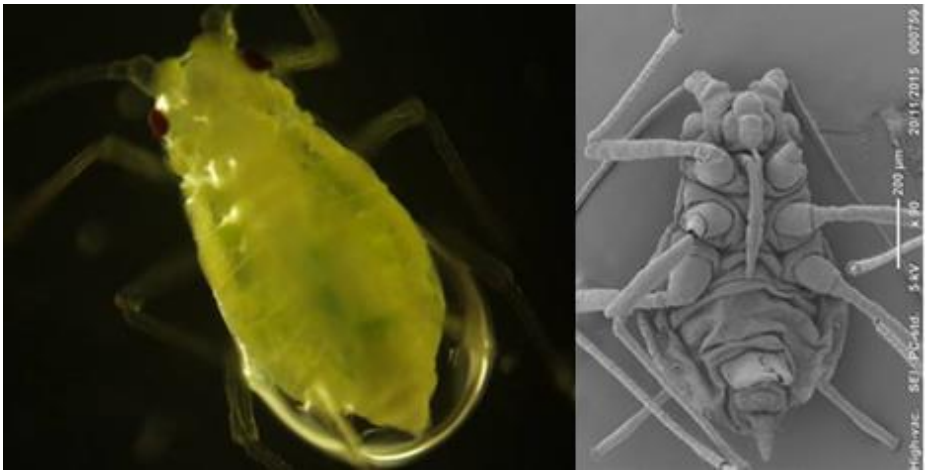
Neuronal signalling molecules as targets for green peach aphid (*Myzus persicae*) control *via* RNA interference



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Abstract

The Green peach aphid (GPA) (*Myzus persicae*) is an important insect pest which causes substantial economic losses to many glasshouse and field crops. Alarmingly, GPAs are becoming resistant to many conventional insecticides, and this trend indicates that there is a real need to develop alternative strategies to protect crops from this insect pest. The aim of this research project was to investigate the potential of RNA interference (RNAi) technology as a strategy to control GPAs. Genes involved in insect neuronal signalling pathways were selected as RNAi targets. Bioinformatic analysis tools were used to identify ESTs putatively encoding sixty-three Neuronal Signalling Molecules (NSMs) from publicly available sequences and from GPA transcriptome data generated in-house. The NSMs included 30 Neuropeptides (NPs), 24 Neuropeptide Receptors (NPRs), and 9 Biogenic Amine Receptors (BARs). From these, transcripts for 24 NSMs were selected for *in vitro* RNAi assays to determine their suitability as targets for host-induced gene silencing (HIGS).

Successful ingestion of dsRNA of target genes by nymphs was confirmed using the presence of a neutral red dye in the body of aphids, incorporated in the dsRNA+30% sucrose diet. Silencing effects of nine genes, e.g. Ecdysis triggering Hormone (*eth*), Capability (*capa*), Juvenille hormone binding protein (*jhbp*), Leucokinin (*lk*), Crustacean Cardioactive Peptide (*ccap*), Octopamine beta 3R (*octβ3r*), Muscarinic acetylcholine receptor 3 (*mAChrM3*), Short NPF (*snpf*) and Insulin-related peptide 2/3 (*irp2/3*) were obvious 24 hours after feeding on the dsRNA diet. RNAi phenotypes included incomplete moulting, uncoordinated movement, lethargy, paralysis and lethality, whereas the control GPAs exposed to no-dsRNA and dsRNA of the green fluorescent protein (GFP) gene of the jellyfish, *Aequorea victoria* moved normally, showing no obvious effects of the treatment. For GPAs treated with dsRNAs of six of these genes (*ccap, capa, mAChrM3, lk, octβ3r* and *irp2/3*), silencing also significantly affected

survival and fecundity when the aphids were later transferred to tobacco plants for 12 days. Silencing of *ccap, capa, irp2/3, lk* and *octβ3r* resulted in 100% lethal phenotypes on the tobacco plants. Knockdown of ds*capar1* and *dsnplp1* also affected GPA reproduction although no visible effects were observed 24 hours after ingestion of dsRNA.

The effectiveness of nine of the 24 genes (*ccap, jhbp, nplp1, capar1, irp5, lk, octβ3r, snpf* and *opsin*) as targets for RNAi control of GPAs were evaluated using HIGS, in which two model plants, tobacco and *Arabidopsis thaliana* were used. Transgenic tobacco plants carrying hairpins (hp) of all nine GPA genes were developed of which those for six genes (except for *lk, octβ3r* and *snpf*), were advanced to the T_2 generation, and used for GPA bioassays. In T_1 tobacco, the mean population was reduced by 97% for hp*octβ3r* event 2 and event 5, while significantly lower GPA populations were recorded for all the lines expressing hp*ccap,* hp*nplp1* and hp*lk* after 12 days ($p<0.05$). As for the T_1 generation, most of the T_2 transgenic events also supported significantly fewer GPA nymphs, with reductions in numbers ranging from 3% to 69%. GPAs feeding on events of hp*ccap,* hp*nplp1* and hp*lk* produced fewest nymphs, as was observed for T_1 generation. In addition, an 80% to 100% reduction in GPAs was evident for T_2 transgenic *Arabidopsis* plants expressing ds*ccap,* ds*jhbp* and ds*nplp1,* and complete mortality was recorded for the hp*ccap* event 3. The results obtained from two transgenic generations and two model plants therefore indicate that the genes studied were vital for the GPA life cycle and knocking down of these genes affects their fecundity or survival.

An *in vitro* study was also conducted to evaluate the effects of silencing five different lengths of dsRNA from different regions of the same EST putatively encoding the JHBP protein, as well as siRNAs of the gene generated *in vitro* from digestion with an RNAseIII enzyme. The longest dsRNA (284 bp) was the most effective in inducing RNAi effects on treated nymphs, since there were more restricted movements in aphids 24 hours after exposure, and the fewest

offspring were produced in the longer-term. One of the shorter dsRNAs (86 bp long, not the shortest,70 bp), also significantly reduced GPA movement, survival and reproduction at levels similar to that of the longest dsRNA. These results show that RNAi effects can vary with the target region from which the hp dsRNA is derived and in this case silencing was more effective for one of the sequences derived from the 3´ region. This study indicates that both the length of the dsRNA and the specific sequence chosen can influence the effectiveness of RNAi.

This project provides new information on GPA neuronal genes as novel candidates for its control *via* gene silencing. It also offers additional data to achieve better RNAi effects related to the target sequence selected. The *in planta* RNAi study also demonstrated that RNAi can be used as a new strategy to control this important crop pest, and its use, either alone or in combination with other gene targets, is discussed.

List of Conferences

Oral Presentations

Sharmin Rahman, John Fosu-Nyarko and Michael G.K. Jones (2017). Targeting vital neuronal genes for aphid control by gene silencing technology. Murdoch Agricultural Research Symposium, Murdoch University, September 2017.

Sharmin Rahman, John Fosu-Nyarko and Michael G.K. Jones (2016). RNA interference: a promising natural strategy for crop pest control. ComBio2016, Brisbane, Australia, October 2016.

Sharmin Rahman, John Fosu-Nyarko and Michael G.K. Jones (2016). RNA interference of insect neuronal signalling genes: a promising tool to control green peach aphid. 7th International Crop Science Congress, China, August 2016.

Sharmin Rahman, John Fosu-Nyarko and Michael G.K. Jones (2015). RNA interference of neuronal signalling molecules: an ecofriendly weapon to control green peach aphids. The 17th Annual Postgraduate Symposium, The Royal Society of Western Australia, Murdoch University, October 2015.

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Abbreviations

35S	35S RNA transcriptional promoter of CaMV
BLAST	Basic Local Alignment Search Tool
bp	Base pair
cDNA	Complementary DNA
cv	Cultivar
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide mix
dsRNA	Double-stranded RNA
EDTA	Ethylenediaminetetra-acetate acid disodium salt
Kb	Kilo base
mRNA	Messenger RNA
ng	Nanogram
PCR	Polymerase chain reaction
PTGS	Post transcriptional gene silencing
RNase	Ribonuclease
RT	Reverse transcription
siRNA	Small interfering RNA
TAE	Tris-acetate-EDTA

(Other abbreviations are provided in the text)

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Chapter 1

General introduction and literature review

1.1 Introduction

Aphids (Insecta: Hemiptera) are sap-sucking hemimetabolous (incomplete metamorphosis) insects, belonging to the superfamily Aphidoidea. They are one of the most destructive agricultural pests worldwide and are also crop pests in Australia. They cause considerable economic losses when they infest a wide range of cultivated crops (Blackman and Eastop, 2007; Valenzuela and Hoffmann, 2015). Among aphid species, the green peach aphid (GPA), (*Myzus persicae*), is one of the most important pests of both field and glasshouse plants (Blackman, 1987; Horsfall, 1924). Unlike many other aphid species, the host range of the GPA is quite broad, and also act as vectors for more than 100 plant viruses. GPAs have also developed resistance to many insecticides which are being most widely used as method of control. There is therefore a need to look for new, sustainable control strategies to protect crops against this aphid pest. One possible emerging strategy for their control is gene silencing technology or RNA interference (RNAi), which is based on delivering double-stranded RNA (dsRNA) to down-regulate vital genes in target organisms. RNAi has already been established as a valuable molecular tool for functional characterisation of genes in many organisms including nematodes, plants and insects. The technology is being investigated as an approach to control pests *via* transgenic plants, and through development of novel biopesticides based on spraying dsRNA onto plants.

The effectiveness of RNAi very much depends on the selection of appropriate target genes. The recent availability of insect genomic data provides new opportunities to identify vital genes for insect control, using *in silico* analyses to provide candidate genes to work on (Thompson and Goggin, 2006). Silencing of the essential insect genes may cause a range of phenotypic effects, such as abnormal behaviour or lethality in target organisms. However, so far, little attention has been paid to molecules involved in insect neuronal signalling pathways as

possible targets for biological control. Such targets include mostly Neuropeptides (NPs) and G-protein coupled receptors (GPCRs): these molecules can also be described as neuronal signalling molecules (NSMs), in general. NSMs are functionally diverse and their expression can have pleiotropic effects, as noted from hydrozoans to humans (Nässel, 2000). Insect NPs act mostly *via* membrane specific GPCRs (Broeck, 2001; Hewes and Taghert, 2001; Nässel, 2002). Therefore, interference in the specific signalling cascades they control can result in disruption of important biological processes. In extreme cases, this interference could lead to the death of the organisms. At the start of this project, there was little information on targeting insect NSMs using the gene silencing approach, particularly for aphids. In this work, RNAi technology has been used to evaluate the potential of targeting insect NSMs to develop a new strategy for aphid control for crop protection.

1.1.1 Aphid morphology and distribution

Aphids are pear-shaped and soft-bodied insects exhibiting a wide colour range, such as green, black, brown, yellow, pink or mixed colours (Blackman and Eastop, 2000; Dixon, 1973). The adult body varies from 1.5 mm to 10 mm in length depending on the species (Dixon, 1973). The female aphid gives birth directly to nymphs that resemble adults but are smaller in size and have no wings. Adults may also be wingless. These insect species have two compound eyes, and piercing and sucking mouthparts, containing a needle-like structure, the stylet through which it can ingest phloem cell contents. Different species can be distinguished by variations in size of body parts, such as antenna, wings, and cauda (aphid's tail) (Dixon, 1973). About 100 aphid species are recognised as agricultural pests because of their significant economic damage to a range of crop plant species (Blackman and Eastop, 2007). Aphids are found in almost all parts of the world, especially in temperate zones of the Northern hemisphere (Blackman and Eastop, 2000). Though they are weak fliers, some species can travel relatively

long distances being carried by winds or major weather systems. Only a small number of aphid species are believed to be native to Australia (Manners, 2016) that might be due to the geographical isolation of Australia.

1.1.2 Life cycle and factors determining aphid polyphenism

Aphids reproduce both sexually and asexually and thus have a comparatively complex life cycle (Dixon, 1973). Depending on the host plant, the aphid life cycle is either heteroecious (host-altering) or autoecious (non-host-altering) (Williams and Dixon, 2007). Heteroecious aphids live on primary host plants in winter, migrate to secondary hosts in summer and finally return to former host species in autumn. In autumn, sexual morphs are produced, and over-wintering eggs are laid by sexual females. The following year when the temperature rises, eggs hatch and several parthenogenetic generations develop (Figure 1.1). GPAs, cowpea aphids (*Craccivora aphis*) and black bean aphids (*Aphis fabae*) are some examples of host-altering aphid species (Williams and Dixon, 2007). Autoecious species usually live either on a single host plant or migrate between closely-related plant species. The pea aphid (*Acyrthosiphon pisum*), the Russian wheat aphid (*Diuraphis noxia*), the soybean aphid (*Aphis glycines*) and the rosy apple aphid (*Dysaphis plantaginae*) are some examples of autoecious species.

Some aphid species alternate parthenogenetic with sexual reproduction (a practice termed as holocyclic), whereas some species never mate and are known as anholocyclic. Factors that determine the development of sexual or asexual morphs include day length, temperature and host plants (Dixon, 1973). Mostly, factors such as overcrowding, poor quality of host plant, the presence of predators, low temperature with a short photoperiod and intrinsic features are crucial for asexual winged aphids (Dixon 1973, Williams and Dixon 2007). On average, an aphid undergoes four moults to become an adult while the nymphs become mature to reproduce within 4-7 days after birth in summer and the average life span is between 20 and 25 days

(Caon and Burfield, 2006). Depending on the aphid spices, each female aphid can produce a total of between 50 to 100 nymphs and become reproductive in four to seven days (Horsfall, 1924; Caon and Burfield, 2006). Aphids rarely live beyond one month (Baumann et al., 1995). The ideal temperature for aphid growth is about 22°C with maximum activity in warmer seasons, whereas no development occurs below 5°C and above 33°C (Caon and Burfield, 2006).

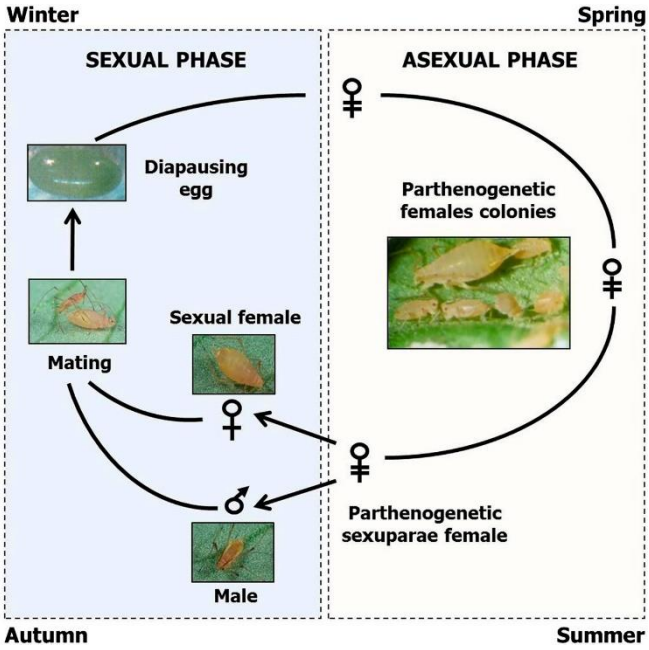


Figure 1.1: Life cycle of *A. pisum* (Tagu et al., 2014)

Polyphenism is one of the important factors for rapid multiplication and successful adaptation of aphids and this occurs in response to environmental changes (seasonal morphs) or temporal differences in living conditions (dispersal morphs). Aphids exhibit wing polyphenism (winged and unwinged) (Simpson et al., 2011) and colour polyphenism (Blackman and Eastop, 2000). Wing polyphenism is common in almost all species, but colour variations are reported in some species such as the cotton aphid and the GPA (Williams and Dixon, 2007).

1.1.3 Feeding habit of aphids

Aphids feed on plant sap and ingest fluids through their stylets from sieve elements in the phloem or mesophyll cells or a combination of these tissues (Chougule and Bonning, 2012). Their mechanism of feeding involves leaf surface penetration, stylet movement through the cell wall space (apoplast) with probing of mesophyll cells, saliva secretion into sieve elements, followed by ingestion of phloem sap (Jaouannet et al., 2014; Tjallingii and Esch, 1993). The aphid stylet is a modified piercing and sucking mouthpart which includes two mandibular and maxillary parts, a salivary canal and nutritional canal. The outer mandibular parts have a nerve canal whereas the inner maxillary parts form a nutritional channel and these merge at the tip of the stylet (Chougule and Bonning, 2012; Will et al., 2013). During probing, their flexible stylets move through the apoplast and transiently puncture the epidermal, mesophyll and parenchyma cells to ingest small amounts of sap without causing any major damage to plants. This behaviour helps the aphid both to sample host plant cell contents and to detect when the stylet reaches a sieve tube (Miles, 1999; Will et al., 2013). It has been suggested that aphids use two main signals: sucrose and pH, to recognise the sieve tube for stylet penetration and feeding (Hewer et al., 2010). During this interaction, aphid species secrete two types of saliva, a gel saliva and a watery saliva, both of which are important during feeding (Miles, 1999). The gel saliva forms the hard layer called salivary sheath, which protects the stylet from chemical and mechanical injury and facilitates stylet movement during ingestion (Tjallingii, 2006; Will et al., 2012). On the other hand, the watery saliva, is secreted either during stylet probing or continuously at the feeding site during sap ingestion (Martin et al., 1997; Miles, 1999; Will et al., 2013). Aphid saliva contains different types of enzymes, predominantly proteases that contain some anti-clogging products. These molecules can interfere with plant defence signalling cascades, so reducing the host's ability to repair the wound *via* clogging (De Vos and Jander, 2009; Goggin, 2007; Will et al., 2013). However, as phloem sap is rich in

carbohydrates and deficient in nitrogenous compounds (Baumann et al., 1995), aphids have to ingest a large amount of this nutritionally 'incomplete plant' sap. As a result, they must process large volumes of dilute plant sap and need to regulate osmotic pressure in the gut and hemolymph. The excess ingested plant sap is excreted as honeydew.

1.1.4 Economic importance of aphids

Aphids can damage host plants both directly and indirectly. Most common symptoms of direct attack which result from feeding are chlorosis, necrosis, stunting or malformation of young leaves, buds, flowers and stems (Goggin, 2007). Some aphid species, such as the spiral gall aphid (*Pemphigus spirothecae*) and the lettuce root aphid (*Pemphigus bursarius*), may cause galls on young leaves (Dixon, 1973). As vectors of plant viruses, aphids can transmit about 275 viruses out of 600, including Potyviruses, the largest plant virus group. Some examples of aphid transmissible viruses are bean common mosaic virus (BCMV), cucumber mosaic virus (CMV), alfalfa mosaic virus (AMV), pea seed-borne mosaic virus (PSbMV) and subterranean clover red leaf virus (SCRLV). Crop yield losses resulting from aphid feeding and associated virus diseases has been estimated and reviewed in many important crops in different geographical regions including Australia (Culliney, 2014; Tatchell, 1989; Valenzuela and Hoffmann, 2015). A summary of yield losses in some crops resulting from aphid infestation is presented in Table 1.1.

Table 1.1: **Crop yield losses in some important crops resulting from aphid infestation**

Crop	Aphid species	Type of aphid injury	% yield loss	Country	References
Wheat	*R. padi, R. maidis*	Direct and indirect	1.5	Australia	Murray et al., 2013
	S. avenae	Direct	10-13	UK	George and Gair, 1979; Rabbinge et al., 1981
	D. noxia	N/A	50-83*	USA	Mirik et al., 2009
	Cereal aphids	Direct	35-40	Pakistan	Khan et al., 2015
		Vector	20-80		
Barley	*R. padi, R. maidis*	N/A	1.24	Australia	Murray et al., 2013
	R. padi	Vector	0-86[1]	UK	Doodson and Saunders, 1970
	Metopolophum dirhodum	Direct	8.8	UK	George, 1974
Oat	*R. padi, R. maidis*	N/A	0.79%	Australia	Murray et al., 2013
Corn	*R. maidis*	N/A	10-53*	USA	Everly, 1960
Canola	*Brevicoryne brassicae, M. persicae, Lipaphis erysimi*	Direct and indirect	2.2	Australia	Murray et al., 2013
Brassicae spp.	*B. brassicae, L. erysimi*	N/A	70-80*	Pakistan	Razaq et al., 2011
	L. erysimi	N/A	10-90*	India	Rana, 2005
Lupin	*Aphis craccivora, Acyrthosiphon kondoi*	N/A	4.3	Australia	Murray et al., 2013
	A. kondoi, M. persicae	Direct	25.3	Australia	Berlandier and Sweetingham, 2003
		Indirect (CMV, BYMV)	15.9	Australia	Jones, 1993
Lentil	Not defined	Direct	4.5	Australia	Birchip Cropping Group. 2001 (Not published)
	A. konodi, A. craccivora, M. persicae	Indirect (AMV, CMV)	84.9	Australia	Latham et al., 2004
Chickpea	Not defined	Indirect (AMV)	98	Australia	Latham et al., 2004
Pea	*A. pisum*	Direct	8-16	UK	Maiteki and Lamb, 1985
		Indirect	13.9	Australia	Coutts et al., 2009
Potato	*M. euphorbiae,*	Direct	5.7	UK	Kolbe, 1970
	M. persicae	Direct	4.4	UK	Southall and Sly, 1976
Sugarcane	*Ceratovacuna lanigera*	N/A	30*	India	Galande et al., 2005

***** under standard cultural practice or without control measure; **1**: subject to variety and infection time

In most cases, aphid damage reports only highlight yield losses due to aphid feeding and/or virus transmission, but do not include other economic losses such as the costs of applying pesticides or other cultural controls practised in the field. In Australia, about $18 million p.a. is spent on insecticides to control various cereal aphids (for feeding damage only), and at least $4 million p.a. for canola (Murray et al., 2013). In Western Australia alone, direct feeding injury from aphids results in mean yield losses of 10.7% in wheat, 9% in canola and 13.1% in lupins (Berlandier and Carmody, 2004; Berlandier and Sweetingham, 2003; Michael, 2003). Some of these data are now out of date, and there is a need to update them. There is also a lack of good data in Europe, where information detailing aphid damage has been limited after 1985 (reported in the UK). There was a single report on aphid feeding damage in Europe in the late 80s, which determined average annual losses of $700K per a tonne of wheat, $850K per a tonne of potatoes and $2,000K per a tonne of sugar beet (Wellings et al., 1987).

