two food colours and the dyes, AF, MB, and FG could not be detected in the aphids. For these dyes, the concentration could not be increased because it could have been detrimental to survival of aphids as demonstrated in the number of aphids that survived after 24 hr of feeding (Table 4.3). However, for CR, NR and AO only a small percentage of aphids were inactive. NR and AO were selected for further investigation based on the number of
aphids that survived after feeding for 24 hr and showed the presence of dye in the stylet, gut, and siphunculi of aphids.

Table 4.3. Survival of GPA nymphs after 24 hr of feeding on eleven dyes.

<table>
<thead>
<tr>
<th>Dye tested with 30% sucrose</th>
<th>Concentration</th>
<th>Total number of GPA fed on dye with 30% sucrose</th>
<th>Number of GPA alive after 24 hr feeding</th>
<th>Presence of dye in aphids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescein isothiocyanate (FITC)</td>
<td>0.5 mg/mL</td>
<td>20</td>
<td>10</td>
<td>Could not be detected due to autofluorescence</td>
</tr>
<tr>
<td></td>
<td>1 mg/mL</td>
<td>20</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 mg/mL</td>
<td>20</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>Fluorescein diacetate (FDA)</td>
<td>0.0001 w/v</td>
<td>20</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.001 w/v</td>
<td>20</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.01 w/v</td>
<td>20</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Red food colour (RFC)</td>
<td>0.5%</td>
<td>20</td>
<td>13</td>
<td>Not detected</td>
</tr>
<tr>
<td>Yellow food colour (YFC)</td>
<td>0.5%</td>
<td>20</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Aqueous acid fuchsin (AF)</td>
<td>0.25%</td>
<td>20</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Aqueous methylene blue (MB)</td>
<td>0.5%</td>
<td>20</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Aqueous fast green (FG)</td>
<td>0.5%</td>
<td>20</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Phloxine B (PB)</td>
<td>0.75 %</td>
<td>20</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>Congo red (CR)</td>
<td>0.5%</td>
<td>20</td>
<td>13</td>
<td>2</td>
</tr>
<tr>
<td>Neutral red (NR)</td>
<td>0.5%</td>
<td>20</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>Acridine orange (AO)</td>
<td>0.05%</td>
<td>20</td>
<td>16</td>
<td>6</td>
</tr>
</tbody>
</table>

Based on the above data for survival after feeding on dyes for 24 hr and the number of aphids detected with dye, five concentrations of AO and NR were analysed further to identify the lowest concentration that could be visualised in aphid bodies when ingested with little or no obvious effects on activity and feeding. This study was done because it was important that the appropriate concentration of dye, duration of exposure and stage of development of aphids be empirically determined before the dye was selected for inclusion in a dsRNA feed. Three replicates of 10 aphids for each concentration were analysed. All concentrations of dyes were taken up by the nymphs. For both the dyes, the highest number of live nymphs
that showed the presence of dye was recorded for the lowest concentrations i.e. 0.0025% for AO and 0.02% for NR (Figure 4.9).

![Graph showing survival of aphids after 24 hr feeding on NR and AO mixed with 30% sucrose.](image)

**Figure 4.9**: Survival of aphids after 24 hr feeding on NR and AO mixed with 30% sucrose. Data represent the proportion of aphids alive after 24 hr ± standard error of the proportion (SEP). (Survival rates for aphids fed 30% sucrose only as well as aphids that did not ingest the feed with the dye are provided for comparison.)

### 4.3.3.2 Effects of NR and AO on the quality of dsRNA

The lowest concentrations of NR (0.02%) and AO (0.0025%) that showed the highest percentage of live aphids after 24 hr feeding were used to determine if the quality of dsRNA was affected 24 hours after the dsRNA was mixed with sucrose. To do this, feed was prepared by mixing 30% sucrose with 500 ng of dsGFP with and without dye. The purity of
nucleic acids was assessed before and 24 hours after setting up feeding sachets using spectrophotometric analysis and gel electrophoresis. The ratio of absorbance at 260 nm and 280 nm was used as a primary measure of nucleic acid purity and at 2.0 is generally accepted as pure for RNA. This ratio for the three kinds of feed was recorded between 2.07 and 2.1 at time zero, while after 24 hours it was between 2.08 and 2.09 thus indicating that there was no major effect on the purity of RNA after 24 hours (Figure 4.10C). The ratio of absorbance at 260 nm and 230 nm is a secondary measure of nucleic acid purity and is generally in the range between 2.0 to 2.2. This ratio for the three kinds of feed was recorded to be 1.9 and 1.78 before and after 24 hours respectively. This slight decrease could perhaps be due to the presence of sucrose and its interaction with dye for 24 hours which may have affected the absorbance at 230 nm (Figure 4.10C). There was no evidence of degradation in the quality of dsRNA mixed with dye and 30% sucrose after 24 hours as compared to zero hours when electrophoresed on a 2% agarose gel at 70V in 1X TAE (Figure 4.10A and 4.10B). Degradation was visualised as smearing of RNA bands however, the intensity of the 524 bp band of dsGFP was sharp at both time-points which indicated that even though there was slight degradation of RNA it essentially remained intact for 24 hr.
4.3.3.3 Migration assay

To test if adding dyes to feed attracted aphids, an experiment was carried out to study their migration towards a feed sachet with and without NR (0.02%) and AO (0.0025%) mixed with 30% sucrose over a period of three hours. It was found that the average distance travelled by aphids provided with sucrose containing NR was greater than those provided with AO at each time-point observed (Figure 4.11). Within the third hour, aphids provided with NR and AO had moved closer to the feed, followed by those presented with sucrose alone. Aphids presented with sucrose only and those not fed moved back and forth over the 3 hr period.
and this is reflected by the high standard errors of the mean (Figure 4.11). This was probably because they were not attracted to move towards the feed as was the case for the aphids provided with NR and AO. During the 3 hr period, no aphid was observed to have fed, but 24 hr after setting up the experiment, NR and AO were clearly present in all the aphids provided with these dyes. These observations suggested that incorporating dyes in feed would not only enable tracing uptake of feed but could also promote feeding.

**Figure 4.11. Migration assay.** The movement of aphids towards the feeding sachets containing NR and AO mixed with 30% sucrose and 30 % sucrose alone was measured every 15 minutes for 3 hr. Data represent ± SEM of six replicates for each treatment.
4.3.4 *In vitro* RNAi using optimised concentrations of vital dye and dsRNA to study the effects of silencing a target gene.

4.3.4.1 Expression of *MpVha-8* expression after 24 hr feeding on 2 µg/µL of *dsMpVha-8* with and without dye.

Transcript abundance of *MpVha-8* in aphids that were fed on target dsRNA mixed in 30% sucrose with and without dye were analysed using semi-quantitative PCRs. RNA was extracted from only those live nymphs that showed the presence of dye after 24 hr (for those fed with NR) and all live nymphs fed without dye. Equal quantities of RNA was used to generate cDNAs of which equal quantities were also used for PCRs. At all PCR cycles the expression of actin was similar for aphids in all the treatments (Figure 4.12A and 4.12B). In both treatments, accumulation of *MpVha-8* transcripts did not differ from that observed in sucrose-only controls suggesting that feeding dsGFP did not affect target gene expression. However, in aphids that had ingested *dsMpVha-8* with dye there was less transcript abundance of the target gene compared to that in aphids fed with *dsMpVha-8* without dye. This shows that there is a pronounced gene knockdown when aphids that had ingested the feed containing dsRNA were assessed compared to aphids fed on dsRNA without dye and then pooled together for analyses.
Figure 4.12. Inverse images of agarose gel electrophoresis results of semi-quantitative PCRs showing transcript abundance *MpVha-8* in aphids after 24 hr feeding of corresponding dsRNA without dye (4.12A) and with dye (4.12B).

### 4.3.4.2 Effects of dsMpVha-8 on survival of GPA

Two bottles, each containing 12 aphids, were used to prepare feeding sachets containing 2 µg/µL of dsMpVha-8 mixed in 30% sucrose with and without NR. For treatments with dye, greater number of nymphs migrated towards the feed within 30 minutes of the experimental set-up, than those without dye. Aphid survival and changes in behaviour were observed after 24 hr feeding. Figure 4.13 shows survival rates for aphids for a period of 24 hr under a variety of diets, suggesting very similar 24 hr survival rates for aphids that consume 30% sucrose with dsGFP and dsMpVha-8, regardless of whether dye is included in the feed or not. A Fisher’s exact test to assess whether there was a significant difference in aphid survival for 24 hr found no significant difference across all treatment groups, including those aphids that consumed sucrose only (*p*-value = 0.4). (This should not be surprising based on Figure 4.13, as doubling the length of error bars should roughly approximate 95% confidence intervals for
these proportions, producing overlap across all treatment groups.) This would suggest that the addition of dye to the feed did not affect activity of aphids, as the proportion of live aphids after 24 hr of feeding in treatments with and without the dye were not significantly different.

Figure 4.13: Survival of aphids after 24 hr feeding on diets of 30% sucrose with 2.0 μg/μL of dsMpVha-8, 30% sucrose with 2.0 μg/μL of dsGFP, 30% sucrose only, and similar diets with 0.02% NR. Data represent the proportion of aphids alive after 24 hr ± standard error of the proportion (SEP). (Survival rates for aphids that did not ingest the feed with the dye and aphids that were given only water are provided for reference.)

4.3.4.3 Effects on long-term survival of aphids in a post-feeding set-up

Five live aphids with dye present in the stylet or gut after feeding on each of dsMpVha-8, dsGFP and sucrose (without dsRNA), and five from each treatment without dye were transferred to tobacco plants, one per plant. Long-term effects of feeding on dsMpVha-8 were assessed by monitoring behaviour, development and reproduction of aphids daily for eight days. Similar to section 4.3.2.2, a survival analysis for a period of 8 days as well as a linear model for population trends for each treatment group, was considered where the
linear model again included a quadratic term for time and time-treatment group interactions. Figure 4.14 shows Kaplan-Meier survival curves for each of the treatment groups, and deaths were only observed for aphids consuming feed containing dsMpVha-8. In line with the Kaplan-Meier survival curves, a log-rank test confirmed that the risk of death is significantly higher for aphids consuming feed with dsMpVha-8 than aphids consuming sucrose only or dsGFP ($p$-value = 5.5e-10).

Figure 4.14: Kaplan-Meier survival curves for aphids fed each of six different feed treatments, consisting of 30% sucrose with 2.0 μg/μL of dsMpVha-8, 30% sucrose with 2.0 μg/μL of dsGFP, 30% sucrose only, and similar diets with 0.02% NR. Survival curves are based on sample sizes of $n = 5$ per feed treatment.

Results for the linear model of number of aphids alive (normalised by initial number of aphids) over time are shown in Table 4.4. Here, differences in sign in time-treatment groups interactions for interaction effects corresponding to a linear effect of time and a quadratic effect of time in part obscure the relationships between population trajectories for the
various treatment groups. However, plots of mean trends with 95% confidence bounds, as shown in Figure 4.15, again quickly highlight clear (and statistically significant) distinctions between population trajectories for aphids fed 30% sucrose with 2.0 μg/μL of dsMpVha-8 (whether with or without dye) from other treatment groups, demonstrating that this concentration of dsMpVha-8 appeared to effectively kill all aphids.

Table 4.4: Summary of model fit for a linear regression of number of aphids alive on time, a quadratic for time, treatment group, and a time-treatment interaction. Number of aphids live have been normalised by the number of aphids alive at the beginning of the study (n = 5 for each treatment group).

|                          | Coef. | Std. Error | t-value | Pr(>|t|) |
|--------------------------|-------|------------|---------|----------|
| (Intercept)              | 1.015 | 0.216      | 4.70    | 1.60e-5*** |
| Time                     | -0.181| 0.084      | -2.16   | 0.035*   |
| 2 μg/μL of dsGFP + NR    | -0.108| 0.305      | -0.35   | 0.726    |
| 2 μg/μL of dsGFP         | -0.413| 0.305      | -1.35   | 0.181    |
| 2 μg/μL of dsMpVha-8 + NR| 0.026 | 0.305      | 0.09    | 0.931    |
| 2 μg/μL of dsMpVha-8     | 0.174 | 0.305      | 0.57    | 0.572    |
| Sucrose + NR             | -0.187| 0.305      | -0.61   | 0.543    |
| Time^2                   | 0.068 | 0.007      | 10.19   | 1.00e-2*** |
| Time:2 μg/μL of dsGFP + NR| 0.143| 0.118      | 1.21    | 0.232    |
| Time:2 μg/μL of dsGFP    | 0.413 | 0.118      | 3.49    | 0.001*** |
| Time:2 μg/μL of dsMpVha-8 + NR| -0.071| 0.118| -0.60   | 0.550    |
| Time:2 μg/μL of dsMpVha-8| -0.094| 0.118      | -0.79   | 0.431    |
| Time:Sucrose + NR        | 0.260 | 0.118      | 2.20    | 0.032*   |
| Time^2:2 μg/μL of dsGFP + NR| -0.026| 0.010| -2.78   | 0.007**  |
| Time^2:2 μg/μL of dsGFP  | -0.070| 0.010      | -7.40   | 5.30e-10*** |
| Time^2:2 μg/μL of dsMpVha-8 + NR| -0.054| 0.010| -5.70   | 3.80e-7*** |
| Time^2:2 μg/μL of dsMpVha-8| -0.053| 0.010      | -5.63   | 5.10e-7*** |
| Time^2:Sucrose + NR      | -0.033| 0.010      | -3.50   | 0.001*** |

Signif. codes: ‘***’ 0.001, ‘**’ 0.01, ‘*’ 0.05.

Residual standard error: 0.3 on 60 degrees of freedom
Multiple R-squared: 0.983, Adjusted R-squared: 0.978
F-statistic: 203 on 17 and 60 df, p-value: <2e-16
Figure 4.15: Normalised proportions of aphids alive by treatment group over a period of 12 days. Lines of best fit (along with 95% confidence bounds) for each group as determined by a linear regression incorporating time, a quadratic for time, treatment group, and a time-treatment interaction are overlaid. (95% confidence bounds corresponding to treatment groups where dye [NR] was added to the feed are shaded in light grey.)

If we treat each aphid as a replicate and combine these results with previous results considering lower concentrations (specifically, 1 and 1.5 μg/μL) of dsGFP and dsMpVha-8, estimated population trajectories for all of the various feed treatments considered is as shown in Figure 4.16. These are based on the linear model summary in Table 4.5, which again considers a quadratic for time and time-treatment group interactions. Once again, we see a clear delineation in population trends for aphids fed 30% sucrose with various concentrations of dsMpVha-8 with aphids fed other feeds. The general trend is one of lower population trajectories with higher concentrations of dsMpVha-8, although the overlap in confidence bounds for aphids fed 1.5 and 2 μg/μL of dsMpVha-8 are large enough for the difference between these two groups not to be statistically significant.
Figure 4.15: Normalised proportions of aphids alive by treatment group over a period of 12 days for all concentrations of dsGFP and dsMpVha-8 and feed with and without NR. Lines of best fit (along with 95% confidence bounds) for each group as determined by a linear regression incorporating time, a quadratic for time, treatment group, and a time-treatment interaction are overlaid.
Table 4.5: Summary of model fit for a linear regression of number of aphids alive on time, a quadratic for time, treatment group, and a time-treatment interaction. Number of aphids live have been normalised by the number of aphids alive at the beginning of the study (n = 5 for each treatment group other than the sucrose only group, which has n = 25).

| Coef.       | Std. Error | t-value | Pr(>|t|) |
|-------------|------------|---------|---------|
| (Intercept) | 0.931      | 0.169   | 5.51    | 2.80e-7   |
| Time        | -0.005     | 0.065   | -0.08   | 0.934     |
| 1 μg/μL of dsGFP | 0.126   | 0.239   | 0.53    | 0.599     |
| 1.5 μg/μL of dsGFP | 0.140   | 0.239   | 0.59    | 0.558     |
| 2 μg/μL of dsGFP | -0.329  | 0.239   | -1.38   | 0.172     |
| 2 μg/μL of dsGFP + NR | -0.023  | 0.239   | -0.10   | 0.923     |
| 1 μg/μL of dsMpVha-8 | 0.168   | 0.239   | 0.70    | 0.484     |
| 1.5 μg/μL of dsMpVha-8 | 0.057   | 0.239   | 0.24    | 0.812     |
| 2 μg/μL of dsMpVha-8 | 0.258   | 0.239   | 1.08    | 0.283     |
| 2 μg/μL of dsMpVha-8 + NR | 0.111   | 0.239   | 0.46    | 0.644     |
| Sucrose + NR | -0.102  | 0.239   | -0.43   | 0.669     |
| Time²        | 0.034      | 0.005   | 6.47    | 3.70e-9   |
| Time:1 μg/μL of dsGFP | -0.116  | 0.092   | -1.26   | 0.211     |
| Time:1.5 μg/μL of dsGFP | -0.169  | 0.092   | -1.83   | 0.071     |
| Time:2 μg/μL of dsGFP | 0.238   | 0.092   | 2.57    | 0.012     |
| Time:2 μg/μL of dsGFP + NR | -0.032  | 0.092   | -0.35   | 0.728     |
| Time:1 μg/μL of dsMpVha-8 | -0.210  | 0.092   | -2.27   | 0.025     |
| Time:1.5 μg/μL of dsMpVha-8 | -0.243  | 0.092   | -2.63   | 0.010     |
| Time:2 μg/μL of dsMpVha-8 | -0.269  | 0.092   | -2.91   | 0.005     |
| Time:2 μg/μL of dsMpVha-8 + NR | -0.246  | 0.092   | -2.66   | 0.009     |
| Time:Sucrose + NR | 0.085   | 0.092   | 0.91    | 0.363     |
| Time²:1 μg/μL of dsGFP | -0.009  | 0.007   | -1.18   | 0.239     |
| Time²:1.5 μg/μL of dsGFP | -0.004  | 0.007   | -0.48   | 0.634     |
| Time²:2 μg/μL of dsGFP | -0.036  | 0.007   | -4.82   | 5.20e-6   |
| Time²:2 μg/μL of dsGFP + NR | 0.008   | 0.007   | 1.09    | 0.279     |
| Time²:1 μg/μL of dsMpVha-8 | -0.018  | 0.007   | -2.48   | 0.015     |
| Time²:1.5 μg/μL of dsMpVha-8 | -0.017  | 0.007   | -2.33   | 0.022     |
| Time²:2 μg/μL of dsMpVha-8 | -0.019  | 0.007   | -2.56   | 0.012     |
| Time²:2 μg/μL of dsMpVha-8 + NR | -0.020  | 0.007   | -2.65   | 0.009     |
| Time²:Sucrose + NR | 0.001   | 0.007   | 0.16    | 0.872     |

Signif. codes: ‘***’ 0.001, ‘**’ 0.01, ‘*’ 0.05.
Residual standard error: 0.235 on 100 degrees of freedom
Multiple R-squared: 0.981, Adjusted R-squared: 0.976
F-statistic: 182 on 29 and 100 df, p-value: <2e-16
4.4 Discussion

In this study, effective knockdown of the target gene *MpVha-8* was demonstrated when the vital dye NR was used to trace uptake of dsRNA. This allowed assessment of only those aphids which had taken up dsRNA. The choice of NR as opposed to other dyes and the results of feeding ds*MpVha-8* with/without dye are discussed.