Alarmingly, in June 2016, in South Australia (SA), 30 ha of land was detected with heavy infestation of *D. noxia* which severely reduced wheat production. Feeding damage generally results in yield losses of up to 10%, but elsewhere *D. noxia* has caused yield losses of more than 80% (https://www.agric.wa. gov.au/barley/biosecurity-alert-russian-wheat-aphid). Infestation with *D. noxia* has now been found to be much more widespread, with its presence confirmed in wider areas of SA, Victoria and New South Wales. It prompted substantial biosecurity measures and research to introduce resistance genes available in the USA and elsewhere into Australian wheat germplasm. Additional losses from aphid infestation include growth of sooty moulds facilitated by honeydew excretion, which interferes with plant photosynthesis in severe cases, and reduces the aesthetic value of ornamental plants (Dixon, 1973; Quisenberry and Ni, 2007).

1.1.5 Common control strategies for aphid infestation

Aphids have a relatively rapid multiplication rate, and it is important to put control strategies in place as soon as they reach or exceed a determined pest threshold. Integrated management practices are commonly used to control early aphid infestation and spread of aphid transmissible viruses, using a combination of biological, mechanical and chemical methods (Quisenberry and Ni, 2007). Different predators and parasites like spiders, birds, flies, wasps, lady bird beetles are used to lower the aphid populations. In Australia, so far, four different biological agents are commercially available: a green lacewing (*Mallada signata*), a pirate bug (*Orius armatus*) and two wasp species (Manners, 2012). *M. signata* has a wide host range including aphids, whereas *O. armatus* can be used to control aphids and thrips. The two commercially introduced wasps are *Aphidius colemani* and *Aphelinus abdominalis,* of which the former species can parasitise more than 40 different aphid species including GPA. However, plant cultivars and sometimes aphid species also affect the effectiveness of parasitic wasps (Kalule and Wright, 2002). Entomopathogenic fungi from the Zygomycota and Ascomycota divisions, are also biological agents that can be used for small scale control of aphid populations (Völkl et al., 2007). Some cultural practices, such as mulching, adjusting crop density, changing sowing time, managing crop rotation are also recommended to keep aphid colonies and infestation under threshold levels (Wratten et al., 2007). To control viruses transmitted by aphids, careful monitoring of aphid populations is often undertaken at specific periods during the year and use of virus-free seed is highly recommended for some crops, e.g. potato seeds (Katis et al., 2007).

Another option for aphid control is to use resistant plant varieties and aphid resistance genes are available for many crops such as legumes, cereals, fruit trees and vegetables (Dogimont et al., 2010). For instance, *RAP1* gene in *Medicago truncatula* for *A. pisum*, multiple Rag genes

(*Rag1*, *Rag2* and *Rag3*) in glycine max for *A. glycines*, *Dn2414* from *T. aestivum* L.for *D. noxia* (Peng et al., 2007; Stewart et al., 2009; Zhang and Wang, 2009; Zhang and Wang, 2010). In WA, a narrow leaf lupin variety, 'Kalya', has been reported to show higher resistance to aphid species (Lawrence, 2001). Similarly, a peach cultivar 'Rubira' which is GPA-resistant carries a single dominant gene (*Rm2* gene) for resistance (Pascal et al., 2002; Sauge et al., 2002). However, most of the resistance genes that have been identified are present in unimproved landraces, wild accessions or related species, and incorporation of these resistance genes into commercial cultivars requires long and complex breeding programs (Dogimont et al., 2010). In commercial agriculture, chemical control is still the most common strategy to control these pests (Bendena, 2010; Dedryver et al., 2010). Because of the non-selective action of chemical insecticides, beneficial insects are also killed, and this raises a question about the general application of the chemicals to field crops for insect control. Many of the insecticides used are not environmentally-friendly or cost-effective. Many aphid species such as *A. gossypii*, GPA, *A. fabae* are known to have developed resistance to some insecticides (Foster et al., 2007). The first widespread resistance to chemical insecticides (organophosphorus insecticides, OP) was reported for GPA in 1955 in Asia, Europe, USA and Australia (Anthon, 1955).

Biotechnological tools like transgenic plants are also being used to control insect pests. For example, *Bt* transgenic crops have been used successfully against Lepidopteran and Coleopteran insects, but these have a very low level of toxicity against the sap-sucking insects. Unfortunately, some resistance has developed against major Lepidopteran insect pests (e.g. strains of *Plutella xylostella*, *Pectinophora gossypiella*, *Helicoverpa armigera* and *Helicoverpa zea*) and they have shown resistance to *Bt* crops in laboratory tests as well as in the field in many countries including Australia (Tabashnik et al., 2013; Tabashnik et al., 2003). Nevertheless, *Bt*-based resistance has been remarkably successful in protecting cotton and

maize against Lepidopteran pests, and the set-aside or refugee areas of non-*Bt* plants has helped ensure the durability of the biotech method of crop protection despite its very wide application worldwide.

1.1.6 Green Peach Aphid

Unlike many other aphid species, GPA is an extremely polyphagous, cosmopolitan insect which demonstrates phenotypic plasticity (different colour morphs and life cycles). In favourable environments, the GPA completes its life cycle in less than two weeks (Moran, 1992; Van Emden et al., 1969). The particular features of GPAs are their ability to adapt rapidly to new host plants, their efficient transmission of many virus diseases and their ability to develop resistance rapidly to chemical insecticides (Anthon, 1955; Foster et al., 2007; Umina, 2016). Although this heteroecious species shows extreme primary host specificity (*Prunus persicae* or genus *Prunus*), their secondary hosts include hundreds of plants from 40 families such as Solanaceae, Chenopodiaceae, Compositae and Cruciferae (Blackman and Eastop, 2000). Like other species, GPA prefers to feed on young plant tissue, and this can result in water stress, wilting, leading to reduction in crop yields and reduced crop quality. This species can transmit about 110 viruses both persistently and non-persistently. In Australia, Cucumber Mosaic Virus (CMV) and Bean Yellow Mosaic virus (BYMV), two non-persistent viruses, are particularly a problem in lupins and are mostly transmitted by GPAs (Berlandier et al., 2010; Blackman and Eastop, 2000). Its wide host range, fast reproductive rate and development of insecticide resistance makes GPA a major crop pest. As recently as late 2016 the GRDC declared that GPA populations in Australia had become resistant to another insecticide group, the nicotinoids. Now, in Australia, GPA populations are resistant to four major chemical groups: organophosphates, carbamates, pyrethroids and recently nicotinoids (Umina, 2016). Figure 1.2 illustrated the present status of populations of GPA and their resistance to

insecticides (except for nicotinoids) in Australia. Taking all these aspects into accounts, it is evident that alternative, sustainable control strategies are needed to protect crops against devastating aphid pests. One potential strategy is to use RNA interference of essential genes as a means to controlling their populations (Price and Gatehouse, 2008; Zhang, Li, et al., 2013).

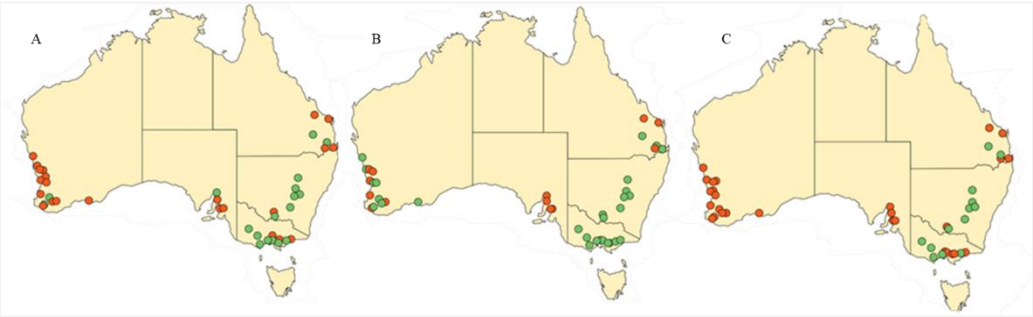


Figure 1.2: Location of GPA insecticide resistance populations in Australia. **A)** GPA resistant to carbamates; **B)** GPA resistant to organophosphate and **C)** GPA resistant to synthetic pyrethroids. Red and green circles indicate populations resistant and susceptible to insecticides) (Umina et al., 2014).

Taking all these aspects into accounts, it is evident that alternative, sustainable control strategies are needed to protect crops against devastating aphid pests. One potential strategy is to use RNAi to control insect of various orders *via* silencing essential genes in target insect pests under study (Price and Gatehouse, 2008; Zhang, Li, et al., 2013).

1.2 History of discovery of RNA interference

RNA interference (RNAi) is a sequence specific down-regulation process that is highly conserved among higher eukaryotes (Carthew and Sontheimer, 2009; Kim et al., 2015; Sen and Blau, 2006). RNAi naturally works as an antiviral defence mechanism in plants, and is now being studied as a control strategy for destructive insect pests of crops.

The RNA silencing mechanism was first observed in 1990 in studies on *Petunia hybrid* to generate violet/purple flowers for commercial purposes, by over-expression of the chalcone

synthase gene in the colour pathway (Napoli et al., 1990). Surprisingly, the flowers of modified plants expressed a surprising range of pigmentation: deep purple, white with pigmented purple and in some cases, flowers were completely white, due to the simultaneous suppression of the transgene as well as the endogenous gene. This phenomenon was initially defined as 'co-suppression'. After two years, a similar phenomenon was found in transformed *Neurospora crassa* (an ascomycete fungus) for two genes, *albino-1* (*al-1*) and *albino-3* (*al-3*) during experiments to intensify the orange colour phenotype of the fungus (Romano and Macino, 1992). Besides getting the intended colouration, a wide range of phenotypes ranging from albino to dark colour were obtained, and the phenomenon was named 'quelling'.

After these reports of co-suppression and quelling, gene silencing was also found in *C. elegans* by Guo and Kemphues (1995). They tried to block the expression of *par-1* (partition 1) gene by introducing antisense RNA, which was thought to bind to the corresponding endogenous mRNA, consequently silencing the gene. In this experiment, they also used sense RNA of the gene as control. Surprisingly, they found that both the sense and antisense strands can induce silencing effectively (Guo and Kemphues, 1995). In parallel, work on plant viruses also suggested involvement of sense and anti-sense RNA to induce RNAi (Waterhouse et al., 1998). The question of how the sense strand induced the silencing effect, as it could not hybridise to the endogenous sense mRNA, was solved in a breakthrough publication by Fire et al. in 1998. They hypothesised that the initial trigger for gene silencing was double stranded RNA (dsRNA) and not the single stranded RNA (ssRNA). They explained the results of Guo & Kemphues (1995) by suggesting that their experiment may have been contaminated by dsRNA during the preparation of the sense and antisense RNA. In the same year (1998), RNAi was also first applied to *Drosophila melanogaster* to investigate the efficiency of dsRNA to inhibit target gene expression (Kennerdell and Carthew, 1998).

1.2.1 Mechanisms of RNAi

The two major RNAi processes are small interfering RNAs (siRNA)-mediated RNAi in which, mostly, exogenous longer dsRNAs are processed into siRNAs and the microRNA (miRNA)-mediated RNAi pathway, which is mainly directed at controlling expression of endogenous genes. The RNAi process can be divided into two separate steps: an initiation step and an execution step. The initiation phase mainly involves the generation of siRNAs from long dsRNA or mature miRNAs from the primary transcripts (usually 21-25 nucleotide) by the RNAseIII type enzymes, Dicer and Drosha (Bernstein et al., 2001). As siRNAs initiate the RNAi process, these two enzymes can be referred as RNAi initiator enzymes. Hamilton and Baulcombe (1999) first studied the importance of siRNAs in the post transcriptional gene silencing process (PTGS) in detail in tomato plants. Within a cell, dsRNA can be present from artificial introduction, aberrant transgenes, transposons, RNA viruses or short endogenous hairpin RNAs (Hannon, 2002). The processing of dsRNAs into siRNAs in all organisms suggests that they share a common mechanism for the initiation of the RNAi pathway (Bernstein et al., 2001; Geley and Muller, 2004).

To start the execution step, siRNA duplexes generated in the initiation step are assembled into the RISC (RNAi Silencing Complex), the multi protein effector complex. This step involves RISC loading and unwinding of siRNAs. At first, siRNAs are incorporated into AGO proteins which is termed pre-RISC (Kawamata and Tomari, 2010). The double strands are then unwound leading to activation of the RISC. The activated RISC with the single RNA strand is called holo-RISC, mature RISC or simply RISC (Kawamata and Tomari, 2010). Of the two single strands, antisense remains attached to the RISC and is called the “guide strand”, and the discarded strand is called “passenger strand” (Khvorova et al., 2003). The strand selection occurs at the pre-RISC stage following the ‘asymmetry rule’ which is not random and the strand

with less stability at the 5´ end acts as guide strand for the RNAi process (Khvorova et al., 2003; Schwarz et al., 2003). If the pairing of siRNA-target mRNA is matched enough, RISC cleaves the phosphodiester backbone of mRNA (usually in the middle of the complementary region) (Meister and Tuschl, 2004). However, this cleavage step is ATP independent and siRNA remains intact and allow RISC to act continuously. Figure 1.3 illustrated the siRNA-mediated RNAi pathway present in eukaryotes.

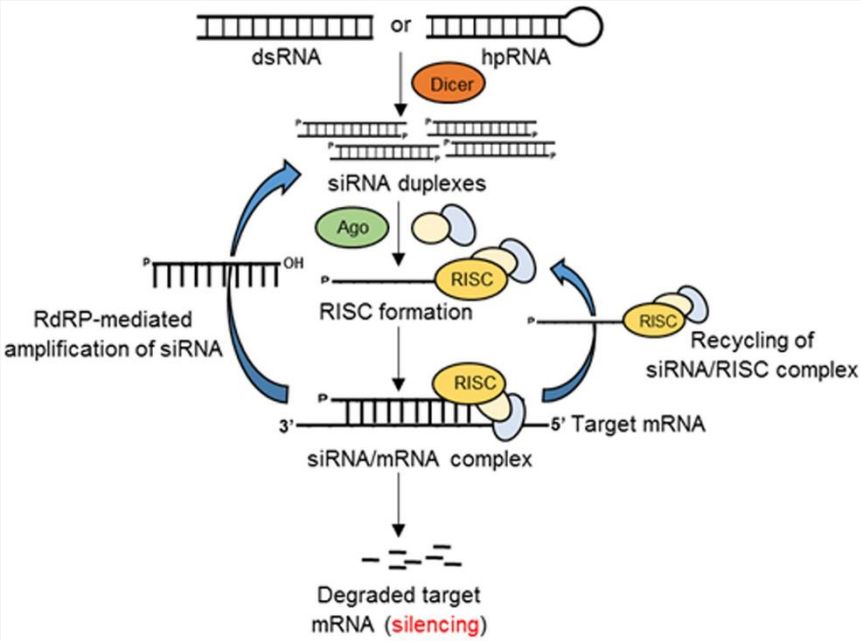


Figure 1.3: Small interfering (siRNA) inducing gene silencing pathway in Eukaryotes (Majumdar et al., 2017).

1.2.2 Core machinery of RNAi

Intensive research had been undertaken using both genetic and biochemical approaches to identify multifunctional proteins now known to be actively involved in the RNAi pathways in many organisms. Two major conserved groups of these proteins, regarded as key regulators for the process, are the Dicer and dicer-like proteins and argonautes, both of which are associated with RNAi involving siRNAs and miRNAs.

Dicer and Dicer-like proteins: The RNAi process is commonly initiated by endogenously or artificially supplied dsRNA to a cell which is cleaved into siRNAs and/or miRNAs (Parrish et al., 2000). The dsRNAs are cleaved by the dsRNA-specific RNaseIII-type endonuclease which is known as Dicer (Bernstein et al., 2001). Dicer or Dicer-like proteins are evolutionarily conserved in many organisms, such as, fission yeast, plants, insects, nematodes, and mammals (Bernstein et al., 2001; Bernstein et al., 2003; Golden et al., 2002; Knight and Bass, 2001; Park et al., 2002; Provost et al., 2002; Volpe et al., 2002). Dicer cleaves dsRNAs with 3´overhangs of 2-3 nucleotides (nt) on both strands and with unphosphorylated hydroxyl groups (Elbashir et al., 2001). Interestingly, Dicers (*dcrs*) have been reported to play other functional roles in many organisms. For instance, *dcr*-1 mutants of *C. elegans* showed resistance to RNAi for germline expressed genes, whereas normal RNAi was reported for somatic genes. The mutant worms were also reported to be sterile, suggesting a crucial role of the *dcr1* gene in germline development along with gene silencing (Knight and Bass, 2001). Dicers have several conserved domains: an amino-terminal helicase domain, dual RNase III motifs, a carboxy-terminal dsRNA binding domain (DBD) and a Piwi/Argonaute/Zwille (PAZ) domain (Bernstein et al., 2001). However, the RNA helicase and PAZ domains are absent in *D. melanogaster drosha,* another RNaseIII-type endonuclease (Filippov et al., 2000). The helicase domain is known to be involved in unwinding of dsRNA, and the RNAse motifs are involved in cleavage of dsRNA into smaller fragments. Blaszczyk et al. (2001) proved that single domain bacterial RNaseIII cut dsRNAs at an 11 nt interval.

Different numbers of dicer (or dicer-like) genes have so far been identified for different species. It appears mouse, human and *C. elegans* genomes encode one dicer gene whereas *Drosophila* and *Arabidopsis* have two and four dicer-like genes, respectively. For insects, *A. pisum,* as well as other aphid species have two copies of *dcr1* genes (Jaubert-Possamai et al., 2010). Similar

to *dcr1*, *Drosophila* also has *drosha* for miRNA biogenesis while *dcr2* is essential for the siRNA-directed pathway (Le et al., 2003; Lee, , et al., 2004). *Drosophila* dicers have other dsRNA binding proteins as cofactors: Loquacious (Loq) for *dcr1* and R2D2 for *dcr2* (Liu et al., 2003; Saito et al., 2005). Although *A. pisum* and *A. glycines* genomes contain most RNAi-related genes, major duplications in the miRNA pathway genes have been reported in *A. pisum*; e.g. four copies for the *drosha* cofactor, i.e. *pasha*, and two copies of miRNA specific *dcr1*). For *A. glycines*, the conserved RNAi genes (such as Dcr2, Ago2 and R2D2) have been found to be constantly expressed in different tissues and nymphal stages suggesting a robust and active RNAi pathway in this species (Bansal and Michel, 2013).

Argonaute proteins: Argonaute (AGO) proteins are key components of the RISC (Song et al., 2004). They have two distinct domains: an amino-terminal PAZ domain that is also present in dicer proteins, and a unique carboxy-terminal Piwi domain (Song and Joshua-Tor, 2006). The PAZ domain is reported to recognise the 5´ ends of ssRNA, and this step is crucial to the gene silencing process (Lingel et al., 2003; Song et al., 2003). The Piwi domain has structural and functional similarity to RNase-H that cleaves target mRNAs by its endonucleolytic activities (Song et al., 2004). The number of AGO proteins identified for different organisms range from one (*Schizosaccharomyces pombe*) to 28 (*C. elegans*) (Carmell et al., 2002, Yigit et al., 2006). *D. melanogaster* and *T. castaneum* genomes encode five AGOs whereas *Arabidopsis* has 10 AGOs (Hunter et al., 2003, Tomoyasu et al., 2008). Most of the insects possess a single *ago1* and *ago2* gene; there are two copies of the *ago1* gene reported for *A. pisum* (Jaubert-Possamai et al., 2010). In *Drosophila,* AGO1 is suggested to be involved in the miRNA pathway while AGO2 is necessary for the siRNA-mediated pathway (Okamura et al., 2004).

1.2.3 Factors determining successful RNAi effects in insect pests

Although the RNAi machinery is conserved in eukaryotes, efficiency of targeted silencing of genes *via* introduction of exogenous dsRNA can vary considerably between different organisms (Scott et al., 2013). For insects, several factors that influence the level of silencing of target genes have been investigated. These include the target gene sequence, dsRNA concentration and length, life stage of target insects and the mode of delivery of the dsRNA or silencing triggers. Different concentrations of dsRNA have been reported to induce similar levels of gene silencing of the same gene (e.g. *v-ATPaseA, v-ATPaseE or snf7*) in different organims (Baum et al., 2007; Baumann et al., 2009; Bolognesi et al., 2012; Coy et al., 2012; Li et al., 2011; Upadhyay et al., 2011; Whyard et al., 2009). However, as reported for *D. virgifera* and *A. pisum,* increasing the concentration of the silencing triggers does not necessarily result a corresponding change in the observed phenotypes (Meyering-Vos and Müller, 2007; Shakesby et al., 2009).

Another crucial aspect which affects RNAi in insects is the choice of sequence for dsRNA synthesis, especially dsRNA lengths and sequence homology. Available reports indicate that dsRNA sizes from 134 bp to 1842 bp can induce successful gene knockdown in insects *via in vitro* feeding, although generally 300 - 520 bp long dsRNAs are used (Baum et al., 2007; Huvenne and Smagghe, 2010). Although there is less information regarding RNAi in aphids, most reports indicate that longer dsRNA (≥ 50 to 200 bp) performed better than short dsRNA/siRNAs as observed for other insects (Bolognesi et al., 2012; Huvenne and Smagghe, 2010; Kumar et al., 2009; Li et al., 2015; Mutti et al., 2006). However, to obtain specificity, in other words, to avoid any off-target effects, it may be important to design shorter dsRNAs. In addition to the length of dsRNAs used, different gene regions (5´ or 3´ end) have also generated variable silencing results in insects. While the RNAi trigger designed from the 3´ end of a target

gene yielded greater mortality in *A. aegypti* and *D. virgifera*; no differences between sequence of the 5´ ends or 3´ ends was found for *A. pisum* (Li et al., 2015; Mao and Zeng, 2012; Pridgeon et al., 2008). These variable observations demonstrate that it is necessary to screen a range of sequences for a target gene, and this activity may also help to reduce the development of resistance by pests by varying the target sequence used to silence the gene of interest.