4.4.1 Identifying a suitable dye and its concentration

For insects where oral delivery of dsRNA is chosen for delivery, there must be an economical and effective means of identifying those insects that actually take up dsRNA to allow accurate assessment of gene silencing, since using feeding sachets insects feed *ad libitum*. The suitability of 11 inexpensive dyes for tracing uptake of dsRNA by aphids was assessed. It was demonstrated that ingestion of four vital dyes were observable in the aphid body and which had no obvious detrimental effects on development and reproduction. In addition, at the optimum concentrations of two of these dyes, they also attracted aphids to the artificial feed containing dsRNA and possible encouraged them to feed more, resulting in a more pronounced gene silencing, at least for the *MpVha-8* gene studied.

It was demonstrated that three of the 11 dyes, AO, CR, and NR could be seen within the aphid body 24 hours after ingestion. The intensities of these dyes inside the aphid body differed, and even for the various concentrations of AO and NR there was no correlation between the concentrations of dye and the intensities in the body: these differences probably reflect the random feeding behaviour of aphids rather than a property of the dyes. Generally, larger numbers of aphids fed on AO and NR than on feed with the other dyes, and at all concentrations tested, these two dyes were ingested as they were visible in aphid bodies. Moreover, the activity of aphids fed on these dyes was not affected and this suggests that the dyes did not have any visible detrimental effects of the aphids. AO has been used as a fluorescence marker for insect tissues, and at concentrations of 0.001, 0.01, 0.1, 1, 2 and 5 mg/mL has been shown to have no deleterious effects on growth, development, longevity, mating or oviposition of insect species of the order Diptera and Hymenoptera (Brenner, 1984; Ribble *et al.*, 2005; Rodrigues *et al.*, 2009; Strand *et al.*, 1990). It appears that the
lower concentrations of NR used in this study were appropriate: 0.1% of NR could be
detrimental to GPA (Mittler and Dadd, 1963).

The dyes MB, FG, AF and the food colours were not visible in the aphid body: either the
concentrations were too low to allow visualisation or the aphids did not feed on these dyes
because of the colour intensity, surface tension, refraction, odour or taste of the dyes and
this may have led to starvation, because in general fewer aphids presented with these dyes
were active after 24 hours. Like most insects, aphids rely on well-developed chemosensory
and olfactory systems to detect semiochemicals in the environment and any undesirable
components of these dyes could disrupt feeding, leading to possible starvation and even
death observed after 24 hours (van Naters and Carlson, 2006).

For the two fluorescein dyes, FITC and FDA, there were no significant differences in the level
of fluorescence observed between unfed aphids and those fed with them. The
autofluorescence in unfed aphids made it difficult to decide whether or not the aphids had
actually ingested the dyes. When concentrations of 0.01, 0.1 and 1 mg/mL of FITC in sucrose
solutions were fed to screwworm for evaluating the ease and reliability of marking sperm,
similarly there was no strong signal in the reproductive tissues (Brenner, 1984). In the
current study 0.5, 1, and 2 mg/mL FITC was used, however none of the concentrations were
found to be suitable to mark the aphids internally.

Even though both NR and AO were both visualised in the aphid body, NR was selected for
further studies because it provided the ease in method of viewing aphids by compound
microscope, instead of needing to use a fluorescence microscope as required for detecting
AO. At optimum concentrations of AO and NR, no damaging effects on development and
reproduction were found; the presence of dye in the feeding sachet attracted insects
towards the artificial feed and perhaps encouraged them to feed more, resulting in a more
pronounced gene silencing of MpVha-8. Aphids migrated towards the feed within thirty
minutes of the set-up indicating that the presence of the colour in the sucrose solution was
responsible for attracting them.
4.4.2 Identifying a suitable concentration of dsMpVha-8

Aphids were fed on 1 µg/µL and 1.5 µg/µL dsRNA resuspended in 30% sucrose without any dye and effects on behaviour and gene expression were studied. When aphids were assessed for changes in behaviour and immediate effects on survival after feeding on dsMpVha-8 for 24 hours, no significant differences were observed compared to control aphids. The expression of MpVha-8 in aphids fed on 1 µg/µL dsMpVha-8 showed only a slight reduction in transcript abundance (at PCR cycle 30) when compared to their respective controls: 30% sucrose-only and dsGFP. It was expected that feeding aphids with an increased concentration (1.5 µg/µL) of dsRNA would result in a greater effect on MpVha-8 silencing and there would be less transcript abundance. However, it was found that the transcript abundance in aphids fed on dsMpVha-8 did not differ much from the control aphids. A comparison of the intensities of DNA bands of the target gene in aphids fed with 1 µg/µL and 1.5 µg/µL dsRNA of MpVha-8 revealed no reduction in transcript abundance, except at cycle 30, in aphids fed with the higher dsRNA concentration. Since there was no obvious difference in these results they could not be used to determine a suitable concentration of dsRNA. Aphids that were assessed for the long-term effects on survival in a post-feeding set-up showed a decrease in numbers in the first few days followed by an increase as a result of reproduction suggesting that perhaps aphids had recovered from the effects of feeding on dsRNA and possibly there was a transient effect of gene knockdown. To confirm this, if the set-up was kept longer than 12 days, it would have been possible to record the increase in aphid numbers, however, this was not possible since beyond day 12 the growing leaves of tobacco forced open the lids of the cups they were planted in.

In considering the nature of the result on feeding aphids at two concentrations of dsRNA, two possible explanations are discussed. Firstly, the concentration of dsMpVha-8 might have not have been enough to cause apparent differences in gene expression and affect long-term survival of aphids on tobacco over 12 days. Secondly, there could be a dilution in transcript abundance which may be contributed by aphids that may not have fed. This in turn may have caused inaccurate estimate of transcript abundance. The likelihood of the second possibility to be correct is greater because there were no means to detect if the test insects had ingested the feed containing dsRNA. The hypothesis that assessing only aphids that have ingested dsRNA provided a better estimation of transcript abundance, lead to the study on
dye uptake. It was proposed that firstly, adding dyes that would allow tracing the uptake of feed followed by assessment of only aphids that have ingested the feed, and secondly, increasing the concentration of dsRNA. The hypothesis was tested by comparing the effects on transcript abundance and long-term survival of aphids fed on an increased concentration of dsMpVha-8 with and without dye.

Two possible reasons for limited effects of silencing on reproduction of GPA after feeding for 24 hr that were worth further investigation: the concentration of dsRNA may have been insufficient to deliver efficient RNAi effects or not all aphids may have ingested the feed which may have contributed to greater transcript abundance in the starting template of cDNA. The former reason may be supported by a study in which increasing the concentration (two microinjections of 50 nL of 5 µg/µL) of dsRNA for a central nervous specific gene, Nibeta2, in the brown planthopper, N. lugens, increased the efficiency of RNAi (Liu et al., 2010). Another preliminary study of artificial feeding of dsRNA corresponding to a larval gut carboxylesterase gene, EposCXE1, in the light brown apple moth, Epiphyas postvittana, indicated that different concentrations of dsRNA yielded different efficiencies in initiating RNAi (Turner et al., 2006). Similarly, injecting 500 ng, 400 ng, 50 ng, 5 ng, 500 pg, 50 pg, and 5 pg of dsGFP into a GFP-expressing transgenic red flour beetle line, pu11, resulted in a ‘perfect’ silencing effect with 50 pg and ‘slightly weaker’ with 5 pg suggesting the importance of optimising the dsRNA concentration for a target gene prior to further studies (Tomoyasu and Denell, 2006). Results from the current (preliminary) study warranted the use of testing higher concentration of dsRNA and comparing the effects of RNAi with and without dye. It was observed that feeding on increased dsRNA concentration (2 µg/µL) indeed affected the long-term survival resulting in mortality by day 5 and 6, and a pronounced reduction in gene expression as discussed in section 4.3.4. In retrospect of Figure 4.16 for the lines of best fit, it was clear that population trajectories decreased with higher concentrations (from 1 µg/µL to 1.5 µg/µL and then to 2 µg/µL) of the target gene. In other words, the increasingly lower mean trajectories as the concentration of dsMpVha-8 increased from 1 µg/µL to 2 µg/µL, although not statistically different, do seem to suggest that higher concentrations were more effective in eliminating aphid populations.
4.4.3 Silencing of MpVha-8

V-ATPases are evolutionarily conserved ATP-dependent proton pumps found in the plasma membrane and endomembranes of eukaryotic cells, and which play a role in important functions such as proton translocation, vesicular trafficking, acidification of cell organelles, pH homeostasis and membrane energisation in eukaryotes (Marshansky and Futai, 2008; Stevens and Forgac, 1997; Wieczorek et al., 2000). To test the optimised in vitro feeding system and to compare the efficacy of gene knockdown in aphids fed with dsRNA with and without dye, the V-ATPase gene that was studied for RNAi effects in other insect species was chosen. Silencing of V-ATPase subunits of several insects through RNAi affects growth and development, and important cellular processes and can result in mortality. For example, feeding dsRNA for V-ATPase in western corn rootworm, D. virgifera virgifera (subunits A, D and E) resulted in both larval stunting and mortality while in Colorado potato beetle, L. decemlineata (subunits A and E), D. melanogaster, T. castaneum, A. pism and M. sexta (subunit E), and in B. tabaci (subunit A) resulted in mortality (Baum et al., 2007; Upadhyay et al., 2011; Whyard et al., 2009). When three different sizes, 276, 249 and 498 bp of dsRNA targeting V-ATPase subunit E were fed to N. lugens at 0.05 µg/µL, no significant differences in mortality between nymphs fed with dsGFP and dsRNA of V-ATPase but a reduction in transcript levels of 55% for up to 10 days was observed (Li et al., 2011).

In aphids that had taken up dsMpVha-8 (dye visible), there was a significant reduction in the transcript abundance of the target gene and a decrease in survival of dsRNA-fed aphids on tobacco as compared to controls. It was shown that feeding dsMpVha-8 was effective in inducing RNAi in GPA and negatively affected their long-term survival compared to those fed on dsGFP and no-dsRNA controls. When some of the surviving dye-fed aphids were assessed for the effects of RNAi on target gene expression through semi-quantitative PCRs after 24 hour feeding, there appeared to be a greater gene knockdown in those aphids compared to the ones fed on dsRNA without dye. This study suggests that NR can be used as dye to trace efficient dsRNA uptake and that disruption of the MpVha-8 gene affected the development and reproduction of GPA. The results of this study also suggest that MpVha-8 may serve as a potential candidate target for host-mediated RNAi.
4.5 Conclusions and further work

To my knowledge, this is the first study that makes use of vital dyes to trace the uptake of dsRNA through *in vitro* RNAi in GPA. This study indicates that the use of vital dyes as internal markers in feeding-based RNAi has great potential in that it allows identification and assessment of only those individuals that have taken-up the feed. Both NR and AO are easy to use and inexpensive compared to the fluorescently labelled Cy-3 dye and provide an economic option in studies involving large-scale screening of dsRNA for other target genes. The lowest concentration of 0.02% for NR and 2 µg/µL dsRNA were then used to study the effects of RNAi using nine different target GPA sequences as described in Chapter 05.
CHAPTER 05

Effects of in vitro RNAi on *Myzus persicae*. 
5.0 Introduction

One of the factors that influence the efficacy of target gene silencing is the gene itself. A gene that is involved in vital life processes of GPA may serve as a potential target for RNAi-mediated crop protection. To identify such targets, often a range of genes or nucleotide sequences are screened using *in vitro* RNAi screen before transgenic plants expressing target aphid genes are generated. This strategy enables identification of the best target genes before going to the next step of generating transgenic plants.

*In vitro* RNAi through artificial feeding has been demonstrated successfully in insects belonging to several different orders including Hemiptera (Baum, *et al.*, 2007; Kumar *et al.*, 2009; Shakesby *et al.*, 2009; Whyard *et al.*, 2009). Some examples of hemipterans in which *in vitro* RNAi has been established using artificial feeding are *A. pisum*, *B. tabaci*, and *N. lugens* (Li *et al.*, 2011; Mao & Feng, 2012; Shakesby *et al.*, 2009; Upadhyay *et al.*, 2011; Whyard *et al.*, 2009), and even though there are reports of *in planta* RNAi in GPA (Bhatia *et al.*, 2012; Guo *et al.*, 2014; Mao & Zeng, 2013; Pitino *et al.*, 2011) there are no studies of *in vitro* RNAi in GPA.

Screening of genes using an artificial feeding system to study the effects of *in vitro* RNAi on the growth and development of insects thus provides an opportunity to select target genes before advancing them into plant-mediated RNAi. This approach has been adapted in a study by Baum *et al.* (2007) in which large-scale screening of 290 target sequences using *in vitro* feeding assay was demonstrated in the western corn rootworm (WCR), *D. virgifera virgifera* and target sequences that affected larval mortality and stunting were then tested *in planta.*

5.1 Aims and objectives of Chapter 05

The aims of this chapter were to test the effects of silencing target GPA nucleotide sequences using an *in vitro* feeding system and to assess their suitability for *in planta* RNAi. To do this, concentrations of dsRNA and conditions for using the vital dye, NR as a tracer for feeding that was optimised in Chapter 4 were used. Nine target sequences were screened and observations were made for the effects of *in vitro* RNAi on behavioural changes, immediate
and long-term survival, and transcript abundance of the target gene. Information obtained from this study is useful in assessing the suitability of these sequences in host-derived RNAi.

5.2 Materials and methods

5.2.1 In vitro feeding of dsRNA and post-feeding set-up

To investigate the effects of in vitro RNAi by artificial feeding of dsRNA for target GPA sequences, 2 µg/µL of dsRNA suspended in 30% sucrose with 0.02% NR was fed to nymphs using the system described in section 2.13.2. Two controls were also included: dsGFP suspended in 30% sucrose with 0.02% NR and no-dsRNA control i.e. only 30% sucrose with 0.02% NR. Each experiment had two replicates of feeding chambers containing 12 aphids each and is represented in Figure 4.1. After 24 hr feeding, presence of NR in the aphid body and any changes in behaviour were observed. The number of aphids active (alive) and inactive (dead), with and without dye were recorded and expressed as a percentage of the total number of aphids used. Some of the live aphids were collected for studying the transcript abundance through semi-quantitative PCRs. To assess long-term effects of feeding, five aphids fed on dsRNA showing the presence of NR were transferred on tobacco to assess their reproduction, and the numbers of aphids were recorded daily for 12 days. Proportions ± standard errors of the proportions were used to represent aphid survival after 24 hr for the various feed treatments.

5.2.2 Target GPA sequences

Ten GPA ESTs representing different functional classes were selected to study their effects on survival, growth and reproduction of GPA. These ESTs, their similarities to genes of other insects with known or predicted functions, and their orthologues in C. elegans with RNAi phenotypes are described in Table 3.2. Target regions of the ESTs selected for further studies are also described in Table 2.1. Of the ten sequences, the effects of in vitro RNAi for MpVha-8 have been presented in Chapter 4. In this Chapter, the remaining nine GPA EST sequences were investigated for their in vitro RNAi effects. These were: MpEat-6, MpCct-6, MpPod-2, MpPdi-2, MpLev-11, MpSox-2, MpTnc-2, MpCars-1, and MpAqp-4.
5.2.3 Amplification of target sequences and GFP

RNA was extracted from a mixed stage aphid population using TRIzol Reagent (Life Technologies Corporation, Australia) and cDNA was synthesised using the High Capacity Transcription kit (Applied Biosystems, Australia) as described in section 2.1. Gene specific primers as listed in Table 2.1 were used to amplify the selected regions of the GPA ESTs. PCRs were performed using MyTaq DNA Polymerase (Bioline, Australia) as per the manufacturer’s protocol in a Veriti 96-Well Thermal Cycler (Applied Biosystems) at 95°C for 3 min followed by 35 cycles of denaturation at 95°C for 30 sec, primer annealing at 60°C for 30 sec, extension at 72°C for 30 sec and a final extension at 72°C for 7 min. Amplified fragments were excised from 1.5% agarose gels, purified as per section 2.6 using Wizard® SV Gel and PCR Clean-Up System, Promega, Australia and cloned into pDoubler as described in section 2.7.