A major factor that also influences the effectiveness of RNAi of genes of insects is a characteristic feature of environmental RNAi: the mode of delivery of silencing triggers to insect cells, cellular uptake from outside the cells, and systemic RNAi i.e. the spreading of RNAi signals throughout the insect body. Environmental RNAi has been affected in many insect species through injection of specific amounts of long dsRNA and siRNAs into the insect body or by *ad lib* feeding of dsRNA in a diet mainly made of 30% sucrose or *via* transgenic plants (Bai et al., 2011; Baum et al., 2007; , Gong et al., 2014, Mao et al., 2007; Mutti et al., 2006; Zhang, et al., 2013, Pitino et al., 2011). Once ingested or introduced, dsRNAs or siRNAs are taken by cells through transmembrane channel-mediated uptake or by endocytosis. The first process involves the protein, SID-1 which mediates passive, ATP independent, uptake of dsRNA in many organisms, but not in *D. melanogaster*, the most studied Dipteran insects to date (Saleh et al., 2006). Unlike plants and animals, the presence of a SID-2 protein, also a transmembrane protein required for environmental-mediated RNAi, has not been confirmed in insect genomes so far (Baum and Roberts, 2014; Tomoyasu et al., 2008; Winston et al., 2002; Xu and Han, 2008; Zha et al., 2011). Moreover, *in silico* analysis showed that insect SID-1 protein shares more sequence homology to the *C. elegans* TAG-30 rather than to SID-1 and therefore, this suggests that for insects, the SID-1 may not be essential for dsRNA uptake or systemic RNAi (Tomoyasu et al., 2008).

Compared to other insects, an energy-dependent endocytic mechanism mediated by scavenger receptors (namely, SR-C1 and Eater) has been reported for *D. melanogaster* to be responsible for cellular uptake of dsRNA (Saleh et al., 2006; Ulvila et al., 2006). For some organisms, e.g. plants, nematodes and fungi, after the entry of dsRNAs into cells, silencing signals are amplified leading to perpetuation of RNAi through generation of secondary siRNAs initiated by the unincorporated sense strand of primary siRNAs, in a process termed 'transitive RNAi'. A key protein for transitive RNAi is the RNA-dependent RNA polymerase (RdRP) (Geley and Muller, 2004). Interestingly, the presence and activity of RdRPs have not yet been discovered in any insect genome suggesting that RNAi in these organisms are only mediated by primary siRNAs (Gordon and Waterhouse, 2007; Tomoyasu et al., 2008) or if transitive RNAi exists then it is likely that the role of the RdRP in this process is undertaken by proteins yet to be discovered (Zhang, Li, et al., 2013).

1.2.4 Applications of RNAi as an insect pest control strategy

Suppression of important insect genes *via* RNAi has been demonstrated successfully for insects of several orders (e.g. Coleoptera and Hymenoptera) in the laboratory as well as in the field (Chu et al., 2014; Hunter et al., 2010; Zhang, Li, et al., 2013). *In vitro* delivery of dsRNA to insects and transgenic plant-mediated RNAi has been used to demonstrate the potency of RNAi as a control strategy for important crop pests, e.g *D. virgifera, Diabrotica undecimpunctata, T. castaneum, H. armigera, Nilaparvata lugens, A. pisum* and GPA (Baum et al., 2007; Bhatia et al., 2012; Mao and Zeng, 2012, 2014; Mao et al., 2007; Pitino et al., 2011; Zha et al., 2011). This strategy has also been explored for the protection of beneficial insects from pathogens or parasites, such as *A. mellifera* from Israeli acute paralysis virus (IAPV) in field conditions or the fungal parasite, *Nosema ceranae* (Hunter et al., 2010; Paldi et al., 2010). This review is

focused on the application of RNAi in insects, and an overview of RNAi application in Hemipteran insects is provided in Table 1.2.

Because the development of RNAi transgenic plants is time consuming and are still not widely acceptable, foliar application of dsRNA, as RNAi insecticide, is being investigated as a next generation technology. Successful ectopic application of dsRNA and/or siRNA to control *A. aegypti* and *Ostrinia furnacalis* has been reported indicating the technology is a practical method for pest management (Pridgeon et al., 2008; Wang et al., 2011). Once applied on leaves, dsRNAs are reported to be stable for up to 28 days under greenhouse conditions; it was not readily washed away with water (San Miguel and Scott, 2016). The cost of making RNAi insecticides using long dsRNAs or the shorter siRNAs in large, commercial quantities appear to be much higher at this stage compared to conventional insecticides. Therefore, using shorter lengths of dsRNA if this is as effective as longer dsRNA, would reduce the costs of this approach. More work is needed in this area to make this technology more commercially viable.

As identification of suitable target genes is the key to successful RNAi-based insect control method, most studies have selected either "housekeeping" genes that regulate essential biological processes as targets for their control (Baum et al., 2007; Mao et al., 2007). Another important group of genes which have been prime targets for insect control encode neuropeptides and G-coupled receptors. These neuroactive genes play key roles in the insect life-cycle (e.g. embryonic and post embryonic development, feeding, homeostasis and osmoregulation, oviposition and mating, migration) and are therefore often referred to as 'master regulators' (Bendena, 2010; Masler et al., 1993). NSMs are also considered as good choice as the signalling system also targets as pesticides. Although intensive research have characterised the proteins and their mode of action, they have not yet been studied as potential targets for gene silencing (Altstein et al., 2000). Hence, rather than targeting the functional

peptides, assessing the effects of silencing the genes could be a better approach to interfering with the pathways these neuronal genes regulate in insects. RNAi is certainly one way to achieve this goal.

Table 1.2: An overview of RNAi applications in different Hemipteran insects

Aphid species	Target Genes	dsRNA	References
Pea Aphid (*Acyrthosiphon pisum*)	Angiotensin-converting enzymes	138 ng	Wang et al., 2015
	Salivary protein (C002)	50 ng	Mutti et al., 2006
	Calreticulin, cathepsin-L	138 ng	Jaubert-Possamai et al., 2007
	Aquaporin	1 μg/μL	Shakesby et al., 2009
	Hunchback	750 ng/μL	Mao and Zeng, 2012
Cotton Aphid (*Aphis gossypii*)	Carboxylesterase E	50-500 ng/µL	Gong et al., 2014
	Cytochrome P450	100 ng/µL	Peng et al., 2016
	Odorant binding Protein 2	62.5–250 ng/μL	Rebijith et al., 2016
White fly (*Bembisia tabaci*)	Actin, ADP/ATP translocase; α-tubulin; Ribosomal protein L9; V ATPase	6 ng	Upadhyay et al., 2011
Potato/Tomato Psyllid *(Bactericerca cockerelli)*	BS-Actin	200 nL of 100 ng/ml	Wuriyanghan et al., 2011
Green Peach aphid (*Myzus persica*)	MpC002 and Rack-1	Transgenic Plant	Pitino et al., 2011
	MIF Cytokine (MpMIF1)	2.5ng/nμL	Naessens et al., 2015
Brown plant Hopper (*Nilaparvata lugens*)	Hexose transporter; Carboxypeptidase; Trypsin-like serine protease	Transgenic plants	Zha et al., 2011
	ATP synthase subunit E	70 µL of 0.1 g/mL	Li et al., 2011
	Trehalose phosphate	0.5, 0.1, 0.02 µg/µL	Chan et al., 2010
Milkweed bug (*Oncopeltus fasciatus*)	Nubbin	6 uL (2.5 µg/µL)	Turchyn et al., 2011
Triatomin bug (*Rhodnius prolixus*)	Nitroporin 2	10.5 µg	Araujo et al., 2009
Bird cherry-oat aphid (*Rhopalosiphum padi*)	RpAce1	10 ng	Xiao et al., 2015
Bean Bug *Riptortus pedestris*	Circadian clock gene period; mammalian-type cryptochrome; Nitrophorin 2	2 mg/ml	Inoue et al., 2002
Grain aphid *Sitobion avenae*	Acetylcholinesterase	7.5 ng/µL	Xiao et al., 2015
Greenbug *Schizaphis graminum*	Salivary protein, C002	20 ng/µL	Zhang et al., 2015

1.3 Neuronal Signalling Molecules (NSMs)

NSMs refer to neurohormones, neuromodulators and/or neurotransmitters that are involved in signal transduction and modulation of the central nervous systems (CNS) of many organisms including insects (Blenau and Baumann, 2001; Nässel, 2002). They are ubiquitous in the nervous system from hydrozoans to mammals (Geary and Maule, 2010). Most insect neuropeptides (NPs) are produced by neurosecretory cells and interneurons and are released into the CNS (Nässel and Homberg, 2006; Nässel, 2002). NPs can also be produced by insect neuroendocrine cells for release into the hemolymph (Nässel, 2002; Taghert and Veenstra, 2003). Although insect NPs have been studied mainly as circulatory hormones because they act at a distance from the release site, they are considered as locally released neuromodulators. Insects NPs and biogenic amines (BAs) act as ligands for the GPCR-mediated neuroendocrine signals and produce host effects (Ewer and Reynolds, 2002; Nässel, 2002). Sometimes, NPs also act directly on ion-gates to transduce signals (Nässel, 2002). NPs may also be defined as co-transmitters if they are released with other classical neurotransmitters (Burnstock, 2004; Nässel, 2009).

The first insect neuropeptide was proctolin, identified from the cockroach, *Periplaneta americana* (Starratt and Brown, 1975). A year later, the neuropeptide adipokinetic hormone (AKH) was discovered in a locust species (Stone et al., 1976). Since then, several NPs with diverse functions have been characterised in many insects of different orders as well as in other organisms. Scherkenbeck and Zdobinsky (2009) classified neuropeptides according to their roles in four major functional processes; growth and development; behaviour and reproduction; metabolism and homeostasis and muscle movement. To date, more than 41 genes encoding neuropeptides have been identified in the genome of the free-living nematode, *C. elegans*, 36 in *D. melanogaster*, 42 genes in *A. pisum*, 37 genes *in B. mori*, 32 genes in *R. prolixus*, 48 genes in *N. lugens* and 36 genes in *A. mellifera* (Broeck, 2001; Huybrechts

et al., 2010; Li et al., 2008; Li et al., 1999; Nässel and Winther, 2010; Ons et al., 2011; Roller et al., 2008; Tanaka et al., 2014).

As indicated earlier, most neuropeptides exert their functions through cell surface receptors, GPCRs that are also known as seven-transmembrane domain receptors (7TM) or heptahelical receptors (Broeck, 2001). These GPCRs couple to their specific ligands, such as NPs, biogenic amines or olfactory substances to transduce extracellular signals into intracellular responses (Broeck, 2001). GPCRs are termed NP receptors (NPRs) and biogenic amine receptors (BARs) when they bind to NPs and biogenic amines, respectively. Figure 1.3 is a simple representation of NP-receptor signalling cascades. Both neuropeptides and their respective receptors are vital components for signal transduction and transmission in insects (Nässel and Homberg, 2006). It has been estimated that in the genome of *D. melanogaster*, 1% of the genes encode GPCRs and it is about 6% in *C. elegans* (Broeck, 2001). Major biogenic amines have been reported in insects including dopamine, tyramine, octopamine, serotonin, acetylcholine and histamine (Hauser et al., 2006). At present, 20 genes encoding BARs in *T. castaneum*, 21 in *D. melanogaster*, 19 in *A. mellifera* and 39 in *A. pisum* have been reported, while for NPR-encoded genes the number is 48 for *T. castaneum*, 45 for *D. melanogaster*, 35 for *A. mellifera* and 18 for *A. pisum* (Hauser et al., 2006; Hauser et al., 2008; Hewes and Taghert, 2001; Li, Yun, et al., 2013).

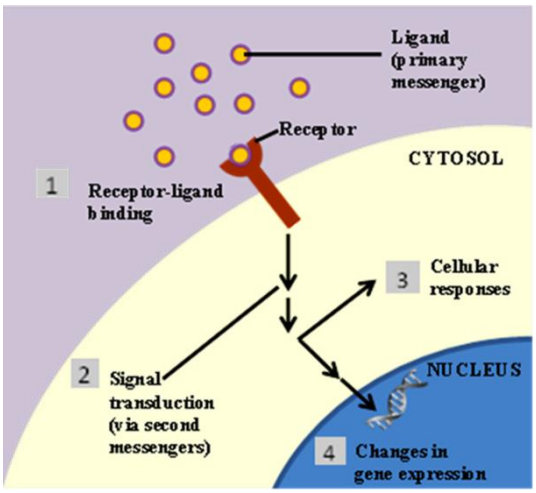


Figure 1. 4: A simple representation of NSM signalling cascade *via* receptors. Ligands include NPs and biogenic amines.

1.3.1 Functions of insect NSMs

Most insect NSMs have multiple functions at various stages of the life cycle. Based on available literature, Table 1.3 and 1.4 summarise the functions of various NSMs reported in different insect species.

Table 1.3: Functions of NSMs in various insect species

Major functional groups	Name of the NPs involved in functional processes	RNAi phenotypes in insects or nematodes	References
Growth and Development	Insulin related peptides (IRPs), Pre-ecdysis triggering hormone (PETH), Ecdysis triggering hormone (ETH), Allatostatin (AST), Alltotropin (AT)	Reduced body size in *irp*-RNAi of *irp*s in *Gryllus chico*; InR RNAi disrupt nymph-adult transition in *Aphis citricidus*, disrupted food searching behaviour in *D. melanogaster* for *ast*-RNAi, an abnormal adult phenotype with reduced egg for *ast* and *at* RNAi in *T. castaneum*	Abdel-Latief and Hoffmann, 2014; Dabour et al., 2011; Ding et al., 2017; Gäde and Hoffmann, 2005; Wang et al., 2012

Major functional groups	Name of the NPs involved in functional processes	RNAi phenotypes in insects or nematodes	References
Moulting	AT, AST, PETH, ETH, EH, Crustacean cardioactive peptide (CCAP), Bursicon (Bur), Corazonin (Crz)	Abnormal wing for bur-RNAi, interrupted ecdysis behaviour and mortality for silencing of *eth*, *eh* and *ccap* in *T. castaneum*; mortality in *R. prolixus*	Arakane et al., 2008
Reproduction	IRPs; PETH, ETH, NPF, Short Neuropeptide F (sNPF); SIFamide; Diuretic hormone (DH); Sex pheromone (PBAN)	Knock down of *irp*s in *Schistocerca gregaria* reduced oocyte growth and vitellogenin synthesis, reduced oviposition rate after silencing of *ast*A in *Spodoptera frugiperda* and *G. bimaculatus*	Badisco et al., 2011; Meyering-Vos et al., 2006
Muscle contraction	Myosuppressin (Msn); AT, Ast, NPF, Sulfakinins (Sk), Proctolin, Pyrokinin (Pk), Tachykinin (Tk); CCAP, Orcokinin (Orc)	Data not available	Altstein and Nassel, 2010; Bendena, 2010; Marciniak et al., 2011; Wasielewski and Skonieczna, 2008
Feeding behaviour	Msn, Tk, NPF, sNPF, Pk	RNAi of *snpf* reduced food consumption in *S. gregaria* and *D. melanogaster*	Badisco et al., 2011; Lee, et al., 2004; Nachman et al., 1997; Van Wielendaele et al., 2013
Water balance	DH (Corticotropin like and Calcitonin-like DH); CAPA family; Leucokinin (Lk); Ion transport peptide (ITP)	Altered *D. melanogaster* fluid secretion if *capar* silenced, RNAi of Lk receptor reduced fluid excretion in *Aedes aegypti*	Davies et al., 2013, Kersch and Pietrantonio, 2011
Energy metabolism	Adipokinetic hormone (AKH); IRPs; Gonadotropin releasing hormone receptor (GnRH-R)	Data not available	Lee and Park, 2004; Stone et al., 1976
Immune response	AKH	Data not available	Nijhout, 1998

From Table 1.3, most of the NPs and/or NPRs are involved in more than one functional class, and this sometimes make it difficult to group NSMs only based on their function(s). For instance, Myosuppressin (MSN) is directly involved in gut motility in the Lepidopteran insect, *Spodoptera littoralisvarious*, and consequently regulates feeding behaviour (Audsley and Weaver, 2009). So, MSN peptides have been classified as important for muscle contraction as well as for insect feeding.

As for NPs, few studies have been undertaken targeting BAR encoding genes in aphid species (Table 1.4). Except for carboxylesterase gene (CarE) in *A. gossypii*, Insulin or their receptor genes in A. *citricidus* or *A. pisum*, no RNAi data for BAR-encoding genes has been available for any other aphid species (Gong et al., 2014; Ding et al., 2017).

Table 1. 4: Functions of BARs reported in different insect species

Biogenic amine	Major function	RNAi phenotypes in insects	References
Octopamine (OA)	Restricted to invertebrates, acts as stress hormone for energy-demanding behaviours; accelerate or inhibit the heart rate	RNAi of OA receptor affected olfactory learning and memory in *A. mellifera*, RNAi of Oβ3R ceased *D. melanogaster* metamorphosis	Farooqui, Vaessin, and Smith, 2004; Ohhara et al., 2015; Roeder, 2005
Tyramine (TH)	Restricted to invertebrates, involve in motor activity regulation, e.g. flight, olfactory stimuli	Data not available	Roeder, 2005
Dopamine (Dop)	Widespread signalling molecules; involve in insect neuronal circuit and flight behaviour	Data not available	Claassen and Kammer, 1986
Serotonin	Insect salivation; heart rate modulations in *R. prolixus*, behavioural gregarisation in locusts,	Data not available	Anstey et al., 2009; Chiang et al., 1992; Colas et al., 1999;

Biogenic amine	Major function	RNAi phenotypes in insects	References
	photoactic behaviour, circadian rhythms, learning and memory		Walz et al., 2006
Acetylcholine	Excitatory synaptic transmission, xenobiotics metabolism, cell differentiation, neuronal formation, probably involve in growth and development, target site for insecticides	Embryonic lethality for RNAi of acetylcholinesterase gene, in *H. armigera*, RNAi phenotypes of carboxylesterase gene increased the insecticide resistance (organophosphate) in *A. gossypii*	Gong et al., 2014; Kumar et al., 2009

1.4 Aims and objectives of this research

The overall aims of this project were to assess the effects of silencing neuronal genes on the development and survival of GPAs, and to identify the most suitable gene(s) as targets for effective GPA control *via* RNAi. Specific objectives of this research were:

1. To apply bioinformatics and comparative genomics to identify genes encoding neuronal genes in GPAs
2. To evaluate the effect of down-regulating selected NSM genes on the behaviour, growth and development of GPAs
3. To assess the efficacy of HIGS of selected NSM genes of GPAs.
4. To compare the efficiency of siRNAs and different lengths of dsRNA in triggering silencing of NSM genes of GPAs

Chapter 2

General materials and methods

2.1 Insect rearing and plants growing

The GPA population (green coloured morph) used in this study was originally identified and maintained by entomologists at the Department of Primary Industries and Regional Development (DPIRD), formerly the Department of Food and Agriculture, Western Australia, (DAFWA) and provided by Dr Roger Jones of DPIRD. The insects were cultured on wild type tobacco, *Nicotiana tabacum* (cv. Wisconsin-98) in insect-proof meshed cages at 22 ± 2°C under a 16:8 hours light: dark cycle. To grow the tobacco plants, soil was prepared with 'Murdoch mixture' (coarse river sand: composted pine bark: coco peat in a 2:2:1 ratio) with addition of the following fertilizers: Grower's blue® (60g), Osmocote® (60g), Calcium carbonate (15g) and dolomite (20g) per 40 L of soil. Soil and fertilizers were fully mixed, pasteurised, and stored in the glasshouse for further use.

2.2 Target gene amplification and confirmation by sequencing

2.2.1 Aphid RNA extraction and cDNA synthesis

RNA from two mg of total GPAs or head capsules (from approximately 2,500 GPA heads in total) were extracted separately from mixed stages of GPA. The GPAs were collected carefully into a 1.5 ml centrifuge tube with a fine paint brush (02 size) and immediately homogenised with liquid nitrogen. Eight hundred microliters of diluted Trizol reagent (Life Technologies Corporation) was added to the homogenate in a fume hood, further homogenised and mixed well by vortexing. After five minutes of incubation at room temperature, 200 µL of chloroform was added and the mixture was shaken vigorously by hand for 15 seconds followed by the incubation at room temperature for three minutes. The emulsion was then centrifuged at 4°C for 15 minutes at 16,000g to separate the phases. The upper aqueous phase was transferred carefully to a fresh 1.5 mL tube for clean-up and DNAse I treatment. To clean-up the total RNA, 1/20th volume of 3M sodium acetate and 2.5 volumes of 100% ethanol was added and incubated at -80°C overnight. The sample was then thawed on ice, centrifuged at 16,000g for 30 minutes at 4°C and the supernatant was carefully discarded. One millilitre of chilled 80% ethanol

was added to the pellet and again centrifuged at 12,000g for 10 minutes. The liquid was discarded. The pellet was air-dried for about 15 minutes and resuspended with 30 µL RNase-free water. DNase treatment of the RNA was carried out following the supplied protocol (RNAse-free DNAse set, Qiagen). The RNA was finally resuspended in 20 µL RNase-free water and quantified with a Nanodrop, ND-1000 Spectrophotometer. For RNA, a 260/280 ratio of ~2.00 was accepted as pure.

Two micrograms of RNA were used for reverse transcription to generate cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) in a standard 20 µL reaction volume. The reaction was incubated at 25^{o}C for 10 minutes, followed by 37^{o}C for 120 minutes and finally 85^{o}C for five seconds. The cDNA concentration was quantified using the Nanodrop spectrophotometer, and the 260/280 ratio was regarded as pure for a value of 1.8.

2.2.2 Primer design

All gene specific primers (GSP) sequences were designed using IDT OligoAnalyzer tool 3.1 (https://sg.idtdna.com/calc/analyzer) and rechecked using the online software OligoCalc (biotools.nubic.northwestern.edu/OligoCalc) for the annealing temperature and secondary structure formation. In total, 24 genes of GPA were amplified with GSPs containing restriction enzyme recognition sites (RE-GSP) (Table 2.1): for these recognition sequences, the restriction enzyme pair *XhoI* and *KpnI* were added to the GSPs except for the *mAChrM3* where *XhoI* and *BamHI* recognition sequences were added. This was necessary to allow amplicons to be ligated to appropriate cloning vectors. Before designing RE-GSPs, restriction site analysis of ESTs of the target genes was done using NEBcutter (V2.0, New England Biolabs).

Table 2. 1: A list of the gene specific primers used to amplify GPA ESTs of target genes

Target gene (s)	Primer (5´- 3´)	Amplicon (bp)
Nicotinic Acetylcholine Receptor subunit beta 1	GGCTACAACAAACTAATCAG	500
	TAGGCGACTCCTGGTATA	
Allatostatin CC	CTAGTTGCACACCAATGCAG	417
	CAGGACACAGCGTTGAAATAAC	
Bursicon beta	CGTACTTGCAGAAGATAAC	290
	CATTTGCAGTCCGCTGGT	
Capa	GAATCCGTTGCAGGATTG	419
	CATCAACACTAACGATTTG	
Capa Receptor R1	GGACGCTCATCGTCGTCCTG	367
	TGACTTGTCCACGGCCGA	
Capa Receptor R2	ATGAATTTGACTGCGGGAA	294
	ATGGACCACATTTCCTGC	
Crustacean Cardio Acceleratory Peptide	GCCATTCATCGACAGCCATG	375
	ACTGGTGGCAAGTGATCGACG	
Ecdysis Triggering Hormone	CGGCAACAGTTGTGGAGTC	453
	TACGGCAAGTCGAAGTCATG	
Eclosion Hormone 2/3	F: CGAAGAAGGAGTAAGAGCAA	323
	R: TAGGCTGGGATGTAAGTCTTT	
Gonadotropin Releasing Hormone Receptor	F: GAAGGTCCAGGGAATCAA	496
	R: CTCATGTGTCTTGTCACTA	
Insulin Related Peptide 2/3	F: GTCTATATTTGAGTGTACTCC	309
	R: TACTGACTTGCACAATAAG	
Insulin Related Peptide 5	F: CCGCGTTGTCCGATACCTC	409
	R: GCAGGCGCGGTGTTATTCCC	
Ion Transport Peptide	F: TGATGGTGTGCATGTCGGCC	489
	R: GGCACAGCTGTTCTGGTC	
Leucine rich repeat containing G-protein coupled receptor 5	F: GCTGATATTTGCCAGGAG	248
	R: CGTGATAGCGTAATTCCG	
Opsin	F: TCTACTGGAGCATCGATGATAG	389
	R: CGCCAAGTTAACTATCAGTAAG	
Lucokinin	F: GATTCCAACGCAATAATACAG	509
	R: CTTCTGGATCCCTTGACCA	
Muscarinic acetylcholine receptor M3	F: GCAGCCGAGCAACTACTTCAT	263
	R: GGTGGAGAACAGGAGAGCC	
Myosuppressin	F: CAATGACAATAAGCAAACC	329
	R: TGTTGTCATAATGGCCAA	
Neuropeptide Like Protein	F: CCAGAATACAAACGATCGCT	530
	R: GGAATCCGTAAGGCAGTC	
Neuropeptide Y Receptor type 2	F: TTTGCGAGGAGACGTGGC	301
	R: CGGAGAAGAAAGAAACAGGTA	
Octopamine receptor beta3R	F: AGCGTCGAGATCACCGGCAA	221
	R: TGCTCGGCAGTCGTATACC	

Target gene (s)	Primer (5´- 3´)	Amplicon (bp)
RR1 cuticle protein 4	F: ATAACTGCTCTGCTCATGGT	402
	R: CTGAGTATGATCCTTGGAC	
Short NeuropeptideF	F: GAAATCCATCGCAGCCGT	469
	R: GAGTAATGTTAATCTGCGGAG	
Tachykinin-like peptides Receptor 99D	F: ATGGAATCTGCCGTCGTTC	321
	R: GAATAGGCCCACGTCATTATC	
Actin	F: ACAGGTCATCACCATCGGAAAGA	436
	TCCACATCTGTTGGAAGGTGGACA	

2.2.3 Polymerase Chain Reaction (PCR) of target sequence amplification

PCRs were carried out in a 20 µL reaction volume using 100-300 ng of cDNA with 0.2-0.3 units of MyTaq™ DNA polymerase, 5X ™ Reaction Buffer Red (Bioline). Ten picomoles (pm) of each forward and reverse primer (Integrated DNA Technologies) were added to the 20 µL reaction volume. The Hot Start Taq DNA Polymerase (New England BioLabs) and GoTaq® DNA Polymerase (Promega) were also used occasionally for touch down PCRs (TD-PCR) in a 20 µL reaction volume. The PCR conditions were an initial denaturation step at 95ºC for five minutes, followed by 35 to 40 cycles of denaturation at 94ºC for one minute, extension at 72ºC for one minute and a final incubation step at 72ºC for seven minutes to ensure complete extension. Primer annealing temperatures were empirical and ranged from 49-60ºC. PCRs were performed in a Perkin Elmer GeneAmp® DNA Thermal Cycler (model 2400) or Applied Biosystems Thermal Cycler (model 7200).

TD-PCR was used to optimise PCR conditions and to increase sensitivity, specificity and yield for the sequences that could not be amplified with the regular PCR programme. For TD-PCR, all conditions were the same as the regular PCRs except the annealing temperatures started from 63°C and then decreased by 1°C for each cycle to a temperature 5°C below the Tm of the primers.

2.2.4 Agarose gel electrophoresis and DNA product purification from gels

PCR products were analysed on 1-2% TAE horizontal agarose gels (Bio-Rad Laboratories) at 60 to 80 Volts for a maximum 90 minutes. To visualise PCR products, the gels were pre-stained with one microliter of SYBR Safe™ DNA gel stain (Invitrogen Corporation) per 10 mL of 1x TAE buffer. One

time TAE buffer was diluted from 50X TAE [242 g Tris, 100 mL 0.5M EDTA (pH 8.0), 57.1 mL Glacial Acetic Acid] that was prepared as described by Sambrook *et al*. (1989). As molecular weight markers, a 100 bp DNA ladder and a 1kb DNA ladder (Fisher Biotech) were used. Gels were visualised with a transilluminator plus camera system (Fisher Biotech) and documented using a BioVision imaging system (www.vilber.com).

2.2.5 DNA clean-up, Sanger sequencing and analysis

Amplicons of the right sizes were excised from gels and transferred to a pre-weighed 1.5 mL microcentrifuge tube. The DNA was isolated and purified using the Wizard SV Gel and PCR clean-up System (Promega) following the manufacturer's protocol. The DNA was eluted in 20 µL nuclease-free water and stored in -20°C until used. The SABC Sanger sequencing facility at Murdoch University (Applied Biosystems Industries; ABI 3730 96 capillary machine) was used to sequence the isolated amplicons, and ABI BigDye terminator (Version 3.1) reagents were used for all the sequencing reactions. The sequencing reactions and clean-up of the dye-incorporated DNA were performed following the BigDye protocol and the SABC sequencing guide prepared by Ms Frances Brigg. Sequencing was also carried out by Ms Frances Brigg, SABC, Murdoch University. The sequenced data were viewed and edited with Finch TV 1.4.0 (Perkin Elmer, Inc.). For further confirmation, edited sequences were rechecked against the insect (taxid 6960) non-redundant nucleotide and non-redundant protein databases using TBLASTX and BLASTX programs on NCBI, respectively.

2.3 Cloning of target inserts using suitable vectors

2.3.1 Ligation

Purified DNA was ligated to respective vectors depending on the purpose. To synthesis dsRNA, target sequences were cloned into an RNAi vector, pDoubler (2.6 kb) (Figure 2.1). Target sequences were ligated into pDoubler at multiple cloning sites using *XhoI* and *KpnI* or *XhoI* and *BamHI* restriction enzymes bordered by T7 promoter sites. For all ligations, insert: vector molar ratio was 3:1 calculated

with the formula, ng of insert = (concentration of vector) x (kilo base (kb) size of the insert) / kb size of vector x 3. All ligation reactions were done in 10 µL volumes and incubated overnight at 4°C. Each mixture contained 1X T4 DNA ligation buffer, 1 Unit of T4 DNA Ligase (Promega) with appropriate amounts of linear vectors and DNA fragments as inserts.

To obtain hairpin constructs (RNAi constructs), sense and antisense strands of the targe gene fragments were sequentially ligated to pCleaver-NosA (4 Kb) (Figure 2.1). The sense (5´- 3´) and antisense (3´-5´) strands of the target gene fragment were digested out from pDoubler in sequence. Sense strand was digested with *KpnI* and *XhoI* from pDoubler and ligated into pCleaver vector linearised with same enzymes. After successful cloning of sense strand into pCleaver following bacterial transformation, positive transformant selection and confirmation (described in Section 2.2.2 and 2.2.3), the modified pCleaver vector was again linearised with *BamHI* and *XbaI* for the ligation of antisense strand which was also digested from pDoubler with *BamHI* and *XbaI*. These sense and antisense strands were ligated at either side of bean catalase gene intron (190 bp) sequentially under the constitutive cauliflower mosaic virus (CaMV) 35S promoter. The cloned pCleaver vector encoded kanamycin resistance with the presence of the nopaline synthase (NOS) gene. The orientation of hairpin cassettes was checked in a separate reaction, and the complete hairpin cassette was then digested out with *NotI* and ligated into pART27 (11 Kb) for plant transformation (Figure 2.1). Before cloning into pART27, the vector was linearized with *NotI* and dephosphorylated with Antarctic Alkaline Phosphatase following the manufacturer's protocol (New England Biolabs, NEB).

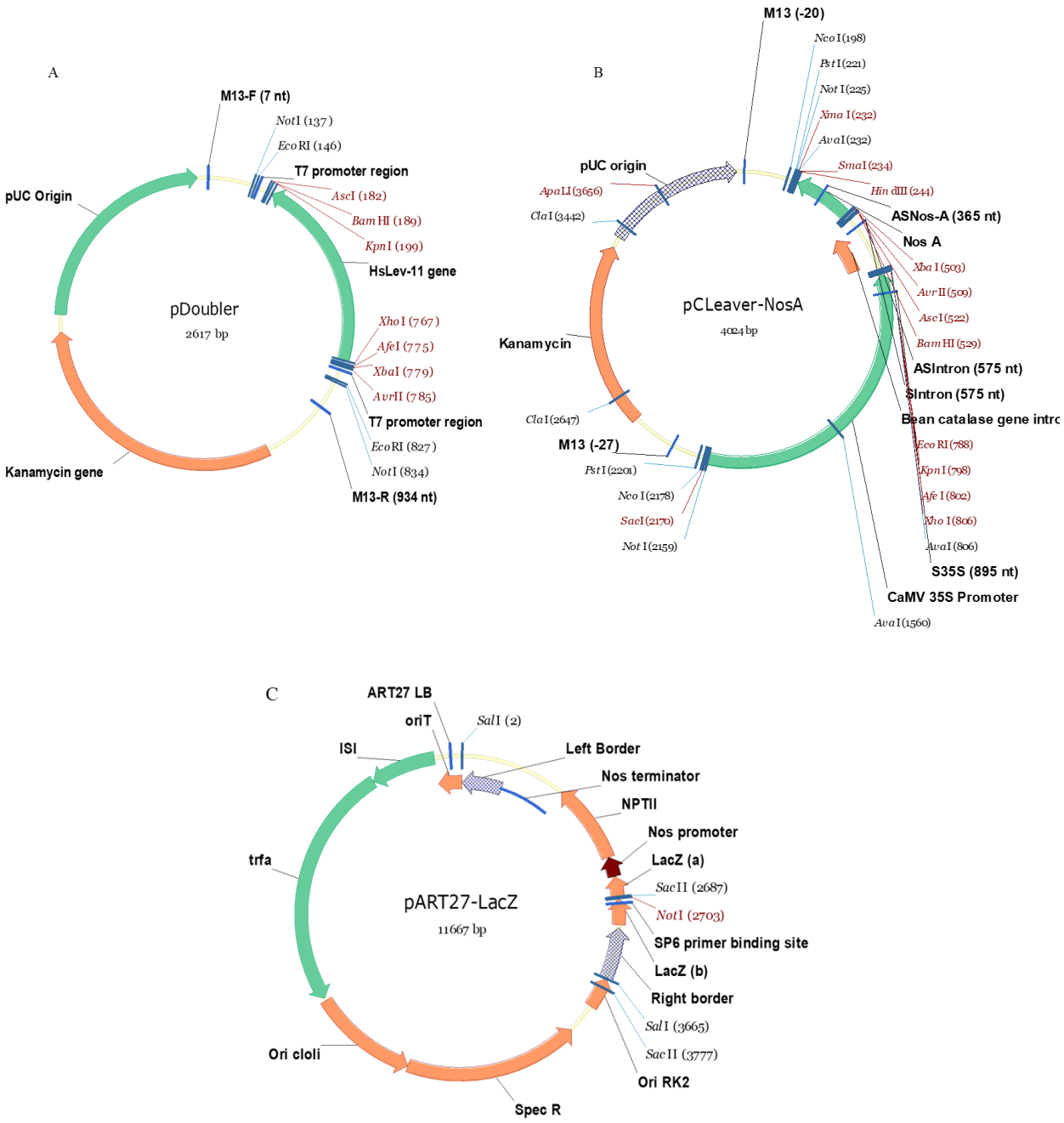


Figure 2.1: Maps of the vectors: pDoubler vector was used to clone target genes for dsRNA synthesis, pCleaver-NosA was used for sense and antisense cloning and pART27 used for cloning of target RNAi hairpin cassettes. Primer binding sites and restriction enzyme sites are indicated.

2.3.2 Bacterial transformation (*Escherichia coli* (*E. coli*) JM109 and *Agrobacterium tumefaciens* (*A. tumefaciens*) GV3101 competent cell transformation)

E. coli JM109 competent cells prepared using the calcium chloride method as described in the Promega Subcloning Notebook (1990) were used to multiply all plasmids required in this project. For transformations, the competent cells were thawed on ice and 25-30 µL was added to five microliters of ligation mixture which was then incubated on ice for at least 30 minutes. The cell-ligation mixture was then incubated at 42°C in a water bath for 45 to 50 seconds and immediately transferred onto ice for two minutes. Seven hundred microliter of LB (Luria-Bertani) broth without any antibiotic was added to the mixture and cultured at 37°C for 90-120 minutes. About 250-300 µL of the bacterial culture was streaked on LB agar media plates with appropriate antibiotics. For pDoubler and pCleaver 25 mg/L kanamycin monosulphate was used for screening the positive transformants. After transformation with *E. coli*, LB plates were incubated at 37°C in the dark for 16-18 hours until colonies grew.

For plant transformation, purified pART27plasmid DNA was again transformed with 50 µL of *A. tumefaciens* GV3101 using the heat shock method. Briefly, after thawing the competent cells on ice, 300 ng plasmid DNA was added and the mixture was left on ice for 20 minutes. The mixture was then incubated at 37°C for five minutes in a water bath, after which one ml of LB broth was added to the mixture for growing the culture. The culture was grown at 28°C in the dark for 3 hours with shaking at 225 rpm. After the incubation period, 200 µL of the grown culture was plated on LB media with rifampicin (25 µg/mL) and spectinomycin dihydrochloride (100 µg/mL) and the plates were incubated at 28°C for 2-3 days in the dark until white colonies started to grow.

2.3.3 Selection of transformants by PCR

After transformation, at least eight individual colonies were picked with sterile 10 microliters pipette tips from selected LB plates and resuspended in 20 µL of PCR grade water. Five microliters of the suspension were used for colony screening by PCR. To select positive colonies for modified pART27

vector transformed with *A. tumefaciens* GV3101, the colony suspension was heated at 96°C for 10 minutes to make the plasmid DNA available in suspension before PCR. To screen desired transformants by colony PCR, the following primer combinations were used. For pDoubler, M13F and M13R primer pair were used (5′-TAAAACGACGGCCAGT-3′ and 5′-CAGGAAACAGCTATGAC-3′, respectively). For sense strand cloning into pCleaver-NosA, S35S and SIntron (5′-GATTGATGTGACATCTCCACTGA-3′ and 5′-TCATCATCATCATAGACACACGA-3′, respectively) and for antisense, ASNosA and ASIntron primers were (5′-CATCTCATAAATAACGTCATGCATT-3′ and 5′-TCGTGTGTCTATGATGATGATGA-3′) used for colony PCR. To select positively transformed colonies for pART27, SP6 and S35ART primers were used (5′-CATACGATTTAGGTGACACTATAGA-3′ and 5′-GTCTTGATGAGACCTGCTGCGTA-3′). Amplicons were 361 bp longer than the inserts for pDoubler positive transformations. For pCleaver-NosA transformants, amplicons from the positive colonies were 316 bp longer for the sense inserts and 184 bp longer for antisense inserts. For the pART27, amplicons from positive colonies amplified about 700 bp as the primers were designed from near the right border of the vector pART27 (for the SP6 binding site) and in the CaMV35S promoter site of the hairpin cassette (for the 35SART binding site). The same PCR conditions wer used for screening transformants from all the vectors: initial denaturation at 96°C for three minutes, 30 cycles of denaturation at 96°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds followed by final extension at 72°C for seven minutes. Each reaction was made of 5x PCR buffer and 0.05 µL of *Taq* polymerase enzyme (Bioline) with 10 pm of forward and reverse primers in a 20 µL of reaction volume.

2.3.4 Plasmid DNA purification and restriction digestion analysis

After screening the positive transformant following appropriate antibiotic selection (as described under Section 2.3.3) and colony PCR, positively transformed bacterial colonies were cultured with appropriate concentrations of antibiotics. To grow the culture, five µL of colony suspension was inoculated to 5-10 mL LB broth in McCartney bottles with suitable antibiotics. For *E. coli*, the cultures were incubated on a shaker (225-250 rpm) at 37°C for 16 to 18 hours. The resulting bacterial cultures

were then purified using the Wizard plus SV minipreps DNA purification system kit (Promega) following the suppliers' protocol. The plasmid DNA was quantified with the Nanodrop spectrophotometer and stored at - 20°C for future work.

Confirmatory restriction enzyme digestion of plasmid DNA was performed to recheck the size of target insets in modified vectors using one to two micrograms of purified plasmid DNA with suitable enzymes incubated for two hours using a thermal cycler. For instance, *XhoI* and *KpnI* enzymes (1U) were used for confirmatory digestion of pDoubler recombinant plasmids, and for pCleaver-NosA recombinant plasmids, *XhoI-KpnI* (for sense strand) or *XbaI-BamHI* (for antisense strand) were used. Confirmatory restriction digestion with *NotI* (NEB) was done for recombinant pART27 to check the successful cloning of the complete hairpin cassette. For regular digestion reactions, 10 µg of recombinant plasmids were digested with 5U of appropriate restriction enzymes following the recommended temperatures of incubation and if needed, inactivation of enzymes. Usually, digestion reactions were incubated at 37°C and heat inactivation was performed at 65°C if required using a thermal cycler.

2.4 *In vitro* RNAi

2.4.1 Synthesis of dsRNA

dsRNAs of target GPA sequences were synthesised for *in vitro* RNAi studies in which dsRNA was mixed with 30% sucrose and supplied to aphids in an artificial feeding set-up. To synthesise dsRNA, target templates with T7 sites at both ends were digested from pDoubler using the restriction enzyme *EcoRI*. The digested fragments were then run on 1% agarose gel, cleaned and quantified as described in Section 2.2.4 and 2.2.5. To generate templates for *in vitro* transcription, the Hi-Scribe T7 *in vitro* transcription kit (New England Biolabs) was used following the manufacturer's protocols. *In vitro* transcription reactions were set up at room temperature in a 20 µL reaction volume in the following order; appropriate volume of nuclease-free water, two microliters of 10x reaction buffer, two microliter each of 100mM of ATP, GTP, UTP and CTP, approximately two micrograms T7 DNA template and

two microliters of T7 RNA polymerase enzyme. The reaction was mixed in a 0.2 mL PCR tube and incubated at 37°C for 16-18 hours in a thermal cycler. After incubation, the synthesised dsRNA was treated with 2.5 µL of DNAse I with 14 µL of RDD buffer (Qiagen) to remove residual DNA. Nuclease-free water was added to make up the total reaction to 200 µL and the reaction was incubated at 20 to 25°C for 10 minutes. The dsRNA was then extracted with chloroform and precipitated with ethanol as described under Section 2.2.1. The pellet of dsRNA was resuspended in 20 µL of nuclease-free water and quantified using the Nanodrop spectrophotometer. The purity of dsRNA was also assessed by running on 1% agarose gel in 1x TAE buffer (details as described in Section 2.2.4).

2.4.2 Artificial feeding set-up with dsRNA

For all artificial dsRNA feeding experiments, dsRNA was mixed with 30% sucrose and 0.02% Neutral Red (NR) dye in a 'feeding sachet' and provided to GPA nymphs (Bilgi et al., 2017). To make the feeding sachet, the parafilm M pieces were first cut into 2cm x 2cm pieces and cleaned with Ambion RNAseZap (Life technologies). The nymphs were gently put into a 5ml yellow-capped bottle (Sarstedt) using a fine paint brush (size 02) and immediately covered with a piece of stretched parafilm to prevent escape. A droplet of 40 µL sucrose diet, with or without dsRNA, was placed on the first layer of the parafilm and carefully covered with another layer of stretched parafilm to avoid any air bubble or spillage. For all feeding studies, 3rd to 4th instars of five to 10 GPAs of similar sizes were carefully selected from GPAs pre-reared on tobacco plants, using a hand magnifier, and starved for about two hours before feeding experiments to encourage uptake of the diet solution. Each nymph was treated as single replicate. The 40 µL of the diet solution consisted of 30% of filter sterilized sucrose, two µg/µL dsRNA, 0.02% NR dye (made with nuclease-free water and filter-sterilised). The complete feeding set-up was kept for 24 hours at room temperature on a lab bench. At the end of the feeding period, any phenotypic and behavioural changes of fed GPA nymphs and survival data were recorded. DsGFP and no dsRNA (30% sucrose only) were used as controls for *in vitro* feeding experiment.