5.2.4 Cloning of nine GPA sequences and GFP and confirmation of transformants

Amplified GFP and GPA EST sequences were cloned at the XhoI/KpnI sites into the in vitro transcription vector, pDoubler developed by Dr. John Fosu-Nyarko as described in section 2.7. Recombinant pDoubler was transformed into competent E. coli JM109 and transformants were screened through antibiotic selection, PCR analyses and restriction digests also as described in section 2.11. Clones were sequenced using ABI 3730 96 capillary machine to confirm their identities as per section 2.12.

5.2.5 Synthesis of dsRNA for nine GPA sequences and GFP

DsRNA corresponding to MpEat-6, MpCct-6, MpPod-2, MpPdi-2, MpLev-11, MpSox-2, MpTnc-2, MpCars-1, and MpAqp-4 sequences were synthesised and used to study the effects of in vitro RNAi by artificial feeding. A GFP fragment as described in section 4.2.5 was used as an external gene control in the feeding experiments. For preparing templates to synthesise dsRNA, recombinant pDoubler was cleaved with Eco RI or Not I to yield the cloned fragment having opposing T7 polymerase promoter regions. Cleaned templates were used to synthesise dsRNA using Hi-Scribe T7 in vitro transcription kit (NEB) as per section 2.13.1.
5.2.6 Semi-quantitative PCRs

To study transcript abundance of target GPA sequences in aphids fed on dsRNA for 24 hr, semi-quantitative PCRs were done. Of the aphids that survived after feeding on dsRNA which showed the presence of dye, five aphids were transferred to tobacco seedlings and the remaining aphids from the two replicates were used for RNA extraction. RNA was extracted from variable numbers of live aphids using Arcturus PicoPure RNA Isolation Kit (Life Technologies Corporation, Australia) followed by an on-column RNase-free DNaseI treatment according to the manufacturer’s protocol (Qiagen, Australia). cDNA was synthesised from 100 ng of RNA and 1:10 dilution of cDNA was used for semi-qPCRs. *MpActin* was amplified using the gene specific primer pairs described in section 4.2.8 and used to normalise target transcript levels. All semi-quantitative PCRs were carried out in triplicates: PCR conditions were as described in section 2.4.2 and for each biological replicate, transcript abundance of target transcript levels was assessed after 30 and 35 cycles and fragment sizes were compared with 100 kb DNA marker.

5.3 Results

5.3.1 Cloning of GPA target sequences

Nine target GPA sequences were amplified from cDNA of GPA and cloned into the *in vitro* transcription vector, pDoubler. The identity of the transformants was confirmed by PCRs first on bacterial colonies and then on plasmid DNA using gene specific primers, which resulted in the expected band sizes (Figure 5.1A and 5.1B). Double digests with *XhoI/KpnI* confirmed the presence of the insert in pDoubler (Figure 5.1C). The identities of the cloned fragments were further confirmed by sequencing and then used to prepare templates for dsRNA.
Figure 5.1. Agarose gel electrophoreses results of colony PCRs, confirmatory PCRs on plasmid DNA, and double digest of pDoubler clones. (5.1A) Colony PCRs of transformed *E. coli* colonies showing the presence of the cloned fragments of the expected sizes. (5.1B) Confirmatory PCR on recombinant pDoubler DNA showing the cloned fragments. (5.1C) Double digest of pDoubler showing the presence of the cloned fragments. Lane 1 contains 100 bp DNA ladder in figures 5.1A, 5.1B and 5.1C.
5.3.2 Effects of in vitro RNAi on GPA

After 24 hr, ingestion of feed containing dsRNA for nine target dsRNA was ascertained by the presence of NR in the aphid body. The numbers of GPAs alive and dead as a result of feeding were recorded, observations on behavioural changes were made, and only fed-aphids were assessed to study the effects of feeding dsRNA on target transcript levels and long-term survival.

5.3.2.1 Immediate effects on behaviour and survival

The proportions of GPA nymphs that ingested dsRNA (presence of NR within the aphid body) and survived 24 hr was recorded for all nine dsRNA, and these, along with standard errors of the proportions, are shown in Figure 5.2. For comparison, similar survival rates are presented for aphids that ingested dsGFP, 30% sucrose with 0.02% NR, and none of the feed (as evidence by the dye being absent from the aphid body). Survival of nymphs fed dsMpLev-11 and dsMpPdi-2 was significantly lower than nymphs fed 30% sucrose with 0.02% NR (Fisher’s exact test Bonferroni-corrected p-values of 0.014 and 0.011, respectively) but not nymphs fed dsGFP. Survival rates were not significantly different across the nine dsRNA. In terms of observed survival, survival of aphids fed on dsMpAqp-4 was the highest (75.0%), while the lowest percentages of survival were found to be in aphids fed on dsMpPdi-2 (47.8%), dsMpLev-11 (50.0%), dsMpSox-2 (58.3%) and dsMpEat-6 (58.3%).
There were apparent differences in behaviour observed in aphids fed on all dsRNA except the ones that fed on dsMpCars-1 when compared to dsGFP and no-dsRNA control (Table 5.1). There were also obvious effects on normal body movement of aphids fed on dsMpCct-6, dsMpLev-11, dsMpSox-2, and dsMpTnc-2 and this could have been because those genes are involved in locomotion (Table 5.1). The most noticeable difference was that some live aphids that fed on dsMpEat-6 and dsMpCct-6 were found lying on their dorsal side with movement of limbs affected (Figure 5.3).

Figure 5.2: Proportions of GPA nymphs ± standard errors of the proportion that survived after 24 hr feeding on nine dsRNA, dsGFP, and 30% sucrose with 0.02% NR.
Table 5.1. Behavioural differences in GPA fed on nine target dsRNAs and dsGFP.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Behavioural differences observed in dsRNA-fed GPA after 24 hr feeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>MpEat-6</td>
<td>Found to be on dorsal side with slow movement in appendages.</td>
</tr>
<tr>
<td>MpCct-6</td>
<td>Found to be on dorsal side with limited movement of limbs.</td>
</tr>
<tr>
<td>MpPod-2</td>
<td>Normal movement but some were sluggish.</td>
</tr>
<tr>
<td>MpPdi-2</td>
<td>Limited movement but some were sluggish.</td>
</tr>
<tr>
<td>MpLev-11</td>
<td>Paralysed with stiff limbs having some local movement.</td>
</tr>
<tr>
<td>MpSox-2</td>
<td>Extremely paralysed, stiff limbs with local movement in only one pair of limbs.</td>
</tr>
<tr>
<td>MpTnc-2</td>
<td>Paralysed, stiff limbs with local movement in one or two limbs.</td>
</tr>
<tr>
<td>MpCars-1</td>
<td>Normal movement.</td>
</tr>
<tr>
<td>MpAqp-4</td>
<td>Body appeared turgid and appeared slow moving.</td>
</tr>
<tr>
<td>GFP</td>
<td>Normal movement.</td>
</tr>
<tr>
<td>30% sucrose + 0.02% NR</td>
<td>Normal movement.</td>
</tr>
</tbody>
</table>

Figure 5.3. GPA nymphs after 24 hr in vitro feeding. Images of dsRNA-fed nymphs found on their dorsal surface at 4X (5.3A) and 20X (5.3B) magnification after feeding on dsMpEat-6, and nymphs fed on no-dsRNA control at 10X (5.3C) and 40X (5.3D).
5.3.2.2 Effects on transcript levels of target genes

Changes in transcript levels of all nine target genes were studied by semi-quantitative PCRs and the house-keeping MpActin gene was used as an internal control. RNA was extracted from nine, eleven, ten, six, seven, nine, ten, thirteen, fourteen and sixteen nymphs fed on dsMpEat-6, dsMpCct-2, dsMpPod-2, dsMpPdi-2, dsMpLev-11, dsMpSox-2, dsMpTnc-2, dsMpCars-1, dsMpAqp-4, dsGFP and no-dsRNA control respectively. At both, 30 and 35 PCR cycles, the transcript abundance of actin in aphids fed on all nine dsRNA was similar and remained unaffected when compared to no-dsRNA control containing only sucrose and NR (Figure 5.4). Transcript abundance of all nine target genes was unaffected in aphids fed on dsGFP when compared to no-dsRNA control. However, there was an apparent decrease in transcript abundance for all nine target genes tested and was evident in PCR cycle 30. Amongst all target genes, transcript abundance of MpCct-6, MpPod-2, MpLev-11 and MpTnc-2 was noticeably reduced (Figure 5.4).
Figure 5.4. Inversed images of agarose gel electrophoreses results of semi-quantitative PCRs. (5.4A) Expression of *MpActin* and (5.4B) target GPA sequences.
5.3.2.3 Long-term effects on survival of GPA

Five live aphids showing the presence of dye after feeding on one of nine target dsRNA or dsGFP for 24 hr were transferred to tobacco plants (50 aphids in total), and long-term effects on growth and reproduction were recorded for 12 days. Again, a survival analysis for a period of eight days and a linear model for population trends for the dsRNA and dsGFP treatment groups was considered.

Figure 5.5 shows Kaplan-Meier survival curves for each of the nine dsRNA treatment groups and the dsGFP treatment group. There was significant variability in survival times among the dsRNA treatment groups, but all aphids in the dsGFP treatment group survived the entirety of the treatment. Population trends with 95% confidence bounds by treatment group presented in Figure 5.6 show the dsGFP group with a significantly higher trajectory than the nine dsRNA feed groups. Results from the linear model used to produce these trends are shown in Table 5.2, and the highly significant and negative coefficients for the time-treatment interactions corresponding to the dsRNA feed groups reflected their significantly lower population trajectories relative to the dsGFP feed group. The trends for the dsRNA feed groups were largely flat or decreasing, the lone exception being for dsMpPdi-2 (yellow). The lowest trajectories, which were largely indistinguishable in the graph due to significant overlap in 95% confidence bounds were for dsMpEat-6, dsMpLev-11, dsMpPod-2, dsMpSox-2, and dsMpTnc-2.
Figure 5.5. Kaplan-Meier survival curves for aphids fed each of nine different dsRNA feeds and a dsGFP feed. Survival curves are based on sample sizes of $n = 5$ per feed treatment.
Figure 5.6: Normalised proportions of aphids alive by dsRNA or dsGFP treatment group over a period of 12 days. Lines of best fit (along with 95% confidence bounds) for each group as determined by a linear regression incorporating time, a quadratic for time, treatment group, and a time-treatment interaction are overlaid.
Table 5.2: Summary of model fit for a linear regression of number of aphids alive on time, a quadratic for time, dsRNA treatment group, and a time-treatment interaction. Number of aphids live were normalised by the number of aphids alive at the beginning of the study (n = 5 for each treatment group).

|                  | Coef. | Std. Error | t-value | Pr(>|t|)  |
|------------------|-------|------------|---------|----------|
| (Intercept)      | 0.908 | 0.132      | 6.89    | 5.10e-10*** |
| Time             | -0.038| 0.051      | -0.74   | 0.462    |
| dsMpAqp-4        | 0.018 | 0.186      | 0.09    | 0.925    |
| dsMpCars-1       | -0.057| 0.186      | -0.31   | 0.760    |
| dsMpCct-6        | -0.132| 0.186      | -0.71   | 0.481    |
| dsMpEat-6        | -0.284| 0.186      | -1.52   | 0.131    |
| dsMpLev-11       | -0.391| 0.186      | -2.10   | 0.038*   |
| dsMpPdi-2        | 0.141 | 0.186      | 0.75    | 0.452    |
| dsMpPod-2        | 0.229 | 0.186      | 1.23    | 0.223    |
| dsMpSox-2        | -0.391| 0.186      | -2.10   | 0.038*   |
| dsMpTnc-2        | -0.154| 0.186      | -0.83   | 0.411    |
| Time^2           | 0.042 | 0.004      | 10.26   | <2.00e-16*** |
| Time:dsMpAqp-4   | -0.209| 0.072      | -2.90   | 0.005**  |
| Time:dsMpCars-1  | 0.039 | 0.072      | 0.54    | 0.591    |
| Time:dsMpCct-6   | -0.016| 0.072      | -0.22   | 0.824    |
| Time:dsMpEat-6   | -0.092| 0.072      | -1.28   | 0.204    |
| Time:dsMpLev-11  | -0.127| 0.072      | -1.76   | 0.081    |
| Time:dsMpPdi-2   | -0.150| 0.072      | -2.08   | 0.040*   |
| Time:dsMpPod-2   | -0.240| 0.072      | -3.32   | 0.001**  |
| Time:dsMpSox-2   | -0.127| 0.072      | -1.76   | 0.081    |
| Time:dsMpTnc-2   | -0.178| 0.072      | -2.47   | 0.015*   |
| Time^2:dsMpAqp-4 | -0.020| 0.006      | -3.38   | 0.001**  |
| Time^2:dsMpCars-1| -0.041| 0.006      | -7.08   | 2.00e-10*** |
| Time^2:dsMpCct-6 | -0.037| 0.006      | -6.32   | 7.20e-9*** |
| Time^2:dsMpEat-6 | -0.035| 0.006      | -6.08   | 2.20e-8*** |
| Time^2:dsMpLev-11| -0.031| 0.006      | -5.36   | 5.40e-7*** |
| Time^2:dsMpPdi-2 | -0.015| 0.006      | -2.59   | 0.011*   |
| Time^2:dsMpPod-2 | -0.026| 0.006      | -4.53   | 1.60e-5*** |
| Time^2:dsMpSox-2 | -0.031| 0.006      | -5.36   | 5.40e-7*** |
| Time^2:dsMpTnc-2 | -0.029| 0.006      | -4.91   | 3.50e-6*** |

Signif. codes: ‘***’ 0.001, ‘**’ 0.01, ‘*’ 0.05.

Residual standard error: 0.183 on 100 degrees of freedom.

Multiple R-squared: 0.976, Adjusted R-squared: 0.969

F-statistic: 139 on 29 and 100 df, p-value: <2e-16
5.4 Discussion

In this study, successful silencing of nine target GPA sequences has been demonstrated through oral delivery of dsRNA. Visualisation of NR within the aphid body provided convenient means to identify individual nymphs that had ingested the feed and allowed assessment of the effects of in vitro RNAi. Silencing of all target GPA sequences affected target transcript levels, immediate and long-term survival and also induced apparent behavioural changes for target genes that affected feeding and locomotion.

The most conspicuous behavioural changes were observed in aphids fed on dsRNA for genes involved in movement and locomotion. Paralysis and extremely limited movement was evident in aphids fed on dsMpLev-11, dsMpSox-2 and dsMpTnc-2, and this was also characterised by a reduction in target transcript abundance (Figure 5.4B). In C. elegans, Lev-11 encodes tropomyosin, an actin-binding contractile structure and Tnc-2 encodes the calcium-binding component of the troponin complex of actin filaments. Both Lev-11 and Tnc-2 play an important role in normal body morphology, embryonic and post-embryonic development, pharyngeal muscle contraction, and functioning of actin filaments that are critical in locomotion (Amin et al., 2007; Culetto & Sattelle, 2000; Frand et al., 2005; Jones et al., 2005; Kamath et al., 2003; Mariol et al., 2007; Ono, 2014; Ono and Ono, 2004; Ruksana et al., 2005; Terami et al., 1999). RNAi effects of silencing the tropomyosin and troponin-C encoding genes, Lev-11 and Tnc-2 respectively in C. elegans included disrupted muscle development and impaired normal movement of the cytoskeleton. Interestingly, silencing orthologous sequences for those genes also negatively affected locomotion in GPA. This suggests that the function of those genes may be conserved. However, to further support this, protein analyses, for example Western blots, to study changes in protein expression upon RNAi silencing may be required. Some of the earliest events of mortality post-feeding were also recorded in aphids fed with dsMpLev-11, dsMpSox-2 and dsMpTnc-2 suggesting that silencing these genes also affected long-term survival.

Another gene which showed an obvious effect on long-term survival after feeding on its corresponding dsRNA was MpEat-6 (EATing defective, abnormal pharyngeal pumping). Even though it resulted in only a slight reduction of transcript abundance when compared to controls after 24 hr, feeding on dsMpEat-6 slowed the limbic movement of GPA and dsRNA-
fed aphids did not survive beyond day eight. In *C. elegans*, *Eat*-6 encodes a sodium-potassium alpha subunit of the sodium pump localised in the plasma membrane and affects relaxation of pharyngeal muscles, fertility, lifespan and body length (Doi and Iwasaki, 2008; Hamilton *et al.*, 2005; Shima *et al.*, 1998). Perhaps, silencing *MpEat*-6 may have affected feeding behaviour that may have inhibited aphids from feeding on wild type tobacco seedlings and led to reduced survival.

An interesting finding was that even though aphids fed on *dsMpPdi*-2 showed the lowest percentage of survival after 24 hr amongst all dsRNA tested, long-term survival was reduced only briefly and after day eight there was an increase in aphid numbers as a result of reproduction. This suggests that aphids may have been able to recover from the effects of feeding *dsMpPdi*-2 and this gene may not have affected reproductive ability of GPA. In *C. elegans*, *Pdi*-2 (protein disulfide isomerase) encodes a protein disulfide isomerase beta subunit with a predicted function in oxidative protein folding in the endoplasmic reticulum. It is required for normal body morphology, locomotion, fertility, and moulting. One of the RNAi phenotypes, sluggishness, has been reported in *C. elegans* and was also observed in some aphids fed on *dsMpPdi*-2 after 24 hr feeding (Kamath *et al.*, 2003; Simmer *et al.*, 2003).

In aphids fed on *dsMpAqp*-4, even though there were some phenotypic differences when compared to control aphids and an apparent reduction in target transcript abundance, survival and reproduction was affected only transiently: aphids began to reproduce after day 8. These results were similar to those observed by Shakesby *et al.*, (2009) in pea aphid. In that study, *dsApAQP1* was delivered orally through an artificial diet to study the effects of *in vitro* RNAi. This resulted in a two-fold reduction of *ApAQP1* expression in 24 hr which was transient and by day 7 there was no suppression of gene expression (Shakesby *et al.*, 2009). At the same time there were also no significant differences observed in mortality or weight gain in aphids fed on *dsApAQP1* and *dsGFP*. An explanation for some of the similarities in the observations recorded in the two studies may perhaps be due to high sequence homologies between *MpAqp*-4 (EE570677) and *ApAQP1* (NM001145905). When the two sequences were compared using TBLASTX, there was a 94% similarity in identities with a query coverage of 98% and an e-value of zero (Table 3.2). One possible explanation for the transient effect may be the involvement of compensatory gene(s) that may have helped overcome the effect
of RNAi since aquaporins belong to a family of major intrinsic proteins (Engel and Stahlberg, 2002). In *C. elegans*, *Aqp*-4 is required for water homeostasis and no apparent RNAi effects has been recorded and is predicted to work in conjunction with *Aqp*-2, *Aqp*-3 and *Aqp*-8 to recover from hypotonic stress.

Investigating differences in the rate of reproduction in aphids fed on ds*MpCct*-6, ds*MpPdi*-2, ds*MpCars*-1 and ds*MpAqp*-4 compared to controls for a period longer than 12 days would have given a better idea of long-term effects of RNAi. This would have helped to determine if there were significant improvements over time in the rate of reproduction indicating recovery from the effects of RNAi. However, it was not possible to maintain tobacco plants in plastic cups longer than 12 days since the lids would be pushed open by the growing leaves.

### 5.5 Conclusions and further work

This study has demonstrated the potential of the selected GPA sequences as suitable targets for *in vitro* RNAi. Differences in behaviour, target transcript levels and survival were observed as a result of RNAi silencing. Some genes representing a functional class, for example, the locomotion genes, *MpLev*-11, *MpSox*-2 and *MpTnc*-2 when silenced not only disrupted function but also affected long-term survival. Based on the results of the current study, it would be interesting to examine the effects of host-derived dsRNA for all 10 GPA genes including *MpVha*-8 and especially *MpLev*-11, *MpSox*-2, *MpTnc*-2, *MpEat*-6, *MpPod*-2, *MpAqp*-4 and *MpPdi*-2 on the survival and reproduction of GPA.
CHAPTER 06

Effects of in planta RNAi on Myzus persicae.
6.0 Introduction

RNAi technology is an important tool to study the functions of genes, and it has been deployed widely in insect functional genomics: its potential as a strategy of crop protection has also been demonstrated successfully. Crop-protection through host-delivered RNAi is achieved by genetically modifying the plant genome to express dsRNA homologous to a target insect gene so that dsRNA molecules when ingested by a pest trigger RNAi and subsequently result in silencing of the target gene. Typically, RNAi constructs consist of cloned target fragment in sense and antisense orientation separated by an intron region, and the whole expression cassette is under the function of a constitutive promoter. After transcription, the resulting intron-spliced hairpin RNA structure with sense and antisense regions complementing each other forms the dsRNA molecule that triggers the RNAi pathway, beginning with the cleavage of dsRNA molecules by the dicer enzymes produced by the plant (Helliwell and Waterhouse, 2003).

Recent studies have shown that transgenic plants engineered to express hairpin dsRNA against target genes resulted in varying degrees of host resistance to herbivorous pests belonging to different insect orders (Baum et al., 2007; Mao et al., 2007, 2011). The feasibility of host-mediated RNAi was first demonstrated in the Coleopteran insect, western corn rootworm (WCR), *D. virgifera virgifera* and the Lepidopteran insect, cotton bollworm larvae, *H. armigera* using *Arabidopsis*, corn and tobacco expressing dsRNA for selected target genes (Baum et al., 2007; Mao et al., 2007). These studies provided proof-of-concept for delivering dsRNA through transgenic crops and subsequently, host-derived RNAi was demonstrated in insects of the order Hemiptera. For example, transgenic rice expressing dsRNA against target genes of the brown planthopper, *N. lugens*, and transgenic *Arabidopsis* and tobacco expressing dsRNA against GPA genes has been demonstrated successfully (Pitino et al., 2011; Zha et al., 2011). So far, four studies on GPA have identified and tested potential target genes through in planta RNAi (Pitino et al., 2011; Bhatia et al., 2012; Mao & Zeng, 2013; Guo et al., 2014).

*In planta* delivery of dsRNA to GPA through transgenic plants offers a ‘natural’ mode of feeding and this approach may also be ideal in protecting crops against aphid pests. For
developing an insecticidal RNAi-based approach it is imperative to demonstrate the efficacy of potential target genes through in planta RNAi studies.

6.1 Aims and objectives of Chapter 06

The broad aims of this chapter were to study the effects of silencing target GPA genes through host-mediated RNAi. Results of in vitro RNAi studies were used as a preliminary screen to determine the efficiency of target genes before advancing them into in planta RNAi studies. Subsequently, ten GPA genes previously tested (Chapters 4 and 5) through in vitro RNAi were transformed into tobacco and transgenic plants expressing dsRNA were developed. Challenge assays were carried out to study the effects of in planta RNAi on phenotype, survival and reproduction of aphids. This study would therefore provide valuable new information on the efficiency of GPA genes as possible targets aphid control.

6.2 Materials and methods

The GPA population was reared on tobacco as described in section 2.0.

6.2.1 Cloning of ten GPA sequences and GFP and confirmation of transformants

Both GFP and GPA EST sequences were cloned at the NotI sites into the binary vector, pART27-LacZ as described in section 2.7. Recombinant pART27-LacZ was transformed into competent A. tumefaciens GV3101 and transformants were screened through antibiotic selection, PCRs analyses and restriction digests also described in section 2.11. Clones were sequenced using ABI 3730 96 capillary machine to confirm their identities as per section 2.12.

6.2.2 Development of transgenic tobacco

Tobacco leaf disc transformation was used to develop primary transformants (T0). T0 events were selected, established and allowed to self-fertilise after which seeds of these plants were selected by screening T1 seedlings using appropriate antibiotics. Steps involved in this process are described below.
6.2.2.1 ‘Leaf disc’ transformation

Tobacco (*N. tabacum* cv. Wisconsin-98) plants were transformed using a leaf disc method adapted with slight modifications from two protocols; one was developed by Dr. John Fosu-Nyarko (Nemgenix Pty. Ltd.) and the other protocol by Clemente (2006). Leaf discs were excised from 3-4 week-old tobacco plants grown *in vitro* on Murashige Skoog (MS) Basal Medium with Gamborg Vitamins (Phytotechlab, USA) medium solidified with agar, after sterilisation as per section 6.2.2.2. For every construct, 50 explants of approximately 5 mm X 5 mm were prepared using a sterile blade and soaked for 2 to 5 min in co-cultivation liquid media (CCLM) comprising of 4.4 gm/L MS Basal Medium with Gamborg Vitamins, 1 mg/L BAP, 0.1 mg/L IAA, 200 uM acetosyringone, 30 gm/L sucrose, adjusted to pH 5.6. Acetosyringone and growth hormones BAP and IAA were filter sterilised and added after autoclaving the medium to prevent their degradation at high temperatures. Up to 25 explants per petridish were placed on co-cultivation solid medium (CCSM: as CCLM supplemented with 8 gm/L agar) with adaxial side up, and incubated at 25°C under a 18:6 light:dark cycle for 3 days.

Cultures of *A. tumefaciens* GV3101 with the recombinant binary vector pART27-LacZ was prepared by inoculating 25 mL LB media containing 100 mg/L spectinomycin and 25 mg/L rifampicin, and allowed to grow overnight at 28°C on a shaker set at 225 rpm. Cell cultures were centrifuged at 4,500 g for 20 min at room temperature and the pellet was resuspended to a final OD₆₀₀ of 0.5 to 1.0 in CCLM. Inoculum was placed on ice and 0.001 % w/v Silwet L-77 was added just before use. Plant transformation involved transferring explants to the *Agrobacterium* inoculum and soaking for 30 min with intermittent swirling. Excess inoculum was blotted on sterile Whatman filter paper and explants were placed on CCSM with adaxial side up. Explants were co-cultivated at 25°C under a 18:6 light:dark cycle for 3 days.

Regeneration and selection of explants was done by transferring them to shoot regeneration medium (4.4 gm/L MS Basal Medium with Gamborg Vitamins, 8 gm agar, 30 gm sucrose, 1 mg/L BAP, 0.1 mg/L IAA) supplemented with 200 mg/L timentin to inhibit the growth of *Agrobacterium* and 150 mg/L kanamycin for selection. Petridishes were incubated at 25°C under a 18:6 light:dark cycle for 2 weeks and explants were transferred to fresh regeneration medium every 2 weeks. After 2 weeks, explants begin to callus and multiple shoots arise from a single callus cluster (Figure 6.1).
Figure 6.1. Tobacco leaf disc transformation, set-up for challenge experiments and growth of transgenic tobacco. (6.1A and 6.1B) Callusing leaf discs on petridishes after inoculation and co-cultivation (weeks 1 and 2). (6.1C and 6.1D) Shoot regeneration from calli (weeks 3 and 4). (6.1E and 6.1F) Rooting of selected shoots in Magenta tubes containing rooting medium (weeks 5 and 6) under controlled conditions. (6.1G) Establishment of transgenic seedling in a PC2 glasshouse with an inverted 620 mL clear plastic cup with mesh-covered window at the top for aphid challenge experiments. (6.1H) Growth of tobacco transgenic events. (6.1I) Brown paper bagging of tobacco inflorescences.

Three mm-long individual shoots were excised and transferred to 25 mL solidified rooting medium (4.4 gm/L MS Basal Medium with Gamborg Vitamins, 8 gm agar, 30 gm sucrose, 0.1 mg/L IAA) supplemented with 200 mg/L timentin and 70 mg/L kanamycin in Magenta boxes at 25°C under a 18:6 light:dark cycle. Once roots were established in 2 to 3 weeks, plants were transferred to pots containing pasteurised Murdoch soil mixture supplemented with
controlled release fertilisers and maintained in a temperature regulated Physical Containment Level 2 (PC2) glasshouse (23 ± 5°C).

6.2.2.2 Tobacco seed sterilisation, selection and germination
For explant preparation, wild type seeds were first sterilised and grown on MS Basal Medium with Gamborg Vitamins (Phytotechlab, USA) without any antibiotics. For seed sterilisation, approximately 50 µL packed volume of tobacco seeds was sterilised in 2 mL centrifuge tubes. Seeds were soaked in 1 mL of 100 % ethanol for 1 min, centrifuged for 15 sec at 16,000 g and ethanol was decanted. Seeds were washed with 3 % sodium hypochlorite with Tween-20 (1 drop per 50 mL), vortexed briefly and allowed to stand for 15 min. Tubes were centrifuged for 30 sec at 16,000 g, supernatant was decanted and seeds were washed with sterile water five times before being plated.

For T1 seed sterilisation, the above method was used however, it was time-consuming and laborious especially when several events were to be screened at the same time. Hence, a vapour-phase sterilisation technique was adapted. This was done by first placing 2 mL centrifuge tubes (lid open) containing 50 µL packed volume of tobacco seeds on a tube holder that was kept in a bell jar. One hundred mL of sodium hypochlorite followed by 3 mL of hydrochloric acid was carefully dispensed into a glass beaker placed next to the tube holder. The bell jar was immediately covered and chlorine gas fumes were allowed to accumulate inside the jar. The entire set-up was kept in a fume-hood for 16 hr. The lid was left cracked open for a few minutes before the tubes were transferred to the laminar airflow bench and the tubes were left for 30 min to allow any remaining chlorine gas to escape. After both sterilisation procedures seeds were transferred into 15 mL Falcon tubes containing 8 to 10 mL of 0.4 % water agar supplemented with 200 mg/L timentin and 150 mg/L kanamycin. Overlay agar was mixed thoroughly and poured in petridishes containing MS Basal Medium with Gamborg Vitamins (Phytotechlab, USA) with the same antibiotics.

For selection of transgenic T1 plants, seeds obtained from T0 events were screened for 2 to 3 weeks on media plates supplemented with antibiotics. Seeds were allowed to germinate at 25°C under a 18:6 light:dark cycle. In vitro selection of T1 seeds was sometimes accompanied
by persistent contamination of fungal colonies on media plate and for some events, vapour-phase sterilised seeds failed to grow. To overcome the delay in the screening process, in solium selection of transgenic T1 seedlings was also done. A method described by Xiang et al. (1999) for selection of transgenic Arabidopsis growing in soil, that made use of increasing concentrations of kanamycin over time, was adapted with slight modifications. To do this, seeds were grown in pasteurised Murdoch soil mix in plastic weigh boats and sprayed with sterile distilled water regularly. At the first true-leaf stage, seedlings were sprayed with 100 mg/L kanamycin for the first two days, followed by 300 mg/L for the third and the fourth days, subsequently a concentration of 300 mg/L was used on the fifth day and finally 500 mg/L on sixth and seventh days. Solutions were supplemented with 0.1% Triton-X and control plants were sprayed with solution without kanamycin. Plants that survived the kanamycin spray were considered ‘selected’ and were subjected to PCR analyses.

6.2.2.3 Analysis of transgenic tobacco plants

For all 12 constructs, between three and 10 T1 tobacco plants from each event were used for molecular analyses to confirm that they were transgenic. Genomic DNA from pooled T1 leaf tissue samples was extracted as described in section 2.2 and PCRs were carried out as described in section 2.4.1 to amplify a 350 bp-long fragment of the npt II gene to confirm the presence of the hairpin cassette. To confirm the expression of transgenes, total RNA from randomly selected T1 plants of any one particular event for all 10 hairpin constructs expressing GPA genes was extracted, cDNA was synthesised as described in sections 2.1 and PCRs with gene specific primers were carried out as described in 2.4.1.

6.2.2.4 Aphid challenge experiments

To study the effects of in vitro RNAi on aphid fecundity, challenge experiments were carried out. Two-leaf stage transgenic T0 events and T1 plants were challenged with 2nd instar GPA nymphs established from a single parthenogenetic adult. Nymphs were collected from wild type tobacco plants and one nymph per plant was carefully placed on each test plant using a fine paint brush. An inverted 650 mL transparent plastic cup with a small window of 2 cm X 2 cm sealed with stretched parafilm was placed on top of the plant, pushed into the soil, and
aphid survival was recorded at regular intervals (Figure 1G). For T0 plants, data were recorded every day for a period of 13 days while for T1 plants data were recorded on days four, eight, 12 and 16. At the end of the experiment, a foliar application of the insecticide, Bayer Confidor Garden Insecticide (Yates, Australia) was applied at a recommended concentration to all plants for controlling aphid populations.

6.2.2.5 Maintenance of transgenic tobacco, seed harvest and storage

Plants were watered regularly and fertilised with an all-purpose soluble fertiliser, Thrive (N:P:K ratio of 25:5:8.8; Yates, Australia) when required. Self-fertilised T0 and T1 plants were allowed to produce seeds. Brown paper bags were placed over the inflorescence, pods were allowed to dry completely on the plant and fully mature (brown) pods were harvested (Figure 1I). Seeds were threshed, sifted through 1.18 mm followed by 850 micron metal sieves and stored in 5 mL yellow capped bottles at room temperature until further use.

6.3 Results

6.3.1 Cloning of GPA target sequences

Hairpin cassettes of both GFP and ten target GPA genes were excised from the plasmid vector, pCleaver-NosT by NotI digestion, cloned into the binary vector, pART27-LacZ at the NotI sites, and transformed into competent cells of A. tumefaciens GV3101. The identity of the transformants was confirmed by PCRs on bacterial colony and plasmid DNA using gene specific primers, and this resulted in presence of the expected band sizes. Double digests with XhoI/XbaI and single digest with NotI also resulted in the expected sizes of hairpin cassettes confirming their presence in the recombinant pART27-LacZ (Figure 6.2A and 6.2B). A total of 625 bp in addition to the expected sizes of cassette was obtained. The identities of the cloned fragments were further confirmed by sequencing and BLAST searches, resulted in very high sequence similarities with the original target GPA EST sequence.
Figure 6.2. Agarose gel electrophoresis results of restriction digests on recombinant pART27-LacZ containing the hairpin expression cassette. (6.2A) Double digest with XhoI/XbaI and (6.2B) Single digest with NotI showing the expected sizes of the hairpin cassettes. The higher molecular weight DNA band is the pART27-LacZ backbone.

6.3.2 Analyses of transgenic tobacco for integration of T-DNA

For all hairpin constructs, a very high transformation efficiency of between 80-90% was observed in the primary transformants that were selected, regenerated and rooted on media containing kanamycin. For the T0 events of the 12 constructs transformed into tobacco, PCR analyses of 252 T0 plants would have been time-consuming and laborious. Hence, as a preliminary proof of their transgenic nature, all the events that grew and developed a well-established root system in the presence of kanamycin were assumed to be transgenic based on their antibiotic resistance (Table 1). All of those T0 events were transplanted, advanced to aphid challenge assays and allowed to produce T1 seeds thereafter. Using MpVha-8 as an example, Figure 6.3 gives an overview of the steps involved in the development of T1 transgenic tobacco plants.
Table 1. Number of T0 events selected based on kanamycin resistance for the 12 hairpin constructs transformed into tobacco. The asterisk denotes the number of events with established root systems out of 30 shoots that were transferred to rooting medium supplemented with kanamycin.