2.4.3 Post feeding set-up

After the 24 hours of feeding, one to two dsRNA-fed GPAs were stored at -80°C for gene expression analysis and three to five dsRNA-fed GPAs (depending on availability) were transferred to wild type tobacco plants. The longer-term effect of ingestion of different dsRNAs was on aphid reproduction was recorded for a period of 12 days, and their longevity was recorded for up to 21 days. All tobacco plants used for this study were at the two to four leaf stage. The tobacco plants were grown in small plastic cups (285 mL) covered with a 450 mL disposable plastic cup, which had a hole at the bottom covered with a nylon mesh or micropore medical tape (3M) to allow gas exchange. Data survival and reproduction of dsRNA-fed GPAs were taken at on the 4^{th}, 8^{th}, and 12^{th} days after *in vitro* feeding. The number of nymphs present on the tobacco plants were carefully counted by checking both sides of each tobacco leaf with hand magnifier. After counting the nymphs in the *in vitro* feeding experiments, new nymphs were removed regularly from the plants: this allowed identification of the original dsRNA-fed GPA, enabling recording the data on survival of the original dsRNA-treated aphids.

2.4.4 Semi quantitative PCR

Transcript abundance was assessed in dsRNA-fed GPAs. The RNA extraction protocol was modified from a method used to extract RNA from a single nematode (Ly et al., 2015). Single dsRNA-fed GPAs or two head capsules of dsRNA-fed GPAs were isolated and transferred to 10 µL of lysis buffer [i.e. 5 mM Tris pH 8.0 (Sigma–Aldrich), 0.5% Triton X-100 (Sigma–Aldrich), 0.5% Tween-20 (Bio-rad), 0.25 mM EDTA (Merck) and 1 mg/mL proteinase K (Roche)] in a 1.5 ml tube, crushed carefully with a sterile plastic pestle followed by a brief centrifugation. After centrifugation, the tube was incubated at 65°C for 10 minutes. The Proteinase K was inactivated by heating the tube at 85°C for 1 minute followed by immediate chilling. The lysate was used immediately for cDNA synthesis or stored below -70°C. DNase I treatment was done using TURBO DNA-free kit (Ambion) following the manufacturer's protocol. Briefly, the volume of total RNA (10 µL) was made up to 14 µL with nuclease-free water and 0.6 µL of Turbo DNAse (~1U) and 0.1 volume of DNAse buffer (1.5 µL) was

added. The sample was then incubated at 37°C for 30 minutes, and 0.1 volume (1.6 µL) of DNAse inactivation reagent was added and incubated at room temperature for 5 minutes with occasionally mixing. Finally, the sample was centrifuged at 4°C at 10,000 g for 1 minute, and RNA was collected in a fresh tube. For cDNA synthesis, 300-400 ng of RNA was used, and 1 µL of cDNA was used for semi-qPCR analysis.

2.5 Preparation of bacterial cultures for plant transformation

To prepare the *A. tumefaciens* GV3101 with the modified vector pART27 for plant transformation, 50-100 mL of LB broth supplemented with two antibiotics: 100 mg/L Spectinomycin dihydrochloride and 25 mg/L rifampicin, was inoculated with 1.5 mL to 2 mL of the stock bacterial culture. The culture was incubated on a shaker at 180 rpm at 28°C in the dark for at least three days, to reach the desired optical density (OD). For tobacco, the final OD was measured at 600 nm to adjust 0.5 to 1.0, and for *Arabidopsis*, it was 0.8 at 660 nm was. To prepare the *A. tumefaciens* inoculum, the bacterial cells were pelleted by centrifugation at room temperature for 20 minutes at 5,500 g. The supernatant was discarded, and the pellet was gently resuspended in the same volume of 5% sucrose solution (for *Arabidopsis*) or CCLM (for tobacco) to reach the desired OD of the cultures. To resuspend the pellet freshly made sucrose solution or CCLM was made every time and sterilised by autoclaving. After preparing the bacterial solution, silwet-77 surfactant was added to the solution before use, at a concentration of 0.05% for Arabidopsis and 0.001% for tobacco, mixed well and used for plant transformation.

2.6 Development of transgenic tobacco (*Nicotiana tabacum*) plants using the floral dip method

N. tabacum (cv. Wisconsin-98) seeds were first sterilised in a laminar flow bench following a protocol developed by Dr John Fosu-Nyarko (NemGenix Pty Ltd.). In brief, one millilitre of 100% ethanol was added to viable tobacco seeds at about 50 µL packed volume in a 1.5 mL tube. After inverting the tube

8-10 times, it was allowed to sit for one minute and then centrifuged at 16,000 g for 15 seconds. The ethanol solution was replaced with one mL of 3% bleach (w/v available chlorine) supplemented with one to two drops of Tween-20. After a brief vortex, the tube was left on the laminar flow bench for 15 minutes with occasional inversion. The liquid was removed with pipettes after centrifugation at 16,000 g for 30 seconds, and the seeds were washed with sterile water 8-10 times. Finally, the water was removed, and one mL of 0.4% water agar was added to the sterile tobacco seeds and mixed well. This mixture was overlaid on MS sucrose medium for germination, and the media plates were kept in tissue culture room under 16-18 hours light regime at 24-25°C until the leaves were ready to use as explants.

Discs were excised from leaves of three to four weeks old tobacco plants, and for each hairpin construct, 25 discs of five to eight mm diameter were cut with a sterile cork borer. The midrib of the leaves was carefully avoided while cutting the explants. The cut leaf discs were immediately soaked in co-cultivation liquid medium (CCLM) to avoid dehydration. CCLM media was prepared with 4.4 gm/L MS basal medium with Gamborg vitamins, one mg/mL BAP, 0.1mg/mL IAA, 200 µM acetosyringone, 30 gm/L sucrose with adjusted pH 5.6. Acetosyringone and other growth hormones (BAP and IAA) were filter sterilised and added after autoclaving the CCLM medium. The leaf discs were transferred with sterile forceps and soaked in a 50 mL sterile Petri dish containing 20-30 mL of bacterial inoculum (described under Section 2.4) for 30 minutes with occasional swirling. External drops of bacterial cultures were blotted carefully from the inoculated leaf discs by gentle pressing with sterile Whatman filter papers, and the explants were placed adaxial side up on co-cultivation solid medium (CCSM), which was CCLM supplemented with 8 g/L agar. The sealed Petri dishes with inoculated leaf discs were kept at 24-25°C 18 hr light for four days for co-cultivation. After co-cultivation, the discs were washed with CCLM supplemented with 200 mg/mL of timentin to kill the *Agrobacterium*, and then transferred to selective shoot regeneration medium (SRM): CCSM supplemented with timentin (200 mg/L) and kanamycin monosulphate (150 mg/L). The media plates were incubated at 24-25°C temperature under 18 hr light for four weeks. Within one to two weeks, multiple callus clusters began

to form from the cut perimeters of the leaf discs. The leaf discs were transferred to fresh SRM plates after two weeks. After four weeks of shoot regeneration, individual shoots were excised (three to five mm long) with sharp, sterile blades and transferred to rooting media (RM) (4.4 gm/L MS basal media with Gamborg's vitamins, 0.1mg/mL IAA, 30 gm/L sucrose, 8 g/L Agar, pH 5.6) supplemented with 200 mg/mL Timentin and 70 mg/mL Kanamycin monosulphate. Roots became established in four weeks, and plantlets were transferred to 0.16 L pots (product code: T60R) filled with pasteurised Murdoch soil mixture (Section 2.0). After transferring to soil, the plants were covered with plastic bags to maintain humidity and kept in a Physical Containment Level 2 (PC2) growth chamber for four days. After four days, the plants were uncovered and transferred to the PC2 glasshouse. Fertilisation and watering was done as necessary. During flowering, the plants were covered with brown bags to avoid any cross-pollination, and fully matured pods were harvested to collect next generation seeds. These seeds are referred to as T_1 seeds that produce T_1 tobacco plants after antibiotic screening to remove any non- transgenic plants.

2.7 Development of transgenic *Arabidopsis* (*Arabidopsis thaliana*) plants following floral dip method

For transformation of *Arabidopsis* (ecotype Columbia-0) the floral dip method was used (Bent, 2006). Wild type seeds were grown in the growth chamber under controlled conditions (23°C and 16:8 hours light: dark cycle) in a tray. For Arabidopsis, the soil was prepared with 3 parts of Murdoch mix and 1 part of Richgro® Seed and Cutting Mix with added fertilisers as described in Section 2.0. After pasteurisation at 60^0C, 0.16 L pots were filled with the soil mixture for growing *Arabidopsis* plants. The initial inflorescences were cut off to encourage the growth of multiple, healthy inflorescences. The healthy multiple inflorescences were used for floral dip transformation.

For each hairpin construct, florets of 8 to 10 plants were dipped in Agrobacterium solution for 10 to 15 seconds. The plants, which were maintained in a PC2 growth chamber, were then covered with a plastic

sheet for 24 hours to have high humidity, and grown at 23°C. The following day the plastic sheets were removed. After one week, the same plants were again inoculated with the bacterial inoculum using the same protocol. Watering and fertilisation was done when needed until the siliques started to mature and dry. 'Aracone' bases were placed in the pots as soon as the inflorescence started to grow: these supported plastic sheets which enclosed the plants to prevent lodging, prevent any mixing of seeds, and to collect dry seeds. Watering was stopped when siliques were started to become brown. Completely dried siliques were collected for threshing, and clean seeds were collected in a 5 ml plastic bottle by separating from debris by passage through a 2 mm sieve. These seeds were referred to as T_1 Arabidopsis seeds, and produced T_1 plants after suitable antibiotic screening.

2.8 Statistical analysis

SPSS v24 software (IBM corporation) was used for analysis of variance (ANOVA), calculation of mean, standard deviation (SD) and standard error (SE) (Norušis, 1990). Significance between treatments was tested at $p<0.05$ and pair-wise comparison was done using Tukey's t-test. Bar charts are provided as mean ± SE for each treatment using Microsoft Excel Analysis Tool pack.

Chapter 3

In silico identification of putative GPA transcripts for neuronal signalling molecules

3.1 Introduction

The development of 'Next generation sequencing (NGS) has provided a mjaor advance over traditional methods for identifying transcripts and full-length genes (Ansorge, 2009; Grabherr et al., 2011; Tanaka et al., 2014). To date, NGS has been used as a rapid, efficient method for analysing large data sets of whole transcriptomes and genomes of both model and non-model organisms (Ansorge, 2009). For insects, NGS analysis of the transcriptome of the wasp, *Polistes metricus* was the first to be reported (Toth et al., 2007). Since then, at least 116 transcriptomes of insects of different orders have been published. Analyses of these transcriptomes have led to identifying putative transcripts of many different proteins, including salivary proteins, transcripts involved in photoperiodism, signalling of other important biological processes (Cortés et al., 2008; Li et al., 2008; Ons et al., 2016; Tanaka et al., 2014; Yin et al., 2016; Zhang, et al., 2013). Similarly, NGS of insect genomes and associated publicly available resources, for example, InsectBase (http://www.insect-genome.com), which has information on 138 insect genomes and 116 insect transcriptomes (as of 2015). These have advanced research in insect gene identification and characterisation. Of the available insect transcriptomes, 12 are from Hemipterans (8.7% of the total number of sequenced insects) (Yin et al., 2016), and include *A. pisum*, whose genome was first published in February 2015 (Consortium, 2010). Since then, transcriptomes and genomes of other related Hemipterans have been published, and this includes a GPA transcriptome and genomes of two GPA clones; G006 (collected from the USA) and O (collected from the UK) (Mathers et al., 2017).

The development of recent advanced bioinformatics tools has also aided analyses of large sequence data sets. This exercise is an essential step for functional characterisation of many important genes. One such group of genes are those for proteins required for control of other cellular processes such as cell-to-cell communication and those for neuroendocrine signals that are essential for insect physiological processes and environmental adaptation (Bendena et al., 2012; Fan et al., 2010). Such

genes can be good targets for insect control but have not been studied in detail for most Hemipteran insects.

3.2 Aims of this Chapter

The aims of this Chapter were: (i) to identify ESTs putatively encoding NSMs *in silico* using GPA ESTs, transcriptome and genome data, (ii) to identify homologous sequences of different aphid species putatively encoding NSMs and compare them with those of GPA, (iii) to amplify a subset of these ESTs from GPA for further RNAi studies.

3.3 Methodology

3.3.1 Identification of GPA transcripts putatively encoding NSMs from EST, transcriptome and genome data

To identify putative GPA transcripts for NSMs, available insect ESTs and genes reported to encode neuroactive proteins were compiled and obtained from the NCBI (National Center for Biotechnology Information). Insects from five major orders were used as a reference set. These included Hemipterans (*A. pisum*, *N. lugens* and *R. prolixus*), Hymenopterans (*Nasonia vitripennis* and *A. mellifera*), Dipterans (*Drosophila melanogaster*, *Aedes aegypti* and *Anopheles gambiae*), a Coleopteran (*Tribolium castaneum*) and one Lepidopteran (*Bombyx mori*). The references sequences were grouped separately as NPs, NPRs and BARs (298 and 311 nucleotides of eight insects and 99 nucleotides of five insects, respectively) for analysis. For all the BLAST searches, available nucleotide sequences of reference insects were used instead of amino acid sequences. This is because the purpose of the analysis was to identify target genes for RNAi studies that act at the mRNA level. Publicly available GPA ESTs (27,721) were used to identify similar transcripts (Ramsey et al., 2007) using TBLASTX (http://blast.ncbi.nlm.nih.gov/) with a threshold expect value (E-value) of 1E-05. GPA hits from the search were confirmed as transcripts for NSMs by further comparing them to homologues of other

insects using TBLASTX and BLASTX in the NCBI insect databases (taxid: 6960) for nucleotide collection (nr/nt) and nonredundant proteins (nr), respectively.

Putative transcripts for NSMs were also identified from mixed developmental stage GPA transcriptomes and publicly available genomic data. RNA extraction and cDNA synthesis, similar to that in Section 2.2, and the NGS using the Ion Torrent platform at the SABC are described by Fosu-Nyarko et al., 2017 (in preparation). The raw reads were assembled *de novo* and mapped back to contigs using CLC Genomic Workbench Version 7.5.1 (Qiagen Pty. Limited) with the following parameters: a mismatch cost of 3, insertion cost of 3, deletion cost of 3, length fraction of 0.4 and similarity fraction of 0.9. A local database was created for the resulting 65,376 transcripts (contigs and singletons) in CLC Genomics Workbench, from which putative transcripts for GPA NSMs were identified using BLAST search. TBLASTX was also used to identify genomic contigs of GPA clone O (3,890 Whole Genome Shotgun contigs, Bio Project accession PRJEB11304, NCBI) which contained putative exons of NSMs. From the identified transcripts (NCBI and transcriptome), 32 transcripts were selected for further studies. The process used for *in silico* identification and selection of GPA NSM transcripts for further study is presented in Figure 3.1.

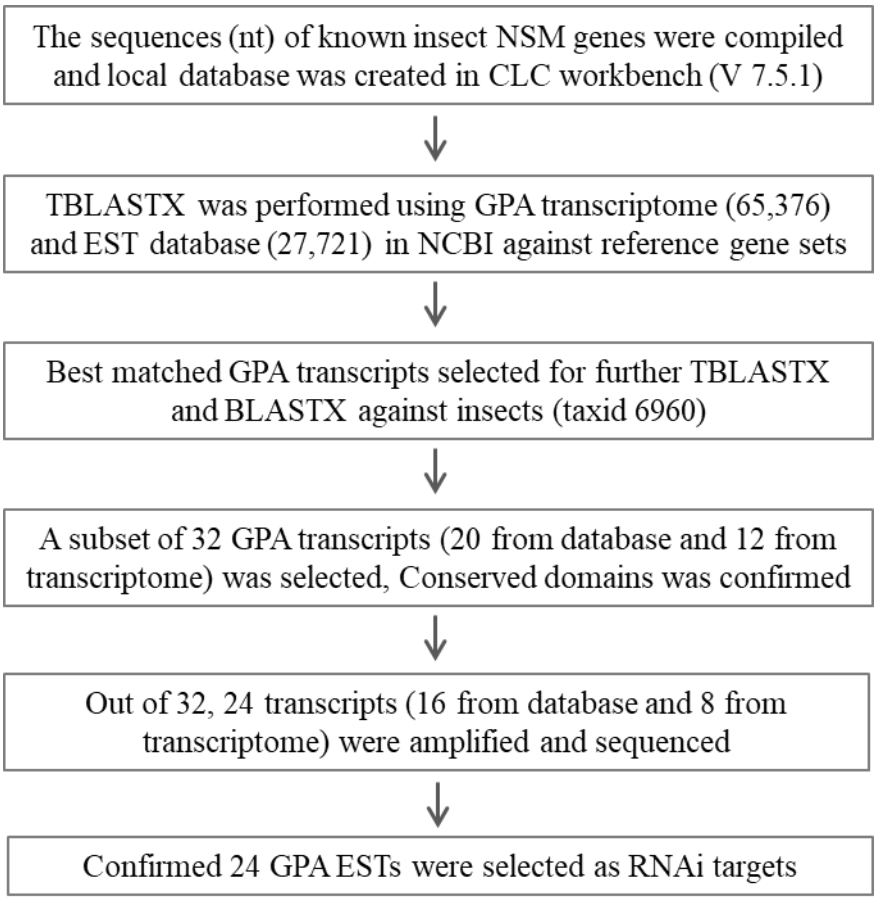


Figure 3. 1: The bioinformatic approach used to identify putative GPA NSM ESTs for RNAi studies.

3.3.2 Comparative analyses of NSMs of aphid species

Homologous NSM sequences from five other aphid species, obtained from the nucleotide, EST and transcriptome shotgun assembly databases (NCBI) were identified using BLAST searches and compared to those of GPA. The five aphid species were *Diuraphis noxia* (Russian wheat aphid, RWA), *Sitobion avenae* (the English grain aphid), *Aphis gossypii* (the cotton aphid), *Aphis glycines* (the soybean aphid) and *Macrosiphum euphorbiae* (the potato aphid). The curated sequences were used for TBLASTX analysis using CLC Genomics Workbench 7.5.1 with an E-value threshold of 1E-05 and bit score of 50. Phylogenetic relationships between sequences of sis NSMs (CAPA, CAPAR, CCAP, LK, NPLP1 and Octβ3R) of insects including aphids were analysed, and all these NSMs were studied further. Phylogenetic analysis was conducted using MEGA6 using neighbour-joining (NJ) tree with 1000 bootstraps (Tamura et al., 2013). Sequences used for this analysis were from similar regions of

genes. These curated sequences were obtained from multiple alignments generated using Geneious v10.0.2 (ClustalW) (Kearse et al., 2012). It should be noted that as *capa* gene-encoding peptides have been described with different names, such as cardio acceleratory peptide (Cap) or Capa or Pyrokinin (Pk) depending on the insect species, all the available nucleotide sequences for these were used to construct the phylogenetic tree. Signal peptides were predicted in translated GPA transcripts using SignalP (Bendtsen et al., 2004).

3.3.3 Amplification of putative ESTs of GPA NSMs

Primers for 32 of NSM transcripts (20 identified from ESTs from NCBI and 12 from the locally generated transcriptome) were designed for PCR from cDNA obtained from the head region of mixed developmental stages of GPAs. The NCBI Conserved Domain tool was used to search the domains in these GPA transcripts and considered during primer designing for further studies (Marchler-Bauer et al., 2014). These transcripts are thought to have roles in insect moulting, growth, reproduction, muscle contraction, energy metabolism and water balance. Of these, 24 transcripts were amplified from cDNA, and the primers used to amplify these 24 transcripts are presented in Table 2.1. The amplicons generated were validated by Sanger sequencing followed by analyses (e.g. multiple alignments using the MultAlin Tool (Corpet, 1988) with the original ESTs from which the primers were designed. RNA extraction, cDNA synthesis and sequencing were as described under Section 2.2.

3.4 Results

3.4.1 Putative transcripts of GPA NPs

During this analysis, GPA transcriptomic and genomic sequences were not available and publicly accessible GPA ESTs were first used for homology searches. Using *A. pisum* NP ESTs (Huybrechts et al., 2010), 19 out of the 27,721 GPA ESTs available at NCBI (Ramsey et al., 2007) were identified as putative transcripts of NPs. Of these, 18 were homologous to transcripts of insect NPs except for the EST, EE262533, which is thought to encode a zinc finger protein. Subsequent analyses used 298

reference sequences of NPs obtained from NCBI to identify for 30 different NP transcripts from the GPA transcriptome (24 NPs) and the genomic contigs (27 NPs) (Table 3.1). Sixty putative transcripts were identified for the 24 NPs whereas 57 GPA genomic sequences (Whole Genome Shotgun contigs, Bio Project accession PRJEB11304, NCBI) appeared to have exons for 27 NPs. Sequences for 21 NPs were identified from both datasets. Homologous sequences for 15 of the 30 NPs were also present in sequence data of four other Hemipteran insects namely *A. pisum, R. prolixus, N. lugens* and *M. persicae* whereas transcripts for 10 NPs (AKH, CCAP, EH, ETH, Insulin, ITP, MSN, SIFamide, sNPF and Tk) were also identified for insects of different orders (Table 3.1). Two contigs of the transcriptome, contig 3064 and contig 666, which encoded an EH and ITP respectively, were predicted to encode cleavable signal peptides. The failure to identify signal peptides in most of the conceptually translated proteins could be attributed to the relatively short lengths of the transcripts - these do not represent full-length sequences of genes.

Sequences for six NPs, Corazonin (Cz), Sulfakinin (Sk), Adipokinetic hormone (AKH), Allatostatin CC (AstCC), CCH-2 amide and Allatotropin (AT), were identified only in the genomic data, with very low sequence similarity scores for all except Allatotropin (bit score of 625.0 and E-value of 2E-178). The BLAST bit scores for the five hits were <52.0 with E values between 2E-06 and 7E-06. Two of these NPs (Cz and Sk) have so far not been identified from available sequences of *A. pisum* (Consortium, 2010; Hauser et al., 2010; Huybrechts et al., 2010). Interestingly, there was no sequence with a significant match to three genes (*astc*, *sifamide* and *gpaα*) in the GPA genomic data for which transcripts with relatively high BLAST scores were present in the transcriptome data. One such transcript matched the *gpaα* of *N. lugens* (AB817258) with a bit score of 178.4 and a low E-value of 2E-47.