<table>
<thead>
<tr>
<th>Hairpin construct containing Mp gene</th>
<th>No. of explants</th>
<th>Average no. of explants resistant to kanamycin</th>
<th>Average no. of shoots/explant</th>
<th>No. of events with an established root system*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eat-6</td>
<td>50</td>
<td>40</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td>Cct-6</td>
<td>50</td>
<td>45</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>Pod-2</td>
<td>50</td>
<td>42</td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td>Pdi-2</td>
<td>50</td>
<td>45</td>
<td>16</td>
<td>21</td>
</tr>
<tr>
<td>Lev-11</td>
<td>50</td>
<td>46</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>Sox-2</td>
<td>50</td>
<td>43</td>
<td>14</td>
<td>23</td>
</tr>
<tr>
<td>Tnc-2</td>
<td>50</td>
<td>47</td>
<td>16</td>
<td>28</td>
</tr>
<tr>
<td>Cars-1</td>
<td>50</td>
<td>44</td>
<td>15</td>
<td>21</td>
</tr>
<tr>
<td>Vha-8</td>
<td>50</td>
<td>43</td>
<td>13</td>
<td>17</td>
</tr>
<tr>
<td>Aqp-4</td>
<td>50</td>
<td>48</td>
<td>14</td>
<td>28</td>
</tr>
<tr>
<td>GFP</td>
<td>50</td>
<td>45</td>
<td>15</td>
<td>23</td>
</tr>
<tr>
<td>Null</td>
<td>50</td>
<td>46</td>
<td>13</td>
<td>26</td>
</tr>
</tbody>
</table>
Figure 6.3. An overview of the process involved in the development of T1 transgenic tobacco expressing the hairpin cassette MpVha-8. All plants (replicates) representing an event were pooled together for each of the five events to analyse genomic DNA whereas for cDNA analyses, randomly selected plants (replicates) for fewer events were used.

Subsequently, germinating T1 seeds resulting from T0 events were selected based on kanamycin resistance and PCR analyses to confirm the integration of T-DNA. For all 10 target genes transformed into tobacco, PCR analyses on genomic DNA from leaf tissue of pooled T1 plants representing each event confirmed the presence of the expected 356 bp-long fragment of the npt II gene (Figure 6.4A and 6.4B). Further confirmation of stable integration and inheritance of T-DNA was obtained by amplification of gene specific regions from cDNA of randomly selected T1 plants representing a specific event for each hairpin construct, except for GFP and empty vector (Figure 6.4C). Amplification of npt II in the genomic DNA and gene specific regions in the cDNA of T1 plants confirmed the transgenic nature of the
primary transformants (T0 events) that were advanced into the next stage of producing T1 progeny.

Figure 6.4. Molecular analyses of transgenic T1 tobacco. (6.4A and 6.4B) Inverse images of agarose gel electrophoreses showing amplification of an npt II fragment in the genomic DNA of pooled T1 plants representing four different events of each hairpin construct. For each hairpin construct-expressing T1 tobacco the events shown in A and B were: MpEat-6 E2, E7, E8 and E12; MpCct-6 E3, E4, E6 and E10; MpPod-2 E2, E6, E7 and E8; MpPdi-2 E4, E6, E7 and E9; MpLev-11 E9, E10, E11 and E12; MpSox-2 E1, E2, E4 and E8; MpTnc-2 E1, E4, E8 and E7; MpCars-1 E7, E8, E14 and E15; MpVha-8 E10, E11, E14 and E15; GFP E1, E2, E4 and E16; Null or empty vector E1, E2, E5 and E26. (6.4C) Agarose gel electrophoresis results showing amplification of gene specific regions of the expected sizes in cDNA of T1 plants from randomly selected plants (replicates) for one event of each hairpin construct. Events selected were: MpEat-6 E2, MpCct-6 E3, MpPod-2 E2, MpPdi-2 E4, MpLev-11 E9, MpSox-2 E1, MpTnc-2 E1, MpCars-1 E7, and MpVha-8 E10.
6.3.3 Effects on aphid fecundity after feeding on transgenic tobacco

To study the effects on aphid fecundity after feeding on dsRNA-expressing tobacco, primary transformants, T0 and selected T1 plants generated after self-fertilisation were challenged with one-2\textsuperscript{nd} instar GPA nymph per plant and aphid numbers were monitored over a period of time. Overall, there was a significant reduction in aphid numbers that fed on target dsRNA-expressing plants when compared to controls in both, T0 events and T1 generation.

6.3.3.1 Aphid fecundity after feeding on transgenic T0 tobacco

A total of 261 plants, including 252 transgenic events and nine replicates of untransformed wild type plants, were used in aphid challenge assays. Aphid survival and reproduction was observed daily for 13 days. Generally, on average, aphids began to reproduce on or after day four in all transgenic and untransformed wild type tobacco. For each dsRNA-expressing tobacco, observations on the number of aphids per day for each event were made and the mean number of aphids present for each time-point was calculated. Observations were subjected to analysis of variance (one-way ANOVA; \( p > 0.05 \)) and there were no significant differences in mean number of aphids on the three controls: untransformed wild type, empty vector- and dsGFP-expressing plants at each time-point. However, on days 10 and 11, the mean number of aphids were significantly higher on empty vector-expressing plants when compared to the other two controls (Figure 6.5).

For all dsRNA-expressing tobacco, aphid numbers were significantly lower compared to the wild-type, empty vector- and dsGFP-expressing plants at each time-point (Figure 6.5). Amongst the dsRNA-expressing T0 events, plants expressing \( \text{dsMpLev-11} \), \( \text{dsMpSox-2} \) and \( \text{dsMpEat-6} \) showed significantly lower number of aphids throughout the period of the experiment with \( \text{dsMpLev-11} \) expressing plants showing the lowest. On the last day of the experiment, day 13, the mean (\( \pm \) SEM) number of aphids observed on wild-type, empty vector- and dsGFP-expressing plants were 16.33 \( \pm \) 0.58, 14.92 \( \pm \) 0.65 and 14.00 \( \pm \) 0.59 respectively. In contrast the mean (\( \pm \) SEM) number of aphids on T0 events expressing \( \text{dsMpLev-11} \), \( \text{dsMpSox-2} \) and \( \text{dsMpEat-6} \) were 3.13 \( \pm \) 0.90, 4.17 \( \pm \) 0.90 and 4.54 \( \pm \) 1.40 respectively, with the other T0 events expressing \( \text{dsMpTnc-2} \), \( \text{dsMpAqp-4} \) and \( \text{dsMpPdi-2} \)
showing the highest mean (± SEM) number of aphids of 8.63 ± 0.96, 6.93 ± 1.01 and 6.20 ± 1.13 respectively (Figure 6.5).
**Figure 6.5. Aphid numbers after feeding on T0 events.** Data represent ± SEM for 9 to 28 replicates. Dotted lines represent tobacco expressing target GPA genes while solid lines are for control tobacco expressing dsGFP, or with empty vector or untransformed wild type.
The distribution of events in comparison to controls and the total number of aphids on day 13 is shown in Figure 6.6. For each dsRNA-expressing tobacco, there were between two and nine events that had no aphids surviving by day 13. Interestingly, dsMpLev-11-expressing tobacco had the most events that had no aphids surviving on the last day. In fact, for all of these nine events, aphids survived only as long as day five or six. Additionally, in dsMpLev-11-expressing tobacco, the most aphids recorded on an event were only ten, which was significantly lower than for events of empty vector- and dsGFP-expressing plants, in which the highest number of aphids present were 22 and 19 respectively.
Figure 6.6. Distribution of all the T0 events showing the total number of aphid surviving on day 13. Event numbers are described against each data point.
6.3.3.2 Aphid fecundity after feeding on transgenic T1 tobacco

A total of 473 plants including 458 replicates of transgenic events and 15 replicates of untransformed wild type plants were used in challenge assays, and aphid survival and reproduction was recorded at five time points: days zero, 4, 8, 12 and 16. Recording aphid numbers each day, as was done with T0 plants was too time consuming for such a large number of T1 plants, and so five time-points with regular intervals were chosen for making observations. Also, the experience from the previous T0 challenge assays was that reproduction generally began after day five and increased over time, and so a final time-point of day 16 was included to obtain a better idea of aphid numbers over a slightly longer timescale.

As observed in T0 challenge assays, an increase in aphid numbers as a result of reproduction was observed for all transgenic and untransformed wild type tobacco plants. For all three control groups, mean number of aphids reproducing at each time-point are shown in Figure 6.7. Survival of aphids on various dsRNA-expressing events was compared to control tobacco containing dsGFP, null vector and untransformed wild type. There were no significant differences (one-way ANOVA; p > 0.05) among the three GFP events observed at the five time-points. This was also true for the four null events. Hence, for subsequent comparisons with target dsRNA-expressing tobacco events, along with untransformed wild type tobacco, the events, GFP E1 and null E26, were selected since these also had the maximum number of replicates of eight and four plants respectively.

Overall, all the events of target dsRNA-expressing tobacco showed lesser mean number of aphids at time-points beyond day zero as compared to the control group of untransformed wild type, GFP and null events. At day 16, among all the various events, MpSox-2 E8 (2.2 ± 0.97) showed the lowest aphid number followed by MpAqp-4 E10 (4.6 ± 2.95), MpLev-11 E12 (6.8 ± 0.94), MpCars-1 E8 (7.6 ± 3.20), MpTnc-2 E8 (8.0 ± 3.30), MpPod-2 E6 (8.4 ± 2.26), MpCct-6 E6 (10.0 ± 4.10), MpEat-6 E8 (10.6 ± 2.02), MpPdi-2 E9 (10.9 ± 2.83), and MpVha-8 E10 (17.8 ± 0.58) (Figures 6.8 to 6.17). Interestingly, six out of the above 10 events, were also the ones that had no surviving aphids by day 13 when their parent (T0) was challenged (Figure 6.6). Those T0 events were dsMpCct-6 E6, MpLev-11 E12, MpSox-2 E8, MpTnc-2 E8, MpVha-8 E10, and MpAqp-4 E10.
Figure 6.7. Aphid numbers on controls: transgenic T1 tobacco harbouring GFP and null vector, and untransformed wild type tobacco.
Figure 6.8. Aphid numbers on transgenic T1 events of *MpEat-6* and control tobacco.
Figure 6.9. Aphid numbers on transgenic T1 events of *MpCct-6* and control tobacco.
Figure 6.10. Aphid numbers on transgenic T1 events of *MpPod-2* and control tobacco.
Figure 6.11. Aphid numbers on transgenic T1 events of *MpPdi-2* and control tobacco.
Figure 6.12. Aphid numbers on transgenic T1 events of *MpLev-11* and control tobacco.
Figure 6.13. Aphid numbers on transgenic T1 events of *MpSox-2* and control tobacco.
Figure 6.14. Aphid numbers on transgenic T1 events of *MpTnc-2* and control tobacco.
Figure 6.15. Aphid numbers on transgenic T1 events of *MpCars-1* and control tobacco.
Figure 6.16. Aphid numbers on transgenic T1 events of *MpVha-8* and control tobacco.
Figure 6.17. Aphid numbers on transgenic T1 events of *MpAqp-4* and control tobacco.
6.4 Discussion

In this study, transgenic tobacco plants expressing dsRNA for ten target GPA genes were generated and studied for their effects on aphid survival and fecundity through host-mediated RNAi. To investigate the efficiency of target genes, transgenic tobacco expressing dsRNA was developed using the ‘leaf disc transformation’ method and T0 plants showing kanamycin resistance on agar media and T1 plants that tested positive for npt II were challenged with aphids. When testing transgenics, generally, stable homozygous populations are subjected to challenge assays however, in this study, primary transformants were also challenged in addition to the segregating T1 generation to get a quick overview on the effects on aphid fecundity after feeding on dsRNA-expressing plants and also to accommodate in planta studies in the time-frame of this research.

The results showed in general that feeding GPA on transgenic plants expressing dsRNA to appropriately chosen target genes can negatively impact aphid on reproduction, and that in planta expression is certainly worth exploring in further details as a strategy for crop protection. Protocols adapted to circumvent problems related to seed selection, challenge assays, effects on aphid fecundity after feeding on T0 and T1 tobacco, and the relationship between the results of in vitro and in planta RNAi in regard to crucial target genes are discussed below.

In selecting T1 seedlings, a recurring problem of fungal contamination was encountered despite careful sterilisation of seeds with ethanol followed by sodium hypochlorite before in vitro selection on kanamycin. This issue resulted not only in loss of probable transformants to contamination but also delayed the procedure of selection and transplantation. To overcome this problem, vapour-phase sterilisation of seeds followed by in solium selection was adapted for tobacco seeds. Both these techniques are routinely used for Arabidopsis selection (Bent, 2006; Xiang, 1999) and this combination proved extremely useful in reducing the time-frame required for selection and also eliminated the problem of contamination. In the study done by Xiang (1999), in solium selection has proved to be reliable with no escapes, and molecular analysis using Southern hybridisation showed that all Arabidopsis transformants screened in solium tested positive for an npt II fragment. Likewise, in the
current study, the amplification of the expected \textit{npt II} fragment in all selected events of the T1 generation confirmed the transgenic nature of the primary transformants.

Using \textit{in vitro} RNAi as a preliminary screen was useful in determining the potential of a target gene prior to \textit{in planta} studies. This was because for some target genes there was a degree of similarity between the results of the \textit{in vitro} and \textit{in vivo} studies with respect to the effects on aphid survival and fecundity. The most noticeable of those genes were \textit{MpSox-2} and \textit{MpLev-11}. When these genes were delivered to aphids through artificial feeding of dsRNA \textit{in vitro} mortality of up to 50% and 42% respectively was observed after 24 hr feeding. This was also accompanied by impaired locomotion, reduction in target gene expression, especially of \textit{MpLev-11}, and the earliest cases of aphid mortality (as early as day one) was observed in ‘long-term survival’ experiments that were conducted post-feeding. Interestingly, these results somewhat mirrored with the results for host-mediated RNAi. Targeting \textit{MpSox-2} and \textit{MpLev-11} significantly reduced aphid fecundity when fed on transgenic plants at the T0 and T1 level, for example, the T1 events \textit{MpSox-2 E8} and \textit{MpLev-11 E12} were among the events that showed highest aphid mortality as compared to controls (Figures 6.6, 6.12 and 6.13).

\textit{Sox} proteins are conserved HMG box superfamily DNA-binding proteins that are essential in diverse developmental processes, and deletions or mutations in them cause developmental defects (Wegner, 1999). RNAi experiments in \textit{C. elegans} suggest that \textit{Sox-2} is important for normal body locomotion, and silencing of this gene results in sterility, slow growth, embryonic and larval lethality (Kagias \textit{et al.}, 2012; Kamath \textit{et al.}, 2003). Similarly, \textit{Lev-11}, has been shown to be essential in normal body movement, and RNAi silencing of this gene in \textit{C. elegans} resulted in impaired movement, paralysis and moulting defects (Frand \textit{et al.}, 2005; Jones \textit{et al.}, 2005). Together with the results of \textit{in vitro} RNAi and host-mediated RNAi, it is clear that oral feeding of ds\textit{MpSox-2} and ds\textit{MpLev-11} had dramatic effects on aphid survival, fecundity and locomotion, and these may serve as potential targets for aphid control in future.

The \textit{V-ATPase} gene has been a selected as a target for gene silencing in many organisms including nematodes and insects, and hence was chosen in this work to function as a ‘validated’ control. The importance of \textit{V-ATPase} in several cellular processes, pH
homeostasis, and normal growth and development was discussed in Chapter 4. RNAi silencing of V-ATPase in *A. pismum, B. cockerelli, B. tabaci* through injection or artificial feeding of dsRNA resulted in mortality of insects (Whyard et al., 2009; Wuriyangan et al., 2011; Upadhyay et al., 2011). Similarly, in this research, results of *in vitro* RNAi demonstrated that feeding dsMpVha-8 for 24 hr resulted in 21% reduction in aphid survival, obvious reduction in target gene expression and mortality by day six (Chapter 4). The promising nature of these results and the fact that this gene has been tested previously encouraged further investigations through host-mediated RNAi studies. In this study, in comparison with untransformed wild type control, reduction in aphid fecundity by day 16 ranged between 26 to 42% among the five T1 events; MpVha-8 E10 T1 exhibited greatest resistance to aphid reproduction (Figure 6.14). These results were similar to a study on *B. tabaci* in which, dsRNA feeding transgenic T1 tobacco lines expressing V-ATPase s. u. A resulted in a 48 to 62% reduction in fecundity (Thakur et al., 2014). Likewise, when *M. persicae* were fed on V-ATPase s. u. E hairpin RNA-expressing transgenic T2 tobacco lines, a 32% reduction of nymphs per adult was observed as compared to untransformed controls (Guo et al., 2014). The results of silencing the V-ATPase gene *in vitro* and *in planta* resemble the studies done by other researchers (Guo et al., 2014; Whyard et al., 2009; Wuriyangan et al., 2011; Thakur et al., 2014; Upadhyay et al., 2011), however, a noteworthy observation was that even though aphid numbers on T1 events of MpVha-8 tobacco were significantly lower than on untransformed wild type they were the highest amongst all the target genes tested by day 16. In any case, results of *in planta* RNAi of dsMpVha-8 like MpSox-2 and MpLev-11 further demonstrated the practicality of using an *in vitro* RNAi screen for selecting genes prior to advancing into host-mediated RNAi.

There were overall differences in the mean number of aphids observed on untransformed wild type plants used as controls in T0 and T1 challenge assays. For example, by day 13 in T0 experiments that were conducted in May there were 16.33 ± 0.58 (n = 9) aphids while in T1 experiments conducted in December the mean number of aphids were 23.87 ± 0.67 (n = 15). These differences could be due to environmental factors such as temperature and day light that are known to affect cyclical parthenogenesis in aphids (Loxdale and Lushai, 2007; Williams and Dixon, 2007). Perhaps, lower temperatures in the winter months of May-June may have affected aphid fecundity in T0 experiments whereas, warm summer months of
December-January may have promoted parthenogenesis thus resulting in higher aphid numbers observed in T1 challenge assays. Even though temperature regulated glasshouses in compliance of statutory regulations for PC2 were used for all transgenic challenge studies, there were problems maintaining ambient temperatures during winter, especially when temperatures dropped at night time.

In previous research, other groups have used either five or 10 nymphs of *M. persicae* at a synchronised life stage to challenge transgenic *Arabidopsis* or tobacco lines (Bhatia *et al.*, 2012; Guo *et al.*, 2014; Mao and Zeng, 2013; Pitino *et al.*, 2011). However, in this work, one 2nd instar nymph per plant was used for challenge assays. This was done for ease in counting aphid numbers daily for 13 days when dealing with 261 T0 plants and at four time-points with 473 T1 plants. Based on previous observations, 2nd instar nymphs generally begin to reproduce four to six days after transfer to tobacco plants, and continue to produce live young from then on throughout its life-cycle. Additionally, by about day 12 or 13, the first progeny began to reproduce, thus rapidly building-up aphid numbers. Hence, to circumvent the time-consuming and labour-intensive process of counting large numbers of aphids on many plants, only one aphid per plant was used. The possibility of nymphs being injured or stressed while being transferred on tobacco leaves was not an issue, since all the aphids transferred to untransformed tobacco plants survived and reproduced.

### 6.5 Conclusions and further work

The study on the effects of host-mediated RNAi on aphid fecundity has provided some interesting and valuable data on the efficiency of the target genes and their potential as candidate genes suitable for RNAi-based crop protection against *M. persicae*. From the research done so far, it is evident that each of the genes selected and tested using RNAi has caused a reduction in aphid population when compared to aphids transferred to untransformed wild type tobacco. The genes that had the most obvious effects were *MpSox-2*, *MpLev-11* and *MpAqp-4*. Except for *V-ATPase*, which has been tested previously for RNAi effects on aphids including *M. persicae*, none of the nine target sequences studied in this thesis have ever been tested in *M. persicae*. All of them are novel targets worth advancing into T2 homozygous populations.
Even though in this research, study to investigate the number of gene copies in the genomic DNA of transgenic plants using Southern blots was not undertaken because of limitations of resources and time, this analysis is one of the aspects that should be done on selected events in the T2 generation. Challenge assays to compare the differences of effects, if any, between one or multiple copy inserts would then provide further knowledge on the level of resistance that may be achieved. Another interesting aspect to this research that can be undertaken in the future is to analyse the sizes of siRNAs produced by transgenic plants using Northern blots, and to examine the sequences from which they were generated and quantify each siRNA sequence. In addition, it would be interesting to undertake transcriptome profiling studies using next generation sequencing technology to investigate the preference of *M. persicae* for uptake of plant-derived dsRNA versus siRNA and compare transcript abundance of plant-derived siRNA versus insect-processed siRNA for each target gene.
CHAPTER 07

General Discussion
Overview

Insect pests, including aphids pose a serious threat to global agriculture and cause severe economic losses worldwide. Some of the problems that are associated with the widely used control strategy of insecticide application include development of resistance, side-effects on non-target organisms, chemical residues and environmental pollution. An RNAi-based strategy of crop protection exploits the natural phenomenon of selectively silencing target genes and functions in insects in a highly specific manner, thus making it an attractive strategy for insect control. The first step in the development of an RNAi-based technology for crop protection that will determine its success is the selection of suitable target genes that play a crucial role in the life-cycle of the organism. When silencing of such genes results in altered behaviour or negatively affects growth, development and reproduction, these can be considered potential targets useful in host-mediated RNAi.

RNAi is a gene regulatory mechanisms present in eukaryotes. Its mechanism was first described in the nematode, *C. elegans* and it has proved to be a powerful tool in functional genomics (Fire et al., 1998). Later, the first successful accounts of RNAi in insects were reported for two species, the light brown apple moth, *E. postvittana* and the triatomine bug, *R. prolixus*, belonging to the orders Lepidoptera and Hemiptera respectively (Araujo et al., 2006; Turner et al., 2006). Since then, RNAi has been demonstrated in a range of insect species of the orders Blattodea, Coleoptera, Dictyoptera, Diptera, Hymenoptera, Neuroptera, Orthoptera and Thysanura (Amdam et al., 2003; Belles, 2010; Bucher et al., 2002; Cruz et al., 2006; Dong and Friedrich, 2005; Misquitta and Paterson, 1999; Mutti et al., 2006; Ohde et al., 2009; Rajagopal et al., 2002).

The current research was undertaken with overall goals of silencing candidate target genes of GPA through artificial feeding of dsRNA and its delivery via transgenic plants. Specifically, the aims were to: identify target genes of GPA, develop an optimised in vitro RNAi screen, evaluate the effectiveness of candidate genes through in vitro RNAi and finally, to study the effects of in planta RNAi. The outcomes of this research provide valuable information on potential target genes useful for generating transgenic plants with enhanced resistance to GPA and potentially other aphids. Some of the major findings, limitations, conclusions of this research and future prospects are discussed below.
7.1 *In silico* identification of target genes in GPA

The first step undertaken in this current research was to identify target genes in GPA using comparative bioinformatics. This study was done to identify suitable candidate genes which play a vital role in biological processes such as feeding, moulting, growth, development and reproduction, and which when silenced via RNAi would disrupt the life-cycle.

One of the major challenges in transgenic plant-mediated RNAi is the efficiency of the target gene and its potential of causing either reduced fecundity or insect mortality. The choice of the target gene is therefore one of the most important factors contributing to the success of RNAi. Identification of target insect genes has been approached in many different ways by various research groups. One of the approaches includes screening of cDNA libraries, and more recently, the use of next generation sequencing technologies to identify stage-specific RNAi target genes (Baum et al., 2007; Mao et al., 2007; Wang 2011). However, the primary choice of approach seems to be selecting genes that have previously been reported as potential targets for RNAi in other insect species or organisms (Mao and Zeng, 2014; Pitino et al., 2011; Zhao et al., 2011; Zhu et al., 2011). For example, Pitino et al. (2011) identified and demonstrated host-mediated RNAi of the MpCOO2 gene in *M. persicae*, the homologue of the salivary transcript that was first identified in *A. pisum* by Mutti et al. (2006). Similarly, Mao and Zeng (2014) identified the Mphb in *M. persicae* which is a homologue of the hunchback (*hb*) gene in *A. pisum* and tested its efficiency for plant-mediated RNAi based on the promising outcome of their work on feeding ds*Aphb* to *A. pisum* through artificial diets (Mao and Zeng, 2012). Both these examples have used the closely related insect species, *A. pisum*, to identify homologues in *M. persicae*.

In the current study, a somewhat different approach was undertaken: instead of mining for genes in insect species, *C. elegans* genes with RNAi phenotypes were used as a reference point to identify GPA ESTs that shared high sequence homologies with *C. elegans* genes. There were two reasons for this choice, first the *C. elegans* genome is the best annotated of any multicellular organism, and second that research supervisors of this project were familiar with *C. elegans* and genomics of plant parasitic nematodes (Jones and Fosu-Nyarko, 2014). The ESTs identified initially in this way were also then compared with genomic resources available for other insect species. Although this is a different approach, the use of *C. elegans*...
for *in silico* identification and studying phylogeny of orthologues, for example, genes involved in the RNAi machinery, in various insects such as *A. glycines*, *T. castaneum*, *D. melanogaster*, *N. lugens* is not new (Bansal and Michel, 2013; Tomayasu *et al*., 2008; Xu *et al*., 2010; Xu and Han, 2008). In fact, *C. elegans* genomic resources have also been used for comparative analyses of nuclear receptors in humans and *D. melanogaster* (Maglich *et al*., 2001).

At the time when this part of the research was initiated (December 2012), there were 27,721 available ESTs of GPA curated by the NCBI. All of these were compared *in silico* to the non-redundant nucleotide database of all other insects and the free-living nematode, *C. elegans*. Since *C. elegans* is the most highly annotated multicellular organism to date and has an extensive information available on the function of genes, it was used as a particularly useful reference for identifying lethal RNAi phenotypes to select target genes in GPA.

Based on the life-cycle of GPA, functional classes of genes that may be involved at specific stages of the life-cycle were chosen. These were in embryogenesis, osmoregulation, moulting, cuticularisation, feeding, and locomotion. Results of the comparative bioinformatics suggested that out of the 27,721 GPA ESTs, a total of 13,099 and 25,962 ESTs shared sequence similarities to *C. elegans* and insects respectively. As might be expected more of these ESTs matched various insect species belonging to the orders Blattodae, Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera, Orthoptera, Phasmida and Phthiraptera. Among them the maximum number of GPA ESTs shared similarities with *A. pisum* and to a certain extent with the Russian wheat aphid, *D. noxia*. The complete genome of the pea aphid was sequenced and became available in 2012 and there is now an abundant sequence information available on the aphid genome database, AphidBase as well as the NCBI (IAGC, 2012). Similarly, there is also much information available on mitochondrial genome sequences for some other insect species including *D. noxia*. The availability of genomic resources for these two insect species and also the fact that they belong to the same tribe, Macrosiphini (sub-family Aphidinae) as the GPA, contributed to the sequence similarities (IAGC, 2012; Lee and Lee 2013; Thau *et al*., 2004; Wang *et al*., 2013a; Zhang *et al*., 2014).
Ten target GPA ESTs representing the six functional classes that shared homologies with *C. elegans* genes were identified based on various BLAST parameters and the RNAi phenotypes they exhibited in *C. elegans*. These genes were: *MpVha-8* (vacuolar H ATPase), *MpEat-6* (EATing: abnormal pharyngeal pumping), *MpCct-6* (Chaperonin Containing TCP-1), *MpLev-11* (LEVamisole resistant or tropomyosin a.k.a *tmy-1*), *MpSox-2* (alternative splicing transcription factors), *MpTnc-2* (Troponin C), *MpAqp-4* (aquaporin or aquaglyceroporin related), *MpPdi-2* (protein disulphide isomerase), *MpPod-2* (polarity and osmotic sensitivity defect), and *MpCars-1* (cysteinyl amino-acyl tRNA synthetase). Some of the phenotypes reported for *C. elegans* were lethality, sterility, larval and embryonic arrest, reproduction-, locomotion- and moult-defective, sluggish and slow-growing. All except three genes, *V-ATPase*, aquaporin and tropomyosin are novel candidates that to my knowledge have not previously been tested in any insect species. Effects of RNAi for *V-ATPase* have been investigated in many hemipterans such as *A. pisum*, *B. tabaci*, *B. cockerelli*, *N. lugens* and *M. persicae*, and aquaporin in *A. pisum*. *V-ATPase* is a popular target that has also been tested in other insect of the order Coleoptera and Lepidoptera, and because it had already been silenced in *M. persicae* through host-mediated RNAi, it served as an established control.

### 7.2 Vital dyes to trace uptake of dsRNA

The second aim of this research was to identify a suitable dye that could be used in artificial feeding solution to trace the uptake of dsRNA and which would allow the identification of only those aphids that had fed, based on visual/microscopic observations. The need for the use of dye is based on the shortcomings of the preliminary studies: optimisation studies to select dye and dsRNA concentrations and the effects of silencing ds*Vha-8* *via in vitro* RNAi in the presence and absence of dye is discussed below.

#### 7.2.1 The need to identify aphids which had taken up dsRNA

Interestingly, the need to incorporate a dye in artificial feeding solutions was a reflection of the results obtained from the preliminary study that was initiated to determine the most appropriate concentration of dsRNA in feeding sachets to study the effects of RNAi on GPA. Since 1 µg/µL and 1.5 µg/µL of ds*Vha-8* did not result in an effective knockdown, it was
reasoned that (1) the concentration of dsRNA may not have been enough to achieve effective gene knockdown or (2) some test aphids may not have fed at all, thus increasing the transcript abundance of the target gene. Two changes were proposed to address those possibilities. The first change was to increase the dsRNA concentration and this reasoning was based on observations from other research which demonstrated the importance of optimisation of dsRNA concentration and a positive correlation between the concentrations of dsRNA and the effects of RNAi (Mutti et al., 2006; Liu et al., 2010; Tomoyasu and Denell, 2004; Turner et al., 2006; Zhang et al., 2013b). The second change was to use a suitable dye that allowed the ‘fed’ aphids to be identified and subsequently to compare the effects on gene expression and long-term survival in aphids fed on dsMpVha-8 with and without dye. This study was undertaken specifically to ensure that only aphids that had ingested dsRNA-feed were assessed for the effects of gene silencing, rather than pooling together test insects which only provides an average measure of knockdown, and which is usually done in studies on insect RNAi (Chen et al., 2010; Li et al., 2011; Mao and Zeng, 2012; Shakesby et al., 2009; Upadhyay et al., 2011). The results obtained in the preliminary study may also suggest that RNAi effects can sometimes be transient. There was a two-fold reduction in ApAQP1 expression at 1 day after feeding A. pism on 1 µg/µL of dsRNA. The reduction was only transient since by day 7 there was no significant reduction (Shakesby et al, 2009). Similarly, Jaubert-Possamai et al. (2007) found that injecting A. pism with dsRNA homologous to calreticulin and cathepsin only led to a transient reduction in target gene expression. Another explanation for the nature of the preliminary results is the lack of an siRNA amplification pathway, which is responsible for systemic RNAi, and has been reported to be present in some other insects (Bucher et al., 2009). Even though sid-1 homologues of C. elegans are reported in aphids (Xu and Han, 2008), their presence does not necessarily confirm the existence of a robust systemic RNAi pathway (Tomayasu et al., 2008). Similarly, RdRPs which are required in the siRNA amplification pathway in C. elegans (Sijen et al., 2001) have not been reported in insects; and for the insect species in which a robust systemic RNAi pathway has been reported, homologues of RdRPs have not been reported (Tomayasu et al., 2008).
7.2.2 The choice of dye

The aims of this research were to identify, optimise and use a suitable dye to trace the uptake of dsRNA for effective assessment of gene silencing in vitro RNAi. Generally, Cy-3™ is used to label dsRNA or siRNA and is marketed as a dye that does not affect stability of dsRNA or siRNA and routinely used in various studies, however, it is expensive. As an alternative to Cy-3™ which has been used previously to investigate uptake of dsRNA in the glassy-winged sharpshooter, *H. vitripennis*, the grain aphid, *S. avenae*, and the potato/tomato psyllid, *B. cockerelli* (Rosa et al., 2012; Zhang et al., 2013b; Wuriyanghan et al., 2011), 11 inexpensive dyes were first evaluated for their suitability in tracing uptake of dsRNA by aphids. Of all the dyes tested, MB, FG, AF and the food colours could not be seen readily in aphids after feeding, and there were also fewer aphids active after 24 hr. Perhaps, the colour intensity, surface tension, refraction, odour or taste of the dyes may have contributed to their starvation. The problem of green-yellow autofluorescence in GPA made FITC and FDA unsuitable as tracers for aphid feeding.

AO, CR, NR and PB were the only dyes readily visible inside the aphid bodies and it seemed that aphids preferred AO and NR more than the others since more of them had fed from feeding sachets. Detailed optimisation studies revealed that concentrations of 0.02% NR and 0.0025% AO were suitable for tracing uptake of feed since these concentrations were easily observed, did not significantly affect aphid survival, and also did not affect the stability of dsGFP at 24 hr in a feeding sachet. The use of AO and NR to mark insect tissues through artificial feeding has been used previously without any negative effects on insect growth, development and reproduction, and this was consistent with the observations in this study (Brenner, 1984; Gast and Landin, 1966; Mittler and Dadd, 1963; Strand et al., 1990). At optimal concentrations, both dyes also encouraged the migration of aphids towards the feed within 30 min and promoted feeding. Due to the prerequisite of a fluorescence microscope when AO was used as a tracer dye, the use of NR seemed logical choice of dye for the work done in this study.

The green food colour, Cy-3™ and GFP PCR products have in fact been used to label dsRNA feeding solution fed to potato/tomato psyllid in sucrose solutions (Wuriyanghan et al., 2011). In that study the researchers also found that the presence of 15% sucrose in artificial diets
did not affect the stability of dsRNA, and with the results obtained from spectrophotometric and electrophoretic analyses in this study confirm their results and the stability of dsGFP in a 30% sucrose solution. Similarly, Li et al. (2011) tested the stability of dsGFP in different media; DEPC-treated ddH2O, 10% sucrose prepared in DEPC-treated ddH2O, artificial feed at various pHs. They reported that dsGFP remained stable in the first two media for 24 hr, and they subsequently used 10% sucrose prepared in DEPC-treated ddH2O for artificial feeding experiments on the brown planthopper, N. lugens. In the research by Wuriyanghan et al. (2011), however, no information was provided on the stability of dsRNA in the presence of the three labels used or any information on the number of insects that had taken up the dye or whether there was any effect on survival. Similarly, in the research on grain aphid and glassy-winged sharpshooter where Cy-3™-labelled dsRNA was fed artificially, there is no particular information on the statistics of aphids that ingested the feed (Rosa et al., 2012; Zhang et al., 2013b). The current study is unique as it provides the first detailed report on the various dyes tested, their concentrations and effects on dsRNA stability, and also assesses gene silencing in aphids fed on dsRNA with and without dye.