There was one transcript and one genomic contig of GPA that appear to be homologous to the neuropeptide like protein 3 gene, *Nplp3* of *N. lugens*. However, the BLAST scores (bit scores of 63.0-

64.0, and E-values of 1E-09 and 1E-13, respectively for the transcript and contig) were not convincing enough to conclude that the GPA encodes such a protein. Interestingly, the list of neuropeptides reportedly encoded by *A. pisum* did not include this peptide (Huybrechts et al., 2010).

Table 3.1 Presence of transcripts for NPs identified (ligands for GPCRs) from sequences of GPA and those reported for nine other insects

GPA Homologous sequences putatively encoding NPs	**Mp**		**Dm**	**Tc**	**Ap**	**Nl**	**Rp**	**Aa**	**Ag**	**Bm**	Am
	Transcripts	Gc									
Adipokinetic Hormone (AKH)	-	+	+	+	+	+	+	+	+	+	+
Allatostatin C (Ast-C)	+	-	+	+	+	-	-	+	+	+	+
Allatostatin CC (Ast-CC)	-	+	-	-	+	+	-	-	-	-	-
Allatotropin (AT)	-	+	-	+	+	+	+	+	+	+	+
Bursicon alpha (Burs α)	+	+	+	+	+	+	-	+	+	+	+
Bursicon beta (Burs β)	+	+	+	+	+	+	-	+	+	+	+
Capability (Capa)	+	+	+	+	+	+	-	+	+	+	+
Crustacean Cardioactive Peptide (CCAP)	+	+	+	+	+	+	+	+	+	+	+
CCH-2 amide	-	+	+	+	+	-	+	-	+	+	+
Corazonin (cz)	-	+	+	-	-	+	+	+	+	+	+
Calcitonin like DH	+	+	+	+	+	-	+	+	+	+	+
Corticotropin releasing factor like DH (CRF-DH)	+	+	+	+	+	+	-	+	+	+	+
Eclosion Hormone (EH)	+	+	+	+	+	+	+	+	+	+	+
Ecdysis Triggering Hormone (ETH)	+	+	+	+	+	+	+	+	+	+	+
Glycoprotein Hormone alpha (GPH α)	+	-	+	+	+	+	-	+	+	+	+
Glycoprotein Hormone beta (GPH β)	+	+	+	+	+	+	-	+	+	+	+

GPA Homologous sequences putatively encoding NPs	**Mp**		**Dm**	**Tc**	**Ap**	**Nl**	**Rp**	**Aa**	**Ag**	**Bm**	Am
	Transcripts	Gc									
Insulin-related peptide (bombyxin like or others) (ILP/IRP)	+	+	+	+	+	+	+	+	+	+	+
Insulin-related peptide (IRP5)	+	+	-	-	+	-	-	+	+	-	-
ITP	+	+	+	+	+	+	+	+	+	+	+
MSN	+	+	+	+	+	+	+	+	+	+	+
NPF	+	+	+	-	+	+	+	+	+	+	+
NPLP1	+	+	+	+	+	-	+	+	+	+	+
NPLP3	+	+	+	-	-	+	-	-	-	-	-
Orcokinin	+	+	-	-	+	+	+	+	+	+	+
Proctolin	+	+	+	+	+	+	-	+	-	+	-
Pyrokinin	+	+	+	+	+	+	+	+	+	-	+
SIFamide	+	-	+	+	+	+	+	+	+	+	+
sNPF	+	+	+	+	+	+	+	+	+	+	+
Sulfakinin	-	+	+	+	-	+	+	+	+	+	+
Tachykinin	+	+	+	+	+	+	+	+	+	+	+

Gc: Genomic contig, Mp: *M. persicae*, Dm: *D. melanogaster* (Hewes and Taghert, 2001), Ap: *A. pisum* (Huybrechts et al., 2010), Rp: *R. prolixus* (Ons et al., 2011), Aa: *A. aegypti* (Predel et al., 2010), Ag: *A. gambiae* (Riehle et al., 2002), Bm: *B. mori* (Roller et al., 2008), Tc: *T. castaneum* (Li et al., 2008), Nl: *N. lugens* (Tanaka et al., 2014) and Am: *A. mellifera* (Ons et al., 2016) (NP genes of *A. mellifera* were not included in the analysis).

3.4.2 Transcripts of the GPA transcriptome and genomic data homologous to GPCRs

Using 17 *A. pisum* sequences for NPRs (Li, et al., 2013), only five GPA ESTs (available in NCBI) were identified. Additionally, using 311 insect sequences as reference, 12 from the transcriptome and 16 from the genomic contigs putatively encoded 24 NPRs were identified (Table 3.2). Sequences for 10 of the 24 NPRs were present in both the transcriptome and genomic data. For 16 of the putative NPRs identified, sequences of the corresponding NPs (i.e. the ligands for these receptors) were also present in the datasets (Table 3.2). Whereas sequences of 20 of the NPRs were also identified from *A. pisum* data, transcripts for nine were among sequences of each of the insect species used in the study.

Table 3. 2: GPA transcripts of NPRs identified in transcriptomic and genomic contigs and a comparison with those reported for nine other insects

GPA Homologous sequences putatively encoding NPRs	**GPA**		**Ligand identified for GPA from this study**	**Dm**	**Tc**	**Ap**	**Rp**	**Aa**	**Ag**	**Bm**	**Nv**	**Am**
	Tr	Gc										
AKH-R	-	+	Y	+	+	+	+	+	+	+	+	+
Allatostatin C receptor	-	+	Y	+	+	+	+	+	+	+	+	+
Allatostatin-A receptor	-	+	Y	+	-	+	+	+	+	+	+	+
Allatotropin receptor (AT-R)	+	+	Y	-	+	+	+	-	+	+	+	+
Arginine vasopressin receptor (avpr)	-	+	-	-	-	-	-	-	-	-	-	+
CAPA receptor	+	+	Y	+	+	+	+	-	+	+	+	+
CCAP Receptor	-	+	Y	+	+	+	+	+	+	+	+	+
CCHamide Receptor 1	-	+	-	+	+	+	+	+	+	+	+	+
CCHamide Receptor 2 (bombesin receptor)	-	+	Y	+	+	+	+	+	+	+	+	+
Calcitonine like DH receptor	+	+	Y	+	+	+	-	+	+	+	+	+

GPA Homologous sequences putatively encoding NPRs	**GPA**		**Ligand identified for GPA from this study**	**Dm**	**Tc**	**Ap**	**Rp**	**Aa**	**Ag**	**Bm**	**Nv**	**Am**
	Tr	Gc										
Corticotropin Releasing Factor DH receptor	+	-	Y	+	+	+	-	+	+	-	+	+
Ecdysis Triggering Hormone receptor	+	+	Y	+	+	+	+	+	+	+	+	+
FMRFamide receptor	-	+	-	+	-	+	-	-	+	+	+	+
Gonadotropin-releasing hormone II receptor-like	+	+	-	+	+	+	-	+	-	-	-	-
G-protein coupled receptor moody-like	+	+	-	+	+	+	-	-	-	+	-	-
Insulin receptor	+	-	Y	+	+	+	-	+	+	+	+	+
Leucine-rich repeat-containing G-protein coupled receptor (LRG)	+	+	-	+	-	+	-	-	+	+	-	-
Myosuppressin receptor (msR)	-	+	Y	+	+	+	+	+	+	+	+	+
Neuropeptide FF receptor / SIFamide receptor	-	+	Y	+	+	+	+	+	+	+	+	+
Neuropeptide F Receptor (NPF-R)	-	+	Y	+	+	+	+	+	+	+	-	-
Neuropeptide Y receptor type (1/2/5-like)	+	+	-	+	-	+	-	+	+	+	+	-
Pyrokininn receptor 1/2 (capa receptor)	+	+	Y	+	+	+	-	+	+	+	+	+
Tachykinin-Receptor (Neuromedin K- R)	+	+	Y	+	+	+	+	+	+	+	+	+
Trissin receptor	-	+	-	+	-	-	-	-	-	-	-	-

Using 99 reference sequences representing five major BAR groups, putative transcripts for nine distinct BARs were identified from the GPA sequences used in the study (Table 3.3). Transcripts for one BAR was identified only in the GPA genomic contigs with relatively higher

sequence homology scores (bit score of 167.0). Homologous transcripts for eight BARs (except dopamine receptor) were identified in both datasets.

Table 3. 3: Presence of putative transcripts of BARs of GPA and those reported for BARs reported for seven other insects

GPA Homologous sequences putatively encoding BARs	**GPA**		**Dm**	**Tc**	**Ap**	**Rp**	**Ag**	**Bm**	**Am**
	Tr	Gc							
Dopamine receptor (DopR)	-	+	+	+	+	+	+	+	+
Muscarinic acetylcholine receptor DM1 (mAChRDM1)	+	+	+	+	+	+	+	+	+
Muscarinic acetylcholine receptor M3 (mAChRM3)	+	+	+	+	+	+	+	+	+
Nicotinic acetylcholine receptor beta1 subunit (nAChRβ1)	+	+	+	+	+	+	+	-	+
Octopamine receptor (Oamb)/ tyramine Receptor	+	+	+	+	+	+	+	+	+
Octopamine receptor beta-1R (Octβ1R)	-	+	+	+	+	+	+	+	+
Octopamine receptor beta-2R (Octβ2R)	-	+	+	+	+	+	+	+	+
Octopamine receptor beta-3R (Octβ3R)	+	+	+	+	+	+	+	+	+
Serotonin receptor	+	+	+	+	+	+	+	+	+

For Table 3.2 and 3.3, Gc: Genomic contig, Mp: *M. persicae*, Dm: *D. melanogaster* (Hauser et al., 2006), Ap: *A. pisum* (Li, et al., 2013), Rp: R. *prolixus* (Ons et al., 2016), Tc: *T. castaneum* (Li et al., 2008), Aa*: A. aegypti* (Caers et al., 2012), Ag: A. *gambiae* (Caers et al., 2012), Bm: B. *mori* (Caers et al., 2012), Nv: *N. vitripennis* (Hauser et al., 2010), and Am: A. *mellifera* (Hauser et al., 2006). (NPR and BAR genes of *R. prolixus* and BAR genes of *A. gambiae* were not included in the analysis).

3.4.3 Comparative analysis of putative transcripts for NSMs of aphid species

Homology searches were conducted to identify sequences putatively encoding NSMs of five aphid species; *A. gossypii, A. glycines, D. noxia, S. avaneae* and *M. euphoribae*. These were then compared with putative transcripts of GPA and *A. pisum* previously reported by

Huybrechts et al., (2010) and Li et al., (2013). Of the five aphid species studied, most NPs were identified for GPA and *A. gossypii*, while for NPRs most (27) NPRs were found for *D. noxia* and *A. glycines* (Figure 3.2). Sequences for nine BARs were found in data for all the aphid species studied. To simplify the nomenclature of NSMs, all serotonin and dopamine GPCR genes with different subunits are referred to as 'serotonin receptors' or 'dopamine receptors' in this thesis.

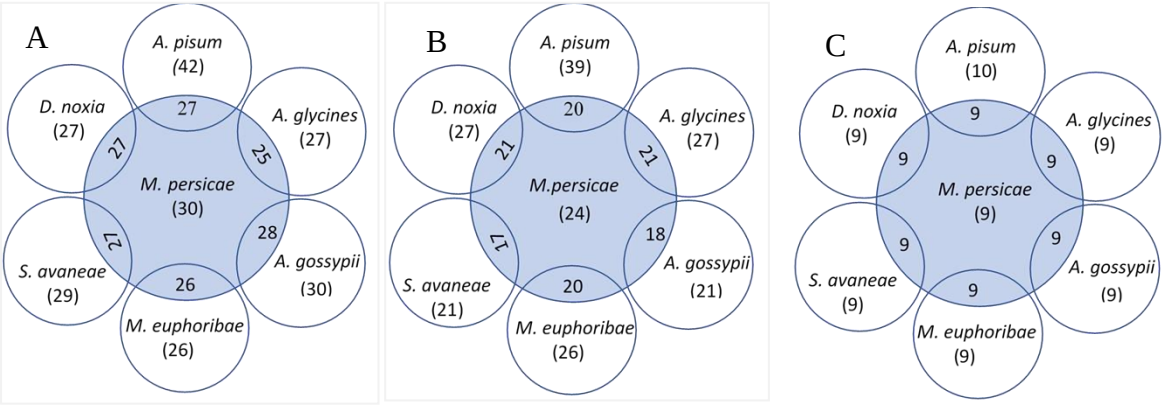


Figure 3.2: Total number of NSMs identified in seven different aphid species; A: NP, B: NPR, and C: BAR (overlapped circle indicates the number of NSMs common for two species).

No direct relationship was observed between the numbers of sequences of individual aphid species studied in the homology searches and the number of NSMs identified (Figure 3.3). For example, 63 NSMs were identified in sequences of GPA, *D. noxia* and *A. glycines*, but the number of sequences available for each organism used for the analysis was significantly different. For *A. gossypii* in which 89,886 sequences were used, only 0.87% were transcripts of 60 NSMs, whereas for *D. noxia* 2.4% of the 23,757 sequences encoded possible NSMs (Figure 3.3). This may reflect the different qualities of transcriptome assemblies of those aphid species.

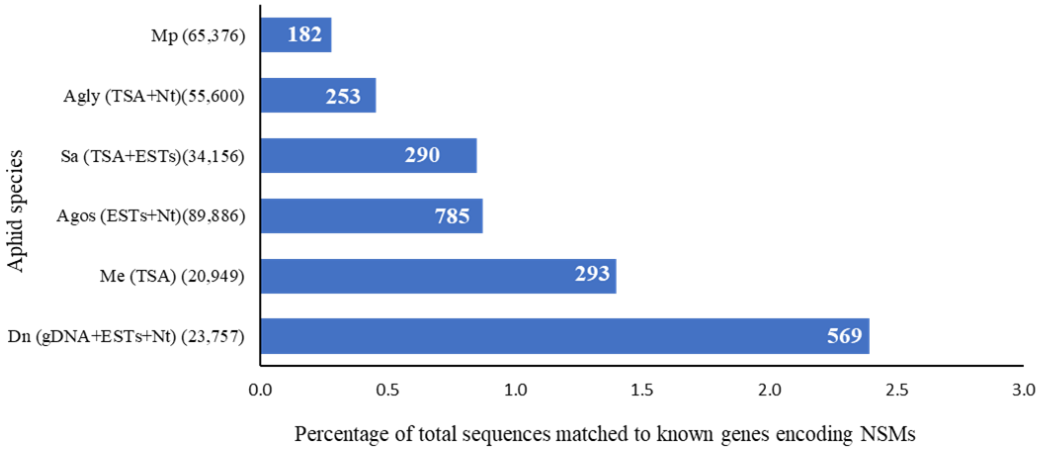
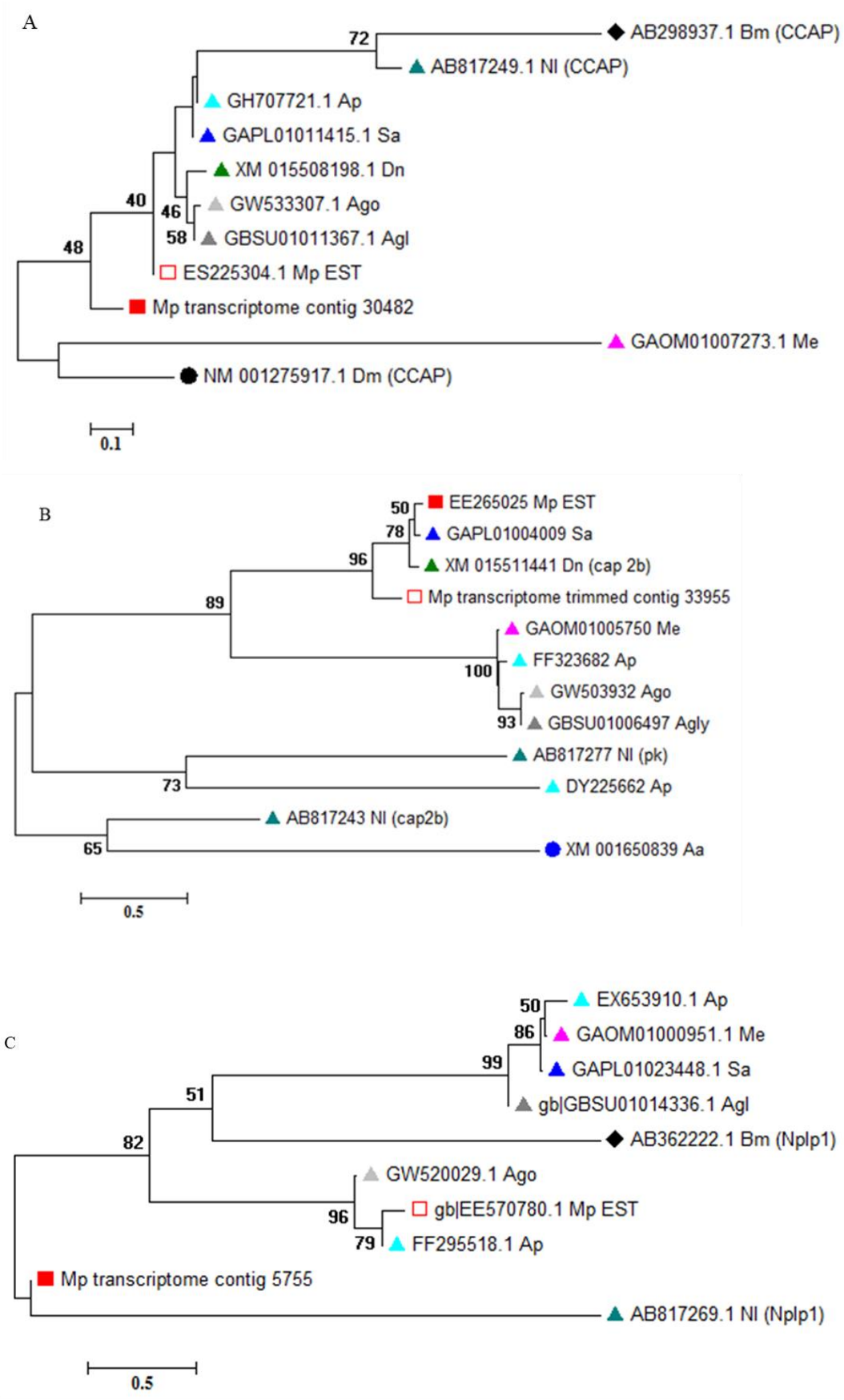


Figure 3.3: The number of sequences of NSMs of six aphid species (the number of homologous sequences for each aphid species is presented in each bar). Me: *M. euphorbia*; Ago: *A. gossypii*, Agly: *A. glycines*, Sa: *S. avenae*, Dn: *D. noxia* and Mp*: M. persicae*.

3.4.4 Phylogenetic analysis of identified sequences of different aphid species thought to encode NSMs

Phylogenetic analysis conducted for six NSM genes using reference genes and homologous sequences identified in different aphid species using similar regions for each of the genes (Figure 3.4). From the phylogenetic trees, higher bootstrap values (up to 100%) were observed for five NSMs using the corresponding nucleotide sequences namely *nplp1, capa, octβ-3R* and *lk*. Most of the GPA transcripts were grouped with the sequences of other Hemipterans rather than those of the distantly related insect orders. For example, sequences encoding Nplp1, Capa, CapaR, CCAP and Octβ-3R were in the same clade as those of *A. pisum*, while Lk clustered with sequences of *N. lugens*. For *Msn* and *Nplp*1, the ESTs identified from NCBI database and from the GPA transcriptome clustered separately, although this might be due to the nucleotide sequence variation. Bootstrap values less than 40% are not shown in Figure 5.4.