7.2.3 Effective silencing of MpVha-8 in aphids that have ingested dsRNA

To test if adding NR to dsRNA feeding solution and assessing only ‘fed’ aphids gave a better idea of gene expression than assessing aphids pooled together (assuming all to have fed), aphids were fed on 2 µg/µL of dsMpVha-8 with and without dye. Even though fewer aphids were found to be active after 24 hr feeding on dsMpVha-8 without dye compared to aphids that fed on dsMpVha-8 with dye, it was not possible to determine that mortality was an effect of dsRNA or whether it was due to starvation, and also how many of the aphids offered feed without dye had ingested dsRNA. Adding dye to dsRNA solution provided an accurate number of aphids that could be confirmed as ‘fed’.

As suspected, it was clear that expression of MpVha-8 in the aphids fed with dye was much lower than in those fed without dye in which some had not fed, indicating a more pronounced gene knockdown in the former based on the results of semi-quantitative PCRs. It would have been interesting to use quantitative real-time PCRs or perhaps even digital PCR to generate a more accurate and absolute quantification of the MpVha-8 transcripts present.
in both samples. This would have given a clearer picture of the level of gene silencing achieved. However, the results of semi-quantitative PCRs were sufficient to demonstrate that there was a greater reduction in the expression of *MpVha-8* in aphids that were fed on and had ingested dsRNA than for the corresponding dsRNA treatment without dye in which all aphids were pooled together.

For both treatments, feeding on ds*MpVha-8* caused mortality of aphids by days 5 and 6 which was observed in post-feeding experiments. These results also indicated that increasing the concentration of dsRNA from 1 µg/µL and 1.5 µg/µL to 2 µg/µL caused a significant impact on immediate as well as long-term survival of aphids. The observations on mortality are in agreement with research on other insects such as *D. virgifera virgifera*, *L. decemlineata*, *D. melanogaster*, *T. castaneum*, *A. pisum*, *M. sexta* and *B. tabaci* in which RNAi silencing of *V*-ATPase has led to significant mortality, thus reaffirming the potential of V-ATPase as a target for insect control and also as an established gene control in RNAi screens (Baum et al., 2007; Upadhyay et al., 2011; Whyard et al., 2009).

### 7.3 Effective target GPA genes based on in vitro RNAi

The main goal of this research was to identify and test potential target genes of *M. persicae* through RNAi. For this purpose, first an RNAi screen with optimised concentrations of target dsRNA and dye was developed. Nine target sequences, *MpEat-6*, *MpCct-6*, *MpPod-2*, *MpPdi-2*, *MpLev-11*, *MpSox-2*, *MpTnc-2*, *MpCars-1*, and *MpAqp-4* were then tested and studied for the effects of in vitro RNAi on behaviour, immediate and long-term survival, and gene expression.

The uniqueness of this research was that all of these genes were identified in and tested for the first time in GPA. Among them, tropomyosin and aquaporin were two genes which have also been investigated for their effects in in vitro RNAi in the insects, *H. vitripennis* and *A. pisum* respectively (Rosa et al., 2012; Shakesby et al., 2009). Effects of silencing these two genes in GPA are discussed in more details below. For all other genes, the information obtained from this research was novel. Abundant information from RNAi studies on
orthologues of all nine genes in *C. elegans* is available on Wormbase and was very useful for comparing some of the RNAi phenotypes observed.

Silencing of all the target genes chosen for study through *in vitro* RNAi affected gene expression: both immediate and long-term survival was affected, and some genes also induced behavioural changes. After 24 hr feeding on dsRNA, the most conspicuous behavioural difference observed was impaired locomotion and movement in aphids that were fed on the locomotion genes, *MpLev-11*, *MpSox-2*, and *MpTnc-2*. There was also an apparent change in movement of aphids that were fed on *dsMpEat-6* and *dsMpCct-6*. *MpEat-6* was identified and categorised in the putative functional gene class responsible for feeding and *MpCct-6* was classified based on its putative gene function in locomotion and osmoregulation.

In this study, feeding *dsMpLev-11* to GPA reduced survival by 50% after 24 hr and the aphids that had fed as determined by presence of dye seemed paralysed with stiff limbs having some local movement. The aphids which fed on *dsGFP* and no-dsRNA solutions displayed normal body movement and also moved freely within the feeding chamber. The results of semi-quantitative PCRs indicate that after 24 hr there was a reduction in target gene expression when compared to the aphids fed on *dsGFP* and no-dsRNA solutions. There was a long-term effect on survival of aphids fed on *dsMpLev-11*; by the second day, the aphids that were transferred on tobacco plants (*n*=5) were dead. Together, these data suggest that *MpLev-11* is a vital gene which when silenced negatively affects movement and locomotion, and also causes aphid mortality. In *C. elegans*, RNAi studies have demonstrated the importance of *lev-11* encoding tropomyosin in muscle arm extension and morphology and has been found to cause paralysis and sterility when silenced (Dixon and Roy, 2005; Ono and Ono, 2002). Tropomyosin also mediates association of the troponin complex with actin filaments (Ebashi and Kodoma, 1966) and the association of troponin-I with thin actin filaments responsible for ovulatory contraction (Obinata *et al.*, 2010). In *C. elegans*, RNAi-mediated silencing of tropomyosin demonstrated its importance in contraction of male sex muscle and pharyngeal muscles, and also plays a role in moulting (Frand, 2005; Gruninger *et al.*, 2006). In *H. vitripennis*, when 800 ng of dsRNA homologous to tropomyosin was microinjected, there was a significant reduction in target mRNA levels and 50% survival of
insects five days post-injection when compared to dsGFP injected controls (Rosa et al., 2012). In the current study, higher concentrations (2 µg/µL) of dsRNA, the delivery method and insect species may have caused differences in observations on survival. In any case, based on the studies of C. elegans and H. vitripennis, even though the concentration and the mode of delivery of dsRNA varied, the effects of silencing tropomyosin have consistently illustrated its importance as a vital gene.

In C. elegans, there are two muscle tissues: pharyngeal tissue for feeding and the body wall tissue for locomotion (Kagawa et al., 2006). Both tropomyosin and troponin C (tnc-2) are associated with the actin filaments and are important for pharyngeal pumping required for feeding, normal body movement and ovarian muscle contraction (Kagawa et al., 2006; Ono and Ono, 2002). Tnc-2 encodes the calcium-binding component of the troponin complex of thin actin filaments (Wormbase). It is specifically expressed in pharyngeal muscles and is responsible for their contraction. Interestingly, in vitro RNAi of MpTnc-2 resulted in 34% mortality after 24 hr feeding, reduction in MpTnc-2 gene expression and also by day 3, all aphids had died. These results were also accompanied by behavioural differences such as paralysis with stiff limbs having slight movement in one or two limbs. These observations suggest that like the MpLev-11, MpTnc-2 also is a crucial gene with desirable RNAi effects and may be a feasible target in RNAi-mediated crop protection.

Another GPA gene worthy of discussion is MpAqp-4, particularly because there are similarities in results of in vitro RNAi with those found in A. pisum (Shakesby et al., 2009). In the current study, feeding of dsMpAqp-4 reduced aphid survival by 25% after 24 hr and this was accompanied by a slight behavioural change such that aphids moved more slowly than control aphids and the bodies appeared somewhat turgid. Also, the target gene expression was only slightly reduced in comparison to controls. These results resemble the results of in vitro RNAi observed in A. pisum in which there was a two-fold reduction in ApAQP1 at day 1 post-feeding, which was then transient, and by day 7 no reduction in gene expression was detected. Similarly, no significant differences were observed in mortality or weight gain in aphids fed on dsApAQP1 and dsGFP (Shakesby et al., 2009).
In long-term survival experiments, the percentage of surviving aphids reduced progressively over time and was significantly different from controls at all time-points. However, on day 12 there seemed to be an increase in the number of aphids as a result of reproduction. Perhaps, if the experiment had included observations for a longer period an increase in aphid numbers would have been recorded. However, this was not possible since the space in the plastic cups used for growing experimental tobacco plants was insufficient and the rapid growth of tobacco plants would have displaced the lids.

One reason for the similarities in results of silencing aquaporin found here and by Shakesby et al. (2009) is the high sequence homologies between *MpAqp-4* (EE570677) and *ApAQP1* (NM001145905). Another reason for the transient silencing effect may be the presence and involvement of other gene(s) that may have compensated for the effects of RNAi, since aquaporins belong to a family of major intrinsic proteins (Engel and Stahlberg, 2002). In *C. elegans*, *Aqp-4* plays a role in osmoregulation, however no obvious defects have been observed via RNAi and this gene is predicted to work in conjunction with *Aqp-2*, *Aqp-3* and *Aqp-8* to recover from hypotonic stress (WormBase). Also, as suggested previously, the transient nature may perhaps be due to the absence of an siRNA amplification pathway required for systemic RNAi.

Last, but not the least, there were some interesting results on the performance of the target genes in comparison to *MpVha-8* which was chosen as an established control based on the promising results of RNAi in other insect species. Even though there was also a noticeable reduction in gene expression accompanied by 22% mortality after 24 hr feeding of ds*MpVha-8*, which also significantly differed from control fed dsGFP and no-dsRNA, it was the lowest among all the target dsRNA tested. However, by day 6 on tobacco, there were no aphids alive. In long-term survival experiments, in addition to aphids that were fed on *MpVha-8*, mortality of aphids also occurred after feeding on dsRNA of *MpLev-11*, *MpSox-2*, *MpTnc-2*, *MpPod-2* and *MpEat-6*. This suggests that based on the lethal effects recorded from in vitro RNAi studies, these five GPA genes are potential candidate targets worthy of further investigation and may be viable targets for host-derived RNAi. It can be said that although all nine targets yielded significant RNAi effects, the above five candidates performed better or at least equally to *MpVha-8*. To confirm whether these genes performed better than *MpVha-8*,
first, the accuracy of the experiments could be checked by repeating them, and also it would be good to use qPCRs and digital PCR techniques to measure the relative and absolute gene expression respectively. In this study however, there were time and financial restrictions and so only semi-quantitative PCR analyses could be done.

An example of novel candidate targets for RNAi that performed better than most published genes is provided by the research done by Zhang et al. (2013b). In that study, transcriptomic profiling of the alimentary canal of S. avenae before and after feeding on wheat was done by Illumina sequencing. Using de novo assembly and gene expression analyses, 5490 unigenes were identified and annotated. Sixteen unigenes which were either up- or down-regulated on feeding were studied for their in vitro RNAi effects using an artificial feeding system. MpCOO2, the homologue of the salivary transcript first identified in A. pisum was used as an established control and 7.5 ng/μL of dsRNA was fed to aphids for 8 days. Five out of the 16 candidate unigenes showed higher mortality, stunting, and significant reduction in gene expression than the aphids fed on MpCOO2.

7.4 Effective target GPA genes studied in planta by RNAi

The final aim of this research was to test the suitability of the candidate target GPA genes through plant-mediated RNAi. Transgenic tobacco expressing the ten target genes, MpEat-6, MpCct-6, MpPod-2, MpPdi-2, MpLev-11, MpSox-2, MpTnc-2, MpCars-1, MpVha-8 and MpAqp-4 were developed and challenged at T0 and T1 generations. The information obtained from this study was useful for comparing the efficacy of genes tested through in vitro and in planta RNAi, determining the reliability of an in vitro RNAi screen and finally ranking the target gene(s) based on their effectiveness in controlling aphid numbers. These aspects are discussed below.

So far, there are four published studies of host-mediated RNAi in GPA (Bhatia et al., 2012; Guo et al., 2014; Mao & Zeng, 2013; Pitino et al., 2011). These studies have tested the effects of various genes including V-ATPase s. u. E (Guo et al., 2014) on target gene expression, aphid fecundity and phenotype. In the study by Guo et al. (2014), transgenic T2 tobacco engineered with hairpin RNA-expressing vector containing the GPA homologue of V-
ATPase s. u. E were challenged and it was found that there was a 32% reduction of nymphs produced per adult by day 14. There was a significant difference in aphid numbers between on wild type control and transgenic plants expressing V-ATPase s. u. E (Guo et al., 2014). In the current study, when T1 tobacco plants expressing V-ATPase s. u. E were challenged with aphids, there were significant differences in aphid numbers for all time-points between wild type and transgenic tobacco. For the five events of T1 tobacco, the reduction in aphid populations at day 16 ranged from 26% and 41%. In comparing the results obtained from in vitro feeding and aphid challenges of T1 transgenic plants, the results were similar in most cases. However, it is important to note that the transgenic tobacco generations being at the T1 generation were still segregating. It would have been better to challenge homozygous T2 transgenic lines, however, due to the time-frame of the research, it was not possible to establish T2 generation plants. In addition, sites of insertion and copy number of transgenes add to the variation in challenge results. From the T1 tobacco events expressing V-ATPase s. u. E, it would be interesting to advance the events MpVha-8 E10, MpVha-8 E16, MpVha-8 E11, MpVha-8 E14 and MpVha-8 E15 and challenge the T2 generation, and to undertake further molecular analyses of copy number and insertion sites.

While V-ATPase s.u. E- expressing tobacco has been studied here and previously, the remaining nine GPA targets were novel and have not been tested by host-mediated RNAi. Four target genes, MpEat-6, MpLev-11, Mp Sox-2 and MpTnc-2 that yielded promising results when tested in vitro and also showed reduction in aphid numbers when T2 tobacco expressing these genes were challenged. In particular, MpLev-11 and Mp Sox-2 showed significant reduction in aphid numbers at not just T0 but also T1 levels. For example, the T1 events Mp Sox-2 E8 and MpLev-11 E12 were among the events that showed highest aphid mortality compared to controls. Similarly, MpEat-6 and MpTnc-2 when tested in vitro are promising candidates worth advancing in to in planta studies based on the significant aphid mortality achieved after 24 hr feeding and in the ‘long-term survival’ experiment. GPA fed on transgenic T1 events of MpEat-6 had fewer aphids than control tobacco. The results of in vitro RNAi for these four genes complemented the results of in planta RNAi, and so using a preliminary in vitro RNAi screen does indeed help to identify potential candidate genes worth advancing into the transgenic research.
For those target genes for which the *in vitro* and *in planta* feeding results differed, *MpPdi-2* when tested *in vitro* RNAi had the lowest aphid survival after 24 hr feeding, but aphid numbers increased by day 12 post-feeding. In contrast, when ds*MpPod-2*, ds*MpAqp-4* and ds*MpVha-8* were fed to GPA, even though the mortality was not high after 24 hr feeding, aphids fed on ds*MpPod-2* and ds*MpVha-8* did not survive beyond days 4 and 6 respectively, while those fed on ds*MpAqp-4* survived and even increased slightly by day 12. When the four genes were tested by host-mediated RNAi, all the T1 events showed significantly lower aphid numbers than controls.

These data suggest that significant aphid mortality observed immediately after 24 hr feeding may not necessarily be followed by long-term effects on survival. There could be several explanations for this. It is possible that the effects of *in vitro* RNAi was transient and this has been reported in pea aphid after feeding on *ApAQP1* (Shakesby *et al.*, 2009) and discussed above. One possibility could be that the target gene may be compensated for its function, for example, it could represent a large multigene family such as aquaporins or SOX proteins (Engel and Stahlberg, 2002; Wegner, 1999). Another possibility is that there may be an efficient feedback mechanism that regulates and counteracts reduction in target mRNA levels by increasing the rate of transcription (Ciudad *et al.*, 2007). Alternatively, the stability of dsRNA may be compromised once inside the aphid gut leading to the degradation of dsRNA, since there is some evidence that dsRNA can be degraded in the aphid gut (Christiaens *et al.*, 2014). It could also be that the target gene chosen, encoding an important function, may not play a vital role in the aphid or there might be a recovery of target gene expression, as required for insects such as pea aphid and triatomine bug and the root lesion nematodes (Araujo *et al.*, 2006; Shakesby *et al.*, 2009; Tan *et al.*, 2013).

It is important to note that even if complete mortality was recorded for certain target genes using *in vitro* RNAi screens, the same may not be observed for host-mediated RNAi. Complete mortality as a result of *in planta* RNAi seems more difficult to achieve. For example, a comparison of results of two studies in *B. tabaci*, in which up to 97% mortality was found by day 6 after continuous feeding on 20 µg/µL of dsV-ATPase A. However, when insects were fed on transgenic lines expressing dsV-ATPase A, there was a significant reduction in mortality (Thakur *et al.*, 2014; Upadhyay *et al.*, 2011).
While comparing results of \textit{in vitro} and \textit{in planta} RNAi it is important to note that aphids were allowed to feed only for 24 h after which they were not provided anymore dsRNA in an \textit{in vitro} RNAi system whereas aphids introduced on transgenic plants were exposed to a continuous supply of dsRNA. Similarly, the amount of dsRNA provided to aphids in an artificial feeding system was controlled, whereas the amount available to aphids feeding on transgenic plants was unknown and may depend on several factors.

Overall, all nine target genes showed significant levels of aphid resistance and some events had strikingly fewer aphid compared to the three controls. Among all 67 transgenic tobacco events, the top ten T1 events with fewest aphids after challenge were \textit{Mpsox-2 E8}, \textit{Mpaqp-4 E10}, \textit{Mppod-2 E8}, \textit{Mpaqp-4 E27}, \textit{Mplev-11 E12}, \textit{Mpaqp-4 E1}, \textit{Mpcars-1 E8}, \textit{Mppod-2 E6} and \textit{Mpaqp-4 E9}. However, the best T1 event for each gene with fewest aphids on challenge were \textit{Mpsox-2 E8}, \textit{Mpaqp-4 E10}, \textit{Mppod-2 E8}, \textit{Mplev-11 E12}, \textit{Mpcars-1 E8}, \textit{Mptnc-2 E8}, \textit{Mpcct-6 E6}, \textit{Mpeat-6 E8}, \textit{Mpdb-2 E9} and \textit{MpVha-8 E10}.