A
AB298937.1 Bm (CCAP)
AB817249.1 Nl (CCAP)
GH707721.1 Ap
GAPL01011415.1 Sa
XM 015508198.1 Dn
GW533307.1 Ago
GBSU01011367.1 Agl
ES225304.1 Mp EST
Mp transcriptome contig 30482
GAOM01007273.1 Me
NM 001275917.1 Dm (CCAP)
72
40
46
58
48
0.1
B
EE265025 Mp EST
GAPL01004009 Sa
XM 015511441 Dn (cap 2b)
Mp transcriptome trimmed contig 33955
GAOM01005750 Me
FF323682 Ap
GW503932 Ago
GBSU01006497 Agly
AB817277 Nl (pk)
DY225662 Ap
AB817243 Nl (cap2b)
XM 001650839 Aa
50
78
96
89
100
93
73
65
0.5
C
EX653910.1 Ap
GAOM01000951.1 Me
GAPL01023448.1 Sa
gb|GBSU01014336.1 Agl
AB362222.1 Bm (Nplp1)
GW520029.1 Ago
gb|EE570780.1 Mp EST
FF295518.1 Ap
Mp transcriptome contig 5755
AB817269.1 Nl (Nplp1)
50
86
99
51
82
96
79
0.5

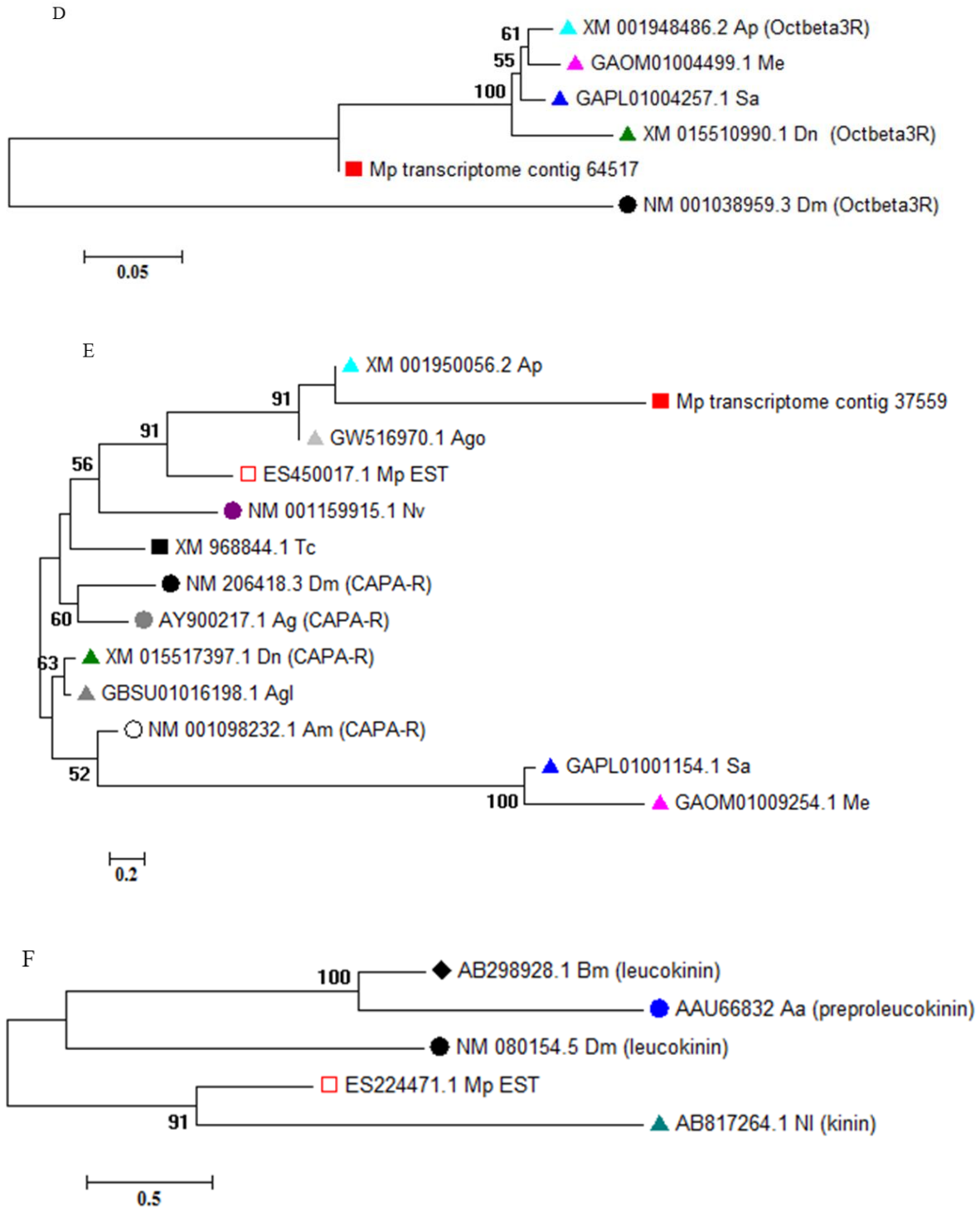


Figure 3.4 (A-F): Phylogenetic analyses of insect nucleotide sequences encoding six NSMs in different insect species. A: CCAP, B: Capa, C: Nplp1, D: Octβ3R, E: CapaR, F: Lk; In the trees, Ap: *A. pisum*; Me: *M. euphorbia*; Ago: *A. gossypii*, Agl/Agly: *A. glycines*, Sa: *S. avenae*, Dn: *D. noxia*, Nl: *N. lugens*, Rp: *R. prolixus*, Dm: *D. melanogaster*, Ag: *A. gambia*, Aa: *A. aegypti*, Bm: *B. mori*, Am:*A. mellifera*, Tc: *T. castaneum*, Nv: *N. vasopressin*.

3.4.5 Transcripts of NSMs not identified in the GPA transcriptome or in genomic data

Transcripts of six putative NPs were not identified in the available GPA transcriptome or genomic sequences. These were Arginin vasopressin (avp), AstA, AstB/ mip, EH1, Pdf, Trissin. Genes encoding a further seven NPRs: CzR, PdfR, BursR, LkR, ProcR, sNPFR and SfkR were also not identified in any of the datasets. While most these genes have been found in the closest Hemipteran species, *A. pisum*, both ligands and receptors namely Avp, Cz, Pdf, Sulfakinin and Trissin were not found in this species (Huybrechts et al., 2010; Li, et al., 2013).

3.4.6 Amplification of GPA transcripts known to encode NSMs

Initially 32 NSM transcripts from GPA thought to be for NSMs (20 from existing databases and 12 from the transcriptome) were selected to be amplified – these were not amplified from cDNA derived from aphid total RNA. All of these genes are known to be involve in essential insect physiological systems, such moulting, reproduction, feeding, water homeostasis and muscle contraction. Using head-derived cDNA, 24 GPA transcripts were amplified (Figure 3.6). Of these 24 transcripts, 14 were NPs transcripts, and 10 were GPCR transcripts (including both NPRs and BARs). The identity of the amplicons generated was confirmed by Sanger sequencing, followed by comparison with the sequences of the original ESTs. Query coverages ranged from 71% to 100%, and E-values were between 3E-129 and 1E-24, and most of the GPA ESTs best matched those of *A. pisum*. Conserved domains were confirmed for 15 ESTs and all putative GPCR ESTs had the 7tm GPCR superfamily domain (Table 3.4). Amplicons of the target genes are presented in Figure 3.5.

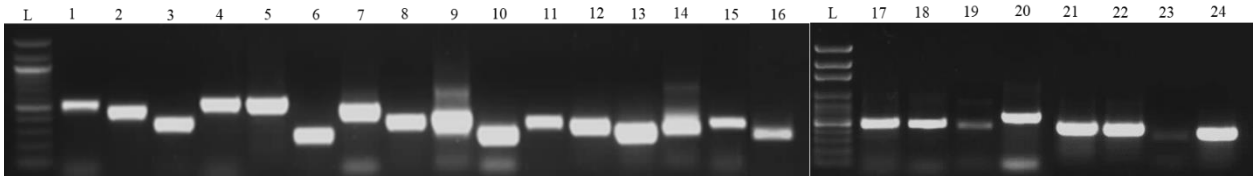


Figure 3.5: Amplified GPA ESTs of target genes; L=100bp ladder, Lane1= LK, Lane 2= Capa, Lane 3= CAPAR1, Lane 4 = NPLP1, Lane 5= nAchRβ1, Lane 6= Lgr5, Lane 7= CP, Lane 8= TkR, Lane 9= EH2/3, Lane 10= OβR 3R, Lane 11= NpY2R, Lane 12= mAChRM3, Lane 13= CAPAR2, Lane 14= Bursβ, Lane 15= ITP, Lane 16= IRP2/3, Lane 17= sNPF, Lane 18= IRP5, Lane 19= Opsin, Lane 20= GRHR, Lane 21= ETH, Lane 22= AstCC, Lane 23= JHBP and Lane 24= CCAP.

Table 3.4: List of GPA ESTs used for *in vitro* RNAi study

Target gene (s)	GPA ESTs (Position on ESTs)	Best matched insect	Best hit matched to other insects	Conserved domain identified in GPA ESTs	Functions in insects
Allatostatin CC (AstCC)	EE570985 (23 - 439)	*A. pisum*	Uncharacterised; XM_003243979	Not found	Growth, energy metabolism
Bursicon β (Burs β)	ES224407 (227 - 559)	*A. pisum*	Bursicon; XM_008181228	Not found	Wing formation, moulting
Capability (CAPA)	EE265025 (356 - 827)	*A. pisum*	CAPA; NM_001162363	Not found	Muscle activity, Water homeostasis
RR1 Cuticle Protein 4 (CpRR1-4)	Contig328	*A. pisum*	CpRR1- 4; NM_001172268	Not found	Cuticle hardening
Eclosion Hormone 2/3 (EH2/3)	Contig392	*A. pisum*	Uncharacterised; XM_001950188	Not found	Moulting
Ecdysis triggering Hormone (ETH)	ES223798 (14 - 459)	*A. pisum*	ETH; NM_001163212	Not found	Moulting
Leucokinin (LK)	ES224471 (82 - 620)	*A. pisum*	Uncharacterised; XM_003244612	Not found	Water homeostasis
Neuropeptide like protein (NPLP1)	EE570780 (59 - 581)	*D. noxia*	Uncharacterised; XM_015522242	Not found	Unknown

Target gene (s)	GPA ESTs (Position on ESTs)	Best matched insect	Best hit matched to other insects	Conserved domain identified in GPA ESTs	Functions in insects
Short NPF (sNPF)	EE570068 (211-686)	*A. pisum*	sNPF; XM_003247202	Not found	Feeding
Tachykinin-like peptides receptor 99D (TKR 99D)	Contig (67516 + 69426)	*A. pisum*	TKR 99D; XM_008183127	7tm GPCRs superfamily	GPCR activity
Capability Receptor (CAPA R1)	ES450017 (264 - 625)	*A. pisum*	Neuromedin-U receptor 2; XM_003241036	7tm GPCRs superfamily	GPCR activity
Capability Receptor (CAPA R2)	Contig 45194	*A. pisum*	Neuromedin-U receptor 2; XM_008188009	7tm GPCRs superfamily	GPCR activity
Gonadotropin-releasing hormone receptor (GRHR)	Contig (53328 + 67150)	*D. noxia*	GRHR; XM_015524328	7tm GPCRs superfamily	GPCR activity
Neuropeptide Y receptor type 2 (NPYR2)	Contig 26232	*D. noxia*	NPY2R; XM_015516771	7tm GPCRs superfamily	GPCR activity
Leucine-rich repeat-containing GPCR 5 (LGR5)	Contig 23523	*A. pisum*	LGR5; XM_001948741	7tm GPCRs superfamily	GPCR activity
Muscarinic acetylcholine receptor 3 (mAchR3)	Contig 44317	*A. pisum*	mAChR M3; XM_016800777	7tm GPCRs superfamily	GPCR activity
Octopamine beta 3R (Octβ3R/Obeta3r)	Contig 41763	*A. pisum*	Oβ3R, XM_001948486	7tm GPCRs superfamily	GPCR activity
Opsin (Long Wavelength)	EE571490 (43 - 433)	*R. padi*	Opsin; FM177114	7tm GPCRs superfamily	Visualisation
Crustacean Cardioactive Peptide (CCAP)	ES225304 (81- 455)	*A. pisum*	CCAP, XM_008189504	CCAP superfamily	Cardio muscle activity
Insulin-related peptide 2/3 (IRP2/3)	EE570268 (260 - 614)	*A. pisum*	Bombyxin C-1; XM_003244078	IlGF like family	Energy metabolism

Target gene (s)	GPA ESTs (Position on ESTs)	Best matched insect	Best hit matched to other insects	Conserved domain identified in GPA ESTs	Functions in insects
Insulin-related peptide 5 (IRP5)	EC387272 (87 - 495)	*A. pisum*	Uncharacterised; FP927759	IlGF like family	Energy metabolism
Juvenile hormone Binding Protein (JHBP)	ES224026 (76 - 358)	*A. pisum*	Uncharacterised; NM_001326644	JHBP superfamily	Embryogenesis, reproduction
Ion transport peptide (ITP)	EC390187 (118 - 660)	*Apis florea*	Predicted apolipoprotein D; XM_012485105	Lipocalin superfamily	Water homeostasis
Nicotinic acetylcholine receptor beta1 (nAchRβ1)	AJ251838 (207 - 750)	*R. padi*	nAchRβ1, KT328076	Neurotransmitter gated ion channel ligand binding	Acetylcholine receptor activity

3.5 Discussion

The homology searches undertaken in this study, making use of sequence data available at the time, revealed 63 putative NSMs or neuronal peptides which were encoded by the GPA genes. Of these, putative transcripts for 30 NPs, 24 NPRs and 9 BARs were identified in the GPA transcriptome and/or genomic sequences. Homologous sequences for most of the NSMs were also present in five other important aphid species studied. Of the aphid species, most were identified for *A. gossypii* (30), whereas the most NPRs were from the sequences of *A. glycines* (27) and *D. noxia* (27). Putative homologous sequences for nine BARs were present in all the aphid species. Phylogenetic analysis of six NSM transcripts showed that those of GPA were mostly closely related to the sequences of *A. pisum*. From the identified NSM sequences, 24 putative GPA transcripts essential for insect physiology were selected for further study.

From the homology searches, it was found that most of the NPs and GPCRs identified in the *A. pisum* genome were also present in the GPA transcriptome and/or genomic contigs. However, the NPs such as Allatostatin B (AstB) or FMRFamide were not identified in the GPA transcriptome or genomic contigs. The cognate receptor for FMRFamide was identified in GPA

sequences suggesting the ligand might be present in GPA, whereas the AstB receptor has not yet been identified from either of these two aphid species (Li, et al., 2013). Another noteworthy observation includes the number of insulin genes present in *A. pisum* and GPA sequences. From the analysis and available literature, transcripts for four insulin genes were identified for GPA while those for at least 10 insulin genes have been reported in the *A. pisum* genome (Huybrechts et al., 2010).

Homologous sequences of four NSMs, namely, *cz, sulfakinin, arginine vasopressin* receptor and *trissin receptor* have not been identified so far in *A. pisum*, but are present in the GPA genomic data, with very low sequence similarity (bit score 51.6 - 53.6), but homologous sequences of the corresponding ligands were not found in any of the GPA datasets. *Trissin receptor* was reported to be lost in Hymenopteran and hemimetabolous insects during evolutionary divergence, and it has been suggested that sulfakinin signal system is not present in *A. pisum* (Amare and Sweedler, 2007; Li, et al., 2013). Therefore, the observed lower homologies for those four GPA transcripts could be the result of the stringency used in the analyses. Among three groups of NSMs, similar number of BARs were found in all aphid species showing that these receptor groups are well conserved, probably reflecting their required function in insect growth and development (Hauser et al., 2006; Li, et al., 2013; Ons et al., 2016).

At the start of this study here was little information on aphid NSMs. However, a set of potential NSM gene targets were identified successfully, and study of their potential for use in aphid control using gene silencing technology is described in the following Chapters.

3.6 Conclusion

The research undertaken in this Chapter on aphid NSMs provides new knowledge for molecular and physiological studies on aphid species and their control targeting NSMs. It

provides a firm basis for the subsequent studies on their functional analysis by gene silencing technology.

Chapter 4

Evaluation of the effects of down-regulation of 24 NSM encoding genes by feeding dsRNA to GPAs using an artificial diet

4.1 Introduction

Since its discovery, RNAi has become recognised as a powerful molecular tool for genetic studies in many insect species. The mechanism is also being explored as a 'next generation' pest management strategy (Arakane et al., 2008; Bellés, 2010; Yu et al., 2016; Zhu, 2013). Screening and selection of suitable target genes is necessary to develop robust RNAi effects (Christiaens et al., 2014; Huvenne and Smagghe, 2010). NSMs are a versatile group of signalling molecules that regulate or initiate many biological processes in insects (Bendena, 2010). The pleiotropic functions of NSMs make them leading targets for control of crop pest insects. In fact, most traditional insecticides have been developed to target components of insect nervous systems (Bendena, 2010). However, there has been little work on gene silencing of insect neuronal genes (Yu et al., 2016; Zhang, et al., 2013).

To screen potential genes as effective RNAi targets, an *in vitro* feeding system offers a simple, and quick method of dsRNA delivery to insects and is less damaging than microinjection (Bai et al., 2011; Baum et al., 2007; Zhang, et al., 2013). This is particularly the case for small, delicate insects like GPAs: for this oral delivery is one of the least disruptive methods to introduce dsRNA (Gong et al., 2014). Although several *in vitro* RNAi studies have been undertaken using artificial feeding in *A. pisum* and other aphid species including GPA, very limited of these has involved study of any genes encoding NSMs (Christiaens and Smagghe, 2014; Ding et al., 2017; Guo et al., 2016; Zhang, et al., 2013). Therefore, this *in vitro* feeding approach will provide new data on the efficiency of neuroactive genes of GPA following oral delivery of target dsRNA.

4.2 Aim of this Chapter

The main objective of the work in this chapter was to identify neuronal genes whose activity is vital for aphid survival, by assessing the effects of gene knockdown on the reproduction or lifespan of GPAs after feeding on diets containing dsRNA to target NSM genes. Silencing of some of these genes resulted in reduced fecundity or longevity 24 hours feeding (short-term effects) and after transfer to plants (as longer-term effects). Promising target genes were identified for further *in planta* studies.

4.3 Experimental procedures

4.3.1 Cloning of target genes using an RNAi vector

After confirmation the chosen gene targets using Sanger sequencing, target products were ligated into the RNAi vector, pDoubler, using suitable restriction sites (*XhoI/KpnI*) as described in Section 2.3. Competent cells of *E. coli* JM109 were then transformed with the modified RNAi vectors using the heat shock method. Positive transformants were screened by PCR using M13 primers in a 20 µL reaction volume. Bacterial colonies of positive transformants were grown to recover cloned plasmids, and confirmatory restriction digestion was done with *XhoI/KpnI*. GFP cloned into pDoubler was provided by Dr John Fosu-Nyarko (Fosu-Nyarko et al., 2016).

4.3.2 Synthesis of DsRNA for selected genes and for GFP

To prepare 25 dsRNA templates (24 target genes and *gfp* as control), target fragments were digested from pDoubler with *EcoRI* to have T7 promoter regions at both ends. Cleaved T7 templates were then checked by run on the gel electrophoresis to confirm that they were the correct size, and then cleaned and quantified (as described in Section 2.2). Finally, 1-2µg of

the T7 templates were used for *in vitro* transcription using the Hi-Scribe T7 *in vitro* transcription kit (NEB) following the supplied described in Section 2.3.1.

4.3.3 Delivery of dsRNA *via* an artificial diet

In vitro feeding was done using 2µg/µL of dsRNA in a total 40 µL of 30% sucrose plus 0.02% neutral red (NR) dye (Section 2.4). NR dye was not used for all the feeding trials because its use had not been fully optimised when these experiments started (Bilgi et al., 2017). In addition, the NR dye was difficult to see in comparatively advanced nymphal stage like late 3rd or 4th instars. Initially, to reduce workload, feeding assays were done using 5 GPA nymphs (3rd to 4th instar), each representing one replicate. For each treatment, these nymphs were collected freshly from pre-reared GPA colonies on tobacco plants and kept in a 5-ml yellow-capped bottle. The artificial diet supplemented with dsRNA was supplied by a parafilm sandwich (as described in Section 2.3.2). Feeding studies were repeated for six target genes which showed lethal phenotypes after 24 hours of dsRNA feeding or fed on the plants, these were *capa*, *ccap*, *octβ3r*, *irp2/3*, *mAChrM3* and *lk*.

4.3.4 Effects of feeding dsRNA on GPA survival and reproduction

After 24 hours feeding on dsRNA, dsRNA-fed GPAs were collected and carefully examined using a compound microscope. Any behavioural or phenotypical changes (e.g. inactive, moulting defects, paralysis or lethal) were observed and assessed using an Olympus BX-51 microscope (4x magnification). To evaluate the longer-term effects of dsRNA feeding on GPA reproduction and survival, dsRNA-fed GPAs (n=3 to 4 GPAs) were transferred to tobacco plants (*Nicotiana tabacum*) (one GPA per plant) and monitored on 2, 4, 6, 8, 10 and 12 days after feeding. To distinguish the original dsRNA-fed GPA, nymphs were removed every time after counting. GPA fecundity and longevity data were collected on the 12th day after feeding with dsRNA. One or two dsRNA-fed GPAs with obvious silencing effects were stored at -80°C

for gene expression analysis. The sucrose diet samples were also collected and run on 1% agarose gels to determine whether there had been any degradation of the dsRNA.

4.3.5 Migration assay for ds*jhbp*-fed GPAs

A migration assay was established to examine the locomotion behaviour of ds*jhbp*-fed GPAs (n=3), using a 12-channel isoelectric focusing tray (Biorad, Australia). The length and width of the individual lanes were 15 cm and 0.5 cm, respectively (Bilgi et al., 2017). Fifty microliters of 30% sucrose diet was placed at one end, and dsRNA-fed GPAs were placed at the other end of the lane. The tray was covered with stretched parafilm so that the GPAs could not escape. The migration assay was monitored for one hour, after which data was taken on aphid movement.

4.3.6 Semi quantitative PCR

Transcript analysis was performed using semi q-PCR as described in section 2.4.4. Since most of the target genes studied were expressed in the insect nervous system (i.e. GPA head capsule), cDNA derived from single GPA head RNA was initially used for semi-qPCR analyses. However, it was not possible to amplify the target genes as well as *actin* (control) from the single head-derived cDNA. Total RNA was then extracted from single GPAs or two head capsules, and the cDNA was used for semi-qPCR with appropriate controls. Initially, silencing of nine genes that showed distinct abnormalities 24 hours after dsRNA ingestion were selected for gene expression analysis. However, only six genes were amplified from the cDNA and so these were used for semi-qPCR study. PCR conditions were as described in Section 2.1.4.

4.3.7 Data analysis

Statistical analysis was performed using SPSSv24 software (IBM Corporation) for analysis of variance (ANOVA) and calculation of means, standard deviation (SD) and standard error (SE) (Norušis, 1990). Significant differences between treatments were tested at $p<0.05$, and pair-

wise comparisons of means were made using Tukey's test. Bar charts are presented as mean ± SE of mean and were constructed using Microsoft Excel Analysis Tool Pack.

4.4 Results

4.4.1 Phenotypic changes immediately after 24 hours of feeding with dsRNA

The set-up used for feeding aphids with dsRNA *in vitro* is presented in Figure 4.1.