If the number of aphids observed by day 16 on all the events of a target gene were averaged then the 10 genes arranged in an ascending order of the aphid numbers would be \textit{Mpaqp-4}, \textit{Mppod-2}, \textit{Mpsox-2}, \textit{Mpcct-6}, \textit{Mpcars-1}, \textit{Mpeat-6}, \textit{Mptnc-2}, \textit{Mplev-11}, \textit{Mpdb-2} and \textit{MpVha-8}. However, averaging aphid numbers across events would not be appropriate because at T1, the transgenic tobacco population is still segregating, and also the number of gene copies inserted is not known. However, it does somewhat give a good idea of how the target genes performed when tested by \textit{in planta} RNAi. Based on all the observations: behavioural changes, survival, and fecundity of aphids after \textit{in vitro} RNAi and the aphid numbers recorded on transgenic T1 events of tobacco expressing target gene, there genes were ranked for their efficiency in aphid control (Table 7.1).

Listed below are some aspects that could have been included in this research, but were not possible either due to limitations in time or financial resources. (1) Selection of homozygous T2 tobacco and conducting replicated aphid challenge experiments, (2) Southern blot analysis for estimating gene copy number (3) measurement of gene silencing in aphids fed on transgenic plants through qPCRs, (4) Northern blot analysis to estimate the sizes of target siRNA in plant tissue and (5) siRNA sequencing of aphid RNA post-feeding, to study if there is
a preference for a specific size and region in the target sequence or hotspots that are responsible for better silencing. In addition to the ten target GPA genes studied in this research, an additional 40 genes were identified in silico but were not advanced in to in vitro or in planta studies. It will also be interesting to investigate the potential of these genes as candidates for aphid control. Screening of more candidate target genes may reveal better targets.

Table 7.1. An overview of the performance and ranking of effectiveness of target genes based on in vitro and in planta results and behavioural changes, survival and aphid fecundity.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Percent mortality after 24 hr feeding dsRNA</th>
<th>Behavioural differences observed after in vitro feeding</th>
<th>Number of days alive on tobacco</th>
<th>Fewest aphids on T1 event</th>
<th>Rating of candidate genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>MpEat-6</td>
<td>41.7</td>
<td>Yes</td>
<td>Day 7</td>
<td>10.6 ± 2.02</td>
<td>5</td>
</tr>
<tr>
<td>MpCct-6</td>
<td>30.3</td>
<td>Yes</td>
<td>Day 12</td>
<td>10.0 ± 4.10</td>
<td>7</td>
</tr>
<tr>
<td>MpPod-2</td>
<td>31.8</td>
<td>Yes</td>
<td>Day 3</td>
<td>6.6 ± 3.15</td>
<td>4</td>
</tr>
<tr>
<td>MpPdi-2</td>
<td>51.9</td>
<td>Yes</td>
<td>Day 12†</td>
<td>10.9 ± 2.83</td>
<td>8</td>
</tr>
<tr>
<td>MpLev-11</td>
<td>50.0</td>
<td>Yes*</td>
<td>Day 1</td>
<td>6.8 ± 0.94</td>
<td>2</td>
</tr>
<tr>
<td>MpSox-2</td>
<td>41.7</td>
<td>Yes*</td>
<td>Day 1</td>
<td>2.2 ± 0.97</td>
<td>1</td>
</tr>
<tr>
<td>MpTnc-2</td>
<td>34.5</td>
<td>Yes*</td>
<td>Day 2</td>
<td>8.0 ± 3.30</td>
<td>3</td>
</tr>
<tr>
<td>MpCars-1</td>
<td>34.9</td>
<td>No</td>
<td>Day 12</td>
<td>7.6 ± 3.20</td>
<td>9</td>
</tr>
<tr>
<td>MpVha-8</td>
<td>21.6</td>
<td>Yes</td>
<td>Day 6</td>
<td>17.8 ± 0.58</td>
<td>10</td>
</tr>
<tr>
<td>MpAqp-4</td>
<td>25.0</td>
<td>Yes</td>
<td>Day 12†</td>
<td>4.6 ± 2.95</td>
<td>6</td>
</tr>
</tbody>
</table>

The symbols * represent very obvious effects on locomotion and movement, and † represents increase in aphid number observed at day 12 post-feeding.

7.5 Future prospects

When compared to conventional breeding, genetically engineering plants can provide an opportunity to broaden the genetic resources of useful genes and also increases the possibility of incorporating several different desirable traits in one event. Since the first GM plant that was produced in 1983 by Fraley et al. much research has been done to produce commercial herbicide and pest resistant crops and even crops with valuable output traits to benefit consumer, such as the Golden Rice.
Because gene silencing by RNAi is such a powerful tool, it is widely used in functional genomics, and also provides potential applications in therapeutics to treat genetic diseases in humans, for crop improvement and protection against insect pests. GM crops expressing the Bt Cry toxins that provide protection against Lepidoptera and Coleoptera, do not protect plants against phloem-feeders such as aphids. However, research such as presented here demonstrates the potential of RNAi-mediated crop protection, and the ‘proof-of-concept’ studies suggest the possibility for control of a range of insect pests, including hemipterans such as aphids. The key to success of host-mediated RNAi is finding the best target genes.

There is tremendous scope for research to expand because more insect genomes are being sequenced, and so there will be greater likelihood of finding more effective target genes. Future research should include using artificial microRNA vectors in addition to intron-spliced hairpin vectors given this may reduce the length of dsRNA expressed or possible off-target effects. Targeting insect genes critical for the detoxification of plant defence compounds, effector genes that modulate host defences, and those involved in insecticide resistance development are also potential areas of future research. Similarly, research to combine RNAi-based control with a different mode of action such as Bt can be explored to increase the effectiveness and durability of aphid control. Even though complete resistance to insects through RNAi may not be possible it can be used to enhance pre-existing levels of resistance in crops. Similarly, by engineering plants expressing insect genes important in resistance development against insecticides, it would be possible to reduce the recommended dosage for chemicals thus reducing the risk to the environment and also achieve a greater control of insects. Another exciting area of research is the development of transgenic plants expressing multiple target sequences for more than one insect or nematode species, given the commonality observed between aphid and nematode effectors that modulate host defences. For this though, the additive effects of gene silencing and also the feasibility of multiple gene stacking would have to be evaluated first. Another area of research that is rapidly gaining interest is investigation of the possibility of genome editing for pest management. Preliminary research on targeted gene disruption using the genome editing approach has been demonstrated in fruit fly, cricket and silkworm and will soon add to the sophistication of genetic manipulation tools to develop insect-resistant plants.
In 2014 there were 185 million hectares of GM crops worldwide, grown in 29 countries (ISAAA, 2015), with the major traits being herbicide tolerance and Bt-based insect resistance. There are many more traits and crops in the pipeline, but commercial use depends on many factors.

The acceptance of GM (RNAi-based) crops may not necessarily be in the immediate future but it is inevitable and will eventually be widely accepted just as Bt crops have been adopted and has been successful for the last 19 years. However, as part of process of implementation of RNAi-based traits, additional research may be needed to address concerns such as possible off-target effects, and effects of transgenes, if any on plant growth and development. Besides this, issues related to intellectual property rights, corporate and political interests, and consumer acceptance have to be addressed. Nevertheless, RNAi-based traits have been approved for commercial release in the USA and Canada, for example, modified soybean oil composition and non-browning apples, and so these issues are clearly surmountable.

7.6 Conclusions
This research was primarily undertaken to identify, validate and test the amenability of target GPA genes for aphid control through \textit{in vitro} and \textit{in planta} RNAi. Most of the aims that were set at the beginning of this research were achieved, and have resulted in novel and valuable information on target genes for control of GPA. Although more research on host-mediated RNAi in subsequent transgenic generations of tobacco is required, this research provides an exciting insight on the efficiency of the candidate genes chosen in controlling GPA. This research has demonstrated the usefulness of an \textit{in vitro} RNAi screen prior to \textit{in planta} studies and also provided optimisation parameters for an \textit{in vitro} RNAi screen that will prove useful in the assessment of target gene expression.
### Appendix 1. Fifty GPA ESTs homologous to *C. elegans* and insect genes shortlisted for RNAi studies.

Listed below are GPA ESTs representing putative functional class, homologous *C. elegans* and *A. pisum* genes and their accession numbers, maximum scores and e-values as a result of TBLASTX against the non-redundant nucleotide collection of *C. elegans* and insects.

<table>
<thead>
<tr>
<th>Functional class of genes</th>
<th>GPA EST</th>
<th><em>C. elegans</em> gene</th>
<th>Accession number</th>
<th>Maximum (Hsp bit) score</th>
<th>E-value</th>
<th><em>A. pisum</em> homologue</th>
<th>Accession number</th>
<th>Maximum (Hsp bit) score</th>
<th>E-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Embryogenesis</td>
<td>DW013146</td>
<td><em>Let</em>-92</td>
<td>NM_069846</td>
<td>407.04</td>
<td>2.15E-113</td>
<td>mRNA, clone: AC10411YM19, full-insert cDNA sequence based on ESTs</td>
<td>FP927095</td>
<td>545.42</td>
<td>1.4E-153</td>
</tr>
<tr>
<td>Embryogenesis</td>
<td>EC387265</td>
<td><strong>Vha-8</strong></td>
<td>NM_068639</td>
<td>142.20</td>
<td>4.72E-61</td>
<td>V-type proton ATPase subunit E-like</td>
<td>NM_001162178</td>
<td>387.34</td>
<td>2.4E-130</td>
</tr>
<tr>
<td>Embryogenesis</td>
<td>ES226510</td>
<td>Vha-1</td>
<td>NM_066764</td>
<td>104.16</td>
<td>1.35E-39</td>
<td>V-type proton ATPase 16 kDa proteolipid subunit (Vha16)</td>
<td>NM_001162059</td>
<td>357.55</td>
<td>3.31E-97</td>
</tr>
<tr>
<td>Embryogenesis</td>
<td>ES223745</td>
<td>Vha-4</td>
<td>AB000919</td>
<td>133.95</td>
<td>6.27E-39</td>
<td>vacuolar ATP synthase 21 kDa proteolipid subunit-like</td>
<td>NM_001162207</td>
<td>405.67</td>
<td>1.5E-111</td>
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<tr>
<td>Embryogenesis</td>
<td>DW011899</td>
<td>Snr-7</td>
<td>NM_058631</td>
<td>121.58</td>
<td>2.79E-27</td>
<td>PREDICTED: probable small nuclear ribonucleoprotein G-like</td>
<td>XM_001949352</td>
<td>188.48</td>
<td>2.74E-46</td>
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<tr>
<td>Embryogenesis</td>
<td>DW014963</td>
<td>Snr-6</td>
<td>NM_067219</td>
<td>114.70</td>
<td>4.96E-25</td>
<td>small nuclear ribonucleoprotein E (NV25)</td>
<td>NM_001163367</td>
<td>254.92</td>
<td>2.61E-66</td>
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<tr>
<td>Embryogenesis</td>
<td>ES217864</td>
<td>Mes-6</td>
<td>NM_001026149</td>
<td>84.46</td>
<td>4.44E-18</td>
<td>PREDICTED: polycomb protein EED-like</td>
<td>XM_001949733</td>
<td>539.46</td>
<td>8.6E-152</td>
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<tr>
<td>Embryogenesis</td>
<td>DW014513</td>
<td>Mg2-1</td>
<td>JQ235186</td>
<td>88.59</td>
<td>6.78E-18</td>
<td>GTP-binding protein YPTM1-like</td>
<td>NM_001162723</td>
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<tr>
<td>Embryogenesis</td>
<td>DW013646</td>
<td>C14A4.11</td>
<td>NM_063889</td>
<td>69.80</td>
<td>4.11E-17</td>
<td>PREDICTED: programmed cell death protein 10-like</td>
<td>XM_001949261</td>
<td>325.48</td>
<td>1.8E-104</td>
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<tr>
<td>Embryogenesis</td>
<td>GW884129</td>
<td>Aos-1</td>
<td>NM_073203</td>
<td>79.10</td>
<td>1.41E-14</td>
<td>mRNA, clone: AC11002048, complete cDNA, full-insert cDNA sequence based on the ESTs</td>
<td>AK341512</td>
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<td>Embryogenesis</td>
<td>ES218884</td>
<td>Aars-2</td>
<td>NM_058880</td>
<td>57.03</td>
<td>5.95E-08</td>
<td>PREDICTED: alanyl-tRNA synthetase, cytoplasmic-like</td>
<td>XM_001950396</td>
<td>219.73</td>
<td>4.57E-56</td>
</tr>
<tr>
<td>Osmoregulation</td>
<td>EE571184</td>
<td>Sca-1</td>
<td>NM_066985</td>
<td>563.52</td>
<td>5.10E-160</td>
<td>PREDICTED: calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type-like, transcript variant 2</td>
<td>XM_003246429</td>
<td>617.39</td>
<td>4.3E-175</td>
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<tr>
<td>Osmoregulation</td>
<td>EC388278</td>
<td>Rpn-11</td>
<td>NM_062311</td>
<td>475.77</td>
<td>1.34E-133</td>
<td>PREDICTED: 26S proteasome non-</td>
<td>XM_001951669</td>
<td>603.61</td>
<td>5.9E-171</td>
</tr>
<tr>
<td>Genotype</td>
<td>Gene ID</td>
<td>Uniprot ID</td>
<td>FPKM</td>
<td>Fold Change</td>
<td>Description</td>
<td>Log2</td>
<td>Log10</td>
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<td></td>
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<tr>
<td>Osmoregulation</td>
<td>EE261593</td>
<td>Agef-1</td>
<td>NM_001136328</td>
<td>314.02</td>
<td>1.19E-93</td>
<td>PREDICTED: brefeldin A-inhibited guanine nucleotide-exchange protein 1-like</td>
<td>XR_119479</td>
<td>690.67</td>
<td>0</td>
</tr>
<tr>
<td>Osmoregulation</td>
<td>EE263803</td>
<td>Cct-6*</td>
<td>NM_171135</td>
<td>146.78</td>
<td>7.70E-91</td>
<td>PREDICTED: t-complex protein 1 subunit zeta-like, transcript variant 1</td>
<td>XM_001946937</td>
<td>438.26</td>
<td>2.3E-143</td>
</tr>
<tr>
<td>Osmoregulation</td>
<td>EC388358</td>
<td>Cct-1</td>
<td>NM_063321</td>
<td>274.62</td>
<td>8.10E-90</td>
<td>PREDICTED: t-complex protein 1 subunit alpha-like</td>
<td>XM_001943033</td>
<td>515.76</td>
<td>1.7E-144</td>
</tr>
<tr>
<td>Osmoregulation</td>
<td>ES217627</td>
<td>Rpn-6.1</td>
<td>NM_001027451</td>
<td>292.03</td>
<td>1.99E-78</td>
<td>PREDICTED: 26S proteasome non-ATPase regulatory subunit 11-like</td>
<td>XM_001945102</td>
<td>468.70</td>
<td>1.5E-130</td>
</tr>
<tr>
<td>Osmoregulation</td>
<td>ES219030</td>
<td>C33H5.18</td>
<td>NM_068897</td>
<td>199.47</td>
<td>1.48E-56</td>
<td>PREDICTED: phosphatidate cytidylyltransferase, photoreceptor-specific-like, transcript variant 2</td>
<td>XM_003240925</td>
<td>446.45</td>
<td>6.9E-124</td>
</tr>
<tr>
<td>Osmoregulation</td>
<td>DW012671</td>
<td>Sdhb-1</td>
<td>NM_063591</td>
<td>211.39</td>
<td>4.37E-54</td>
<td>succinate dehydrogenase B (Sdhb)</td>
<td>NM_001162436</td>
<td>289.74</td>
<td>6.8E-99</td>
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<tr>
<td>Osmoregulation</td>
<td>EC390444</td>
<td>Rheb-1</td>
<td>NM_066678</td>
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<td>1.09E-44</td>
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<td>NM_001162796</td>
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<tr>
<td>Osmoregulation</td>
<td>EE570677</td>
<td>Aqp-4</td>
<td>NM_073111</td>
<td>58.80</td>
<td>1.81E-20</td>
<td>aquaporin (Aqp1), transcript variant 2, mRNA</td>
<td>NM_001145905</td>
<td>587.57</td>
<td>1.3E-168</td>
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<tr>
<td>Moulting</td>
<td>EC388786</td>
<td>Cars-1*</td>
<td>NM_058612</td>
<td>324.104</td>
<td>1.7321E-90</td>
<td>PREDICTED: cysteinyl-tRNA synthetase, cytoplasmic-like, transcript variant 2</td>
<td>XM_003246013</td>
<td>614.609</td>
<td>2.65E-174</td>
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<tr>
<td>Moulting</td>
<td>EE261497</td>
<td>Pptr-1*</td>
<td>NM_074732</td>
<td>274.16</td>
<td>5.9747E-73</td>
<td>PREDICTED: serine/threonine-protein phosphatase 2A 56 kDa regulatory subunit alpha isoform-like, transcript variant 2</td>
<td>LOC100159278, mRNA</td>
<td>XM_003240014</td>
<td>453.777</td>
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<tr>
<td>Moulting</td>
<td>EE261986</td>
<td>Pod-2*</td>
<td>NM_001027229</td>
<td>259.653</td>
<td>1.4098E-68</td>
<td>PREDICTED: acetyl-CoA carboxylase-like, transcript variant 4</td>
<td>XM_003245306</td>
<td>662.454</td>
<td>0</td>
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<tr>
<td>Moulting</td>
<td>ES225480</td>
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* Represents *C. elegans* genes that are cross-listed in two or more functional class.

** Represents target GPA ESTs selected for RNAi studies in the current research.
References


Berlandier, F. 1999. Aphids in lupin crops: their biology and control. DAFWA Farmnote 44.