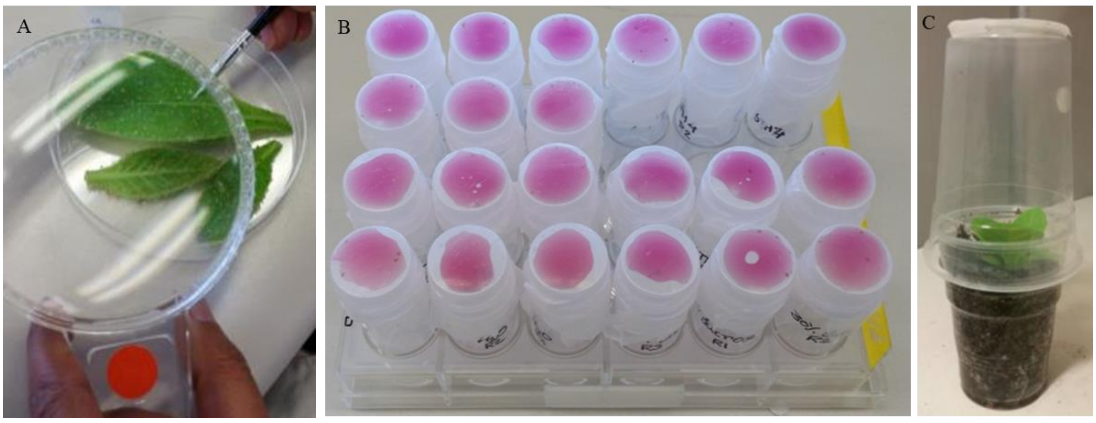


Figure 4.1: Artificial feeding set-up for feeding dsRNA to GPAs *in vitro*. (A) Collecting 3rd – 4th GPA instars (B) 30% sucrose diet with 0.02% NR with or without dsRNA in a parafilm sandwich stretched over the top of tubes, used for *in vitro* feeding (C) dsRNA-fed GPAs on tobacco plants after feeding, with the pot sealed with a plastic cup.

Changes of the behaviour of aphids were studied under a microscope at the end of feeding period. Of the 24 genes tested, GPAs exhibited impaired or slower movement or moulting defects for four genes (*eth, capa, irp2/3* and *irp5*) whereas silencing of four other genes (*ccap, lk, octβ3r* and *mAChrM3*) resulted in lethal effects on fed-GPAs (Table 4.1 and Figure 4.2).

For ds*ccap* treatment, 100% of dsRNA-treated GPAs were paralysed with only very weak movement of their antennae. However, no mortality was recorded after 24 hours feeding. Forty percent mortality was observed for ds*capa*-fed aphids after the treatment. Aphids treated with dsRNA of *lk, octβ3r* and *mAChrM3* were lying on their dorsal side and could not stay upright

even when ‘encouraged’ to do so. GPAs treated with dsRNAs of these three genes were unable to move of normally or were upside down (unlike the control GPAs) when assisted with paintbrush. Also, some lethality was documented for aphids treated with dsRNA of these genes: 40% for ds*octβ3R* and ds*mAChrM3* and 20% for ds*lk*. On the other hand, reduced movement, or sluggish behaviour (with no mortality) was observed for GPAs treated with dsRNA of insulin-related peptide encoding genes (both *irp2/3* and *irp5*). Together with slower movement, 40% of GPAs treated with ds*irp2/3* died. In contrast, no mortality was recorded for the *irp5* gene.

Table 4.1: Phenotypic changes recorded for dsRNA-fed GPAs 24 hours after feeding dsRNA

Gene (s)	**GPA RNAi phenotype observed for different treatments**	**Percentage of GPAs affected**
Ecdysis triggering Hormone (*eth*)	Failed to shed off cuticle and died, the whole body was encapsulated in old skin, body curved	20% died for incomplete moulting
Insulin Related Peptide 5 (*irp5*)	Lethargic with Slower movement	60% less active
Insulin-Related Peptide 2/3 (*irp2/3*)	Lethargic with Slower movement	40% died
Short Neuropeptide (*snpf*)	Slower movement, swollen abdomen	100% less active
Capability (*capa*)	Less active to inactive	40% died
Juvenile hormone binding protein (*jhbp*)	Locomotion was highly affected (uncoordinated movement) but not paralysed	100% affected
Crustacean Cardio Accleratory Peptide (*ccap*)	Entirely paralysed with slow movement of antennae, inactive	100% paralysed

Gene (s)	GPA RNAi phenotype observed for different treatments	Percentage of GPAs affected
Leukokinin (*lk*)	Aphids on dorsal side with restricted movement in one or two limbs, inactive	100% affected, 20% died
Octopamine beta 3R (octβ3r)	Dead aphid turned brown and was on dorsal side; those alive showed extremely paralysed phenotype with rigid limbs	100% affected, 40% dead
Muscarinic acetylcholine receptor M3 like (mAChrM3)	Dead aphid was on dorsal side and alive aphid with very slight local movement in front legs	100% affected with 40% dead
Other 16 genes and control genes: GFP and no dsRNA	Normal movement	Normal phenotype

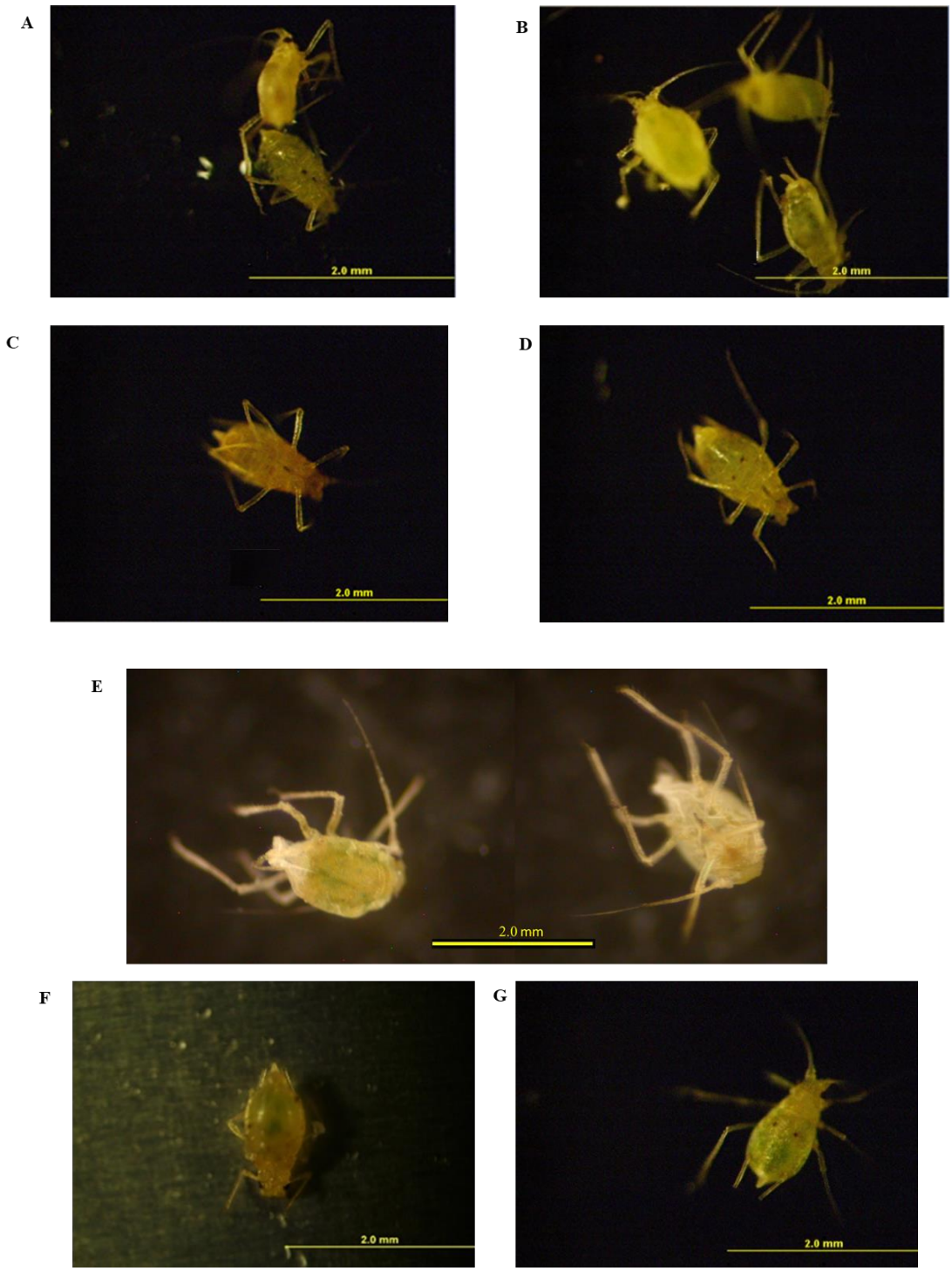


Figure 4.2: Effects of dsRNA treatments on the behaviour and phenotype of the dsRNA-fed GPAs. Paralysed GPAs lying on their dorsal for ds*jhbp* ingestion **(A)**, GPAs with restricted movement and dead GPAs for ds*lk* treatment **(B)**, Lethal phenotype for ds*octβ3r* treatment **(C)**, Lethal phenotype for ds*mAChrM3* treatment **(D)**, Ds*snpf*-fed GPA with swollen abdomen **(E)**,

GPAs failed to moult when *eth* gene was silenced **(F)**, GPA with normal phenotype for dsgfp and no-dsRNA control treatment **(G)**.

Interestingly, a moulting defect was detected in GPAs after feeding with ds*eth* (Ecdysis Triggering Hormone), a gene involved in the insect ecdysis process. For the ds*eth* treatment, single GPA died because of incomplete moulting. The dead GPA was trapped in the old cuticle, and failed to shed its skin. However, since *eth* or expression of other moulting related genes are stage and time specific, the abnormal phenotype was found for only one GPA that probably had undergone ecdysis during the feeding experiment (Figure 4.2E). It seems probable that silencing of the *eth* gene would result in the same phenotype for other aphids in the replicate if they had undergone moulting during the feeding period.

An interesting observation was made for another NP-encoding gene, *snpf* for which all fed GPAs were lethargic rather than showing any obvious detrimental phenotype (Figure 4.2F). The abdomen of ds*snpf*-fed GPAs became large and swollen, and this appeared to make their movements quite slow, although it did not appear to affect their survival but actually increased the fecundity. Aphid mortality was not found for the ds*snpf* treatment. Other than the genes described above, visible changes were not found after treatments with the other 16 target genes studied. All of these genes showed normal phenotypes, as did both ds*gfp*-fed and no dsRNA-fed GPA control treatments. RNAi phenotypes of dsRNA-fed GPA are summarised in Table 4.1 and Figure 4.2.

4.4.2 Assessment of DsRNA integrity in the diet after 24 hours

Most of the dsRNAs were found to be intact in the 30% sucrose diet after the feeding period. Of the collected diets, 12 µL of sucrose diet containing dsRNA was assessed on 1% agarose gels (Figure 4.3). In the artificial diets examined in this way, partial degradation of dsRNA was detected for one gene, namely *npy2r***,** (Figure 4.3B lane 11).

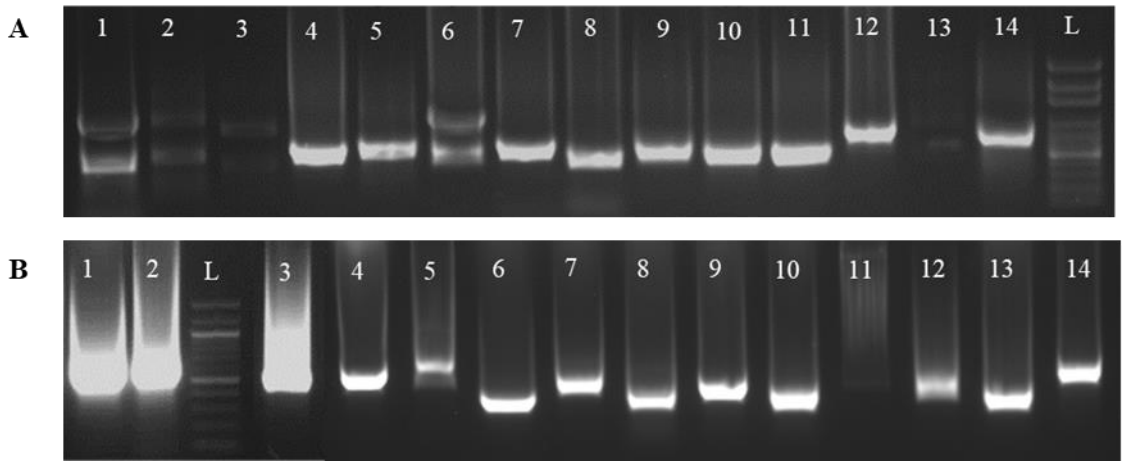


Figure 4.3: 1% Agarose gel showing dsRNA in the 30% sucrose diet before **(A)** and after 24 hours of aphid feeding **(B)**. Lanes: 1: ETH, *2*: Lk, 3: AstCC, 4: Capa, 5: nAChrβ1, 6: CCAP, 7: sNPF, 8: IRP2/3, 9: IRP5, 10: EH2/3, 11: NpY2R, 12: GRH, 13: CapaR1 and 14: GFP and L:100 bp ladder.

4.4.3 Migration assay for GPAs fed on ds*jhbp*

An interesting observation was made for one treatment, after ingestion of dsRNA of *jhbp*, in which all tested GPAs showed impaired and uncoordinated movement. Despite this phenotype, treated aphids did not become paralysed or die. Based on this observation, a migration assay was set up with treated GPAs, for one hour using an isoelectric tray which limited the movement of aphids to individual grooves. The mean distance along which sucrose-fed control aphids moved was taken as 100%. Control treated aphids (no dsRNA and ds*gfp*) were highly active and moved towards the feed at other opposite end of the tray from their initial position although they were statistically significant. In contrast, ds*jhbp*-fed GPAs hardly moved during the assay period. Treatment with ds*gfp* reduced the movement by about 25%, but compared to the no-dsRNA control, movement of aphids treated with ds*jhbp* was reduced 12 fold. (Figure 4.4).

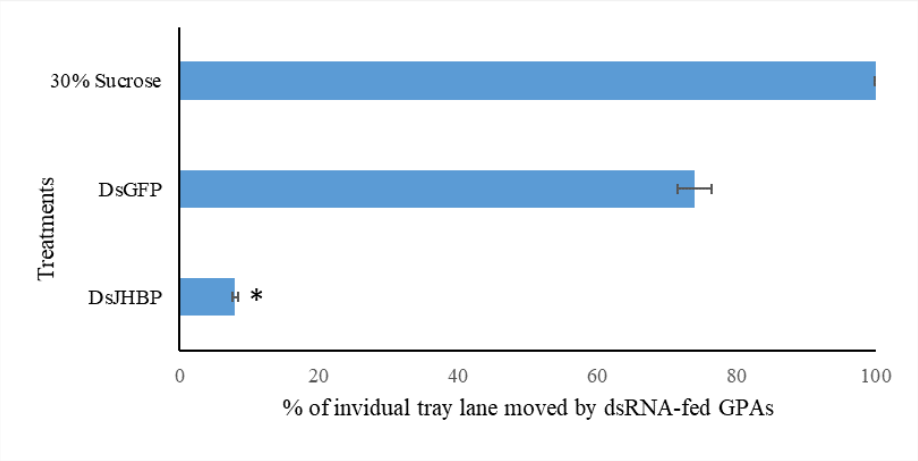


Figure 4.4: Movement of control and ds*jhbp* -fed GPAs over 60 minutes (n=3) to 30% sucrose feed. The significance is represented by * at p<0.05compared to no dsRNA control. Bars represent mean ± SE of the mean.

4.4.4 Effects of gene silencing on GPA reproduction after 24 hours feeding on dsRNA

GPAs which were still alive 24 hours after dsRNA treatment (n=3-4; based on availability) were transferred to individual tobacco plants to record their fecundity data for 12 days. The results obtained could be grouped into three broad classes, these were (i) no fecundity recorded, (ii) 50% or less fecundity comparing to controls and (iii) a similar or higher fecundity than controls (Figure 4.5). (Additional information is provided in the Appendix as Figures S1 and S2 and Tables S1 and S2 which show details of GPA reproduction at four different time points). Of the 24 genes studied, GPAs treated with dsRNA of *ccap, capa, irp2/3 and octβ3r* belonged to the first group. DsRNA-fed GPAs for all these genes showed abnormal phenotypes after dsRNA ingestion and consequently died on plants. As a result, no data on their reproduction was recorded.

A significantly lower reproduction (p<0.05) was also found after feeding two receptor genes and two neuropeptide genes, compared to the no-dsRNA control (30% sucrose diet), The

fecundity was reduced to one-third to a quarter for aphids provided with dsRNAs of *capar1*, *mAChrM3*, *nplp1* and *lk*: specifically, to 33.4%, 26%, 23% and 11.7% of the control treatments, respectively. For the treatment ds*opsin*, the fecundity of GPAs was recorded by 53.5% and 58.6% to no-dsRNA treatment and ds*gfp* treatment, respectively. GPA treated with ds*irp5* produced about 30% fewer progeny than the controls after 12 days on the plants. However, the reproduction of ds*opsin* (p=0.29) and ds*irp5* (p=0.51) treated GPAs was not statistically different from control treatments.

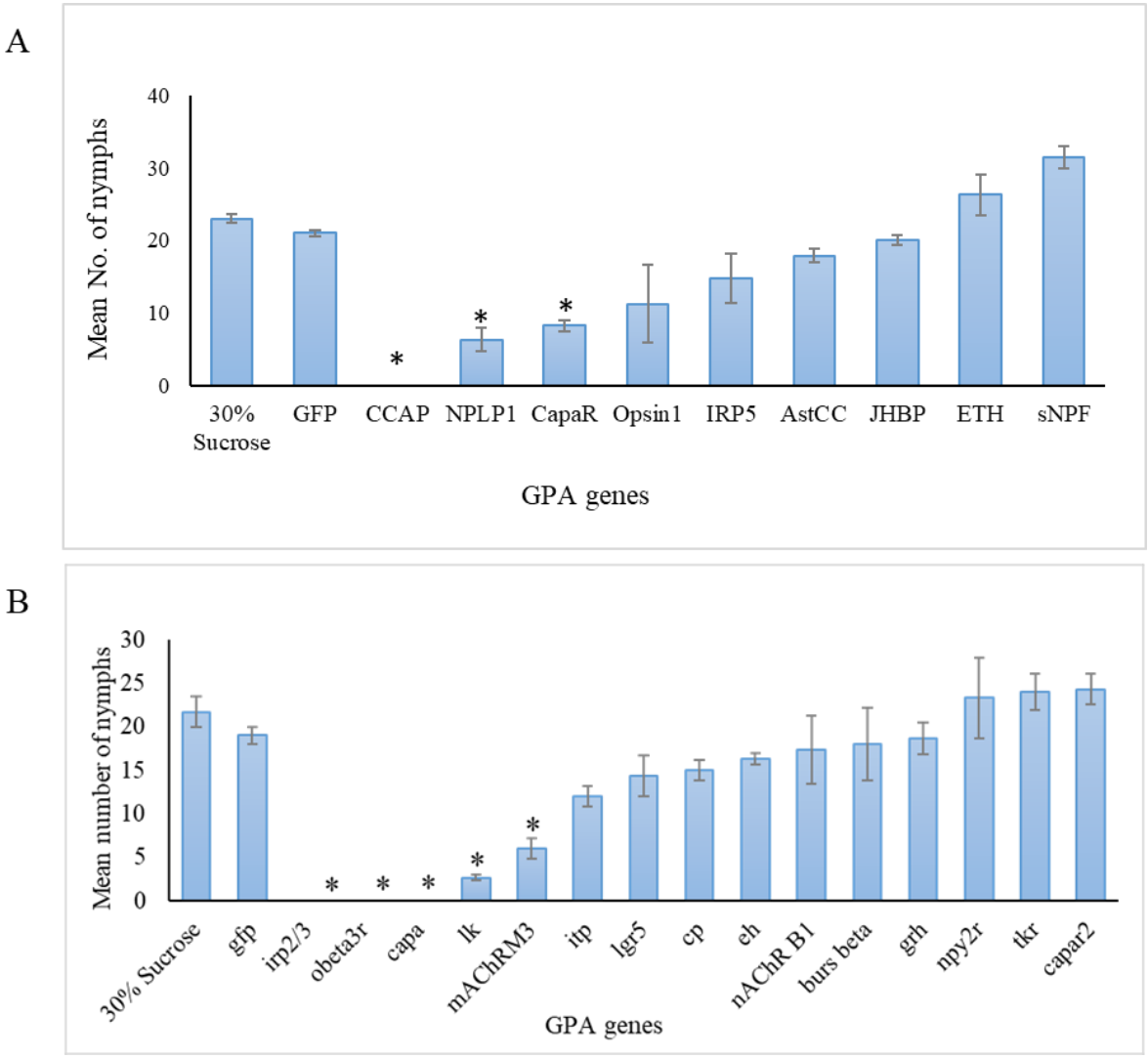


Figure 4.5 (A, B): Average number of aphid nymphs produced by dsRNA-fed GPAs 12 days after transfer to plants. The significance is represented by * at p<0.05 compared to no dsRNA control. Bars represented mean (n=3-4) ± SE of mean.

In contrast, silencing of some genes showed similar or even higher nymph production than that of controls. For instance, silencing of moulting genes (*eth, eh*) or juvenile hormone inhibiting gene (*ast-cc*) did not affect GPA reproduction and a similar fecundity to control treatments was observed on plants after 12 days. The highest reproduction was recorded for ds*snpf*-fed GPAs, which produced 50% more nymphs than the no-dsRNA control. It is interesting to note that although aphids had impaired movement (ds*jhbp*) or less active (ds*snpf*) phenotypes after ingestion of dsRNA, these effects did not impact aphid fecundity after transfer to tobacco plants.

4.4.5 Effects of gene silencing on GPA survival after 24 hours feeding on dsRNA

To assess longer-term effects of dsRNA feeding, the longevity of treated GPAs was recorded until the control (no-dsRNA fed GPA) died after transfer to plants (up to 21 days). Mean GPA longevity is presented in Figure 4.6 (A and B), while the survival trend is presented only for those genes for which silencing of target genes resulted in a significantly shorter lifespan of ten days or less ($p<0.05$) in Figure 4.6 (C).

Aphids that showed abnormal phenotypes after dsRNA ingestion of *ccap*, *octβ3r* and *irp2/3* were not found on plants when checked for several days. Therefore, it was assumed that knockdown of these genes was lethal for GPAs. Silencing of these genes caused 100% mortality of aphids within two days after transfer to the plants. The ds*ccap* and ds*octβ3r*-fed GPAs (except for ds *irp2/3*) were extremely paralysed after 24 hours feeding and died after transfer to the tobacco plants. In contrast, all replicates (n=3) of ds*irp2/3*-fed GPAs died within 48 hours on the plants having shown 40% mortality after dsRNA feeding.

All replicates of ds*capa* and ds*lk*-fed GPAs died within four days of transfer to tobacco plants (Figure 4.6, B); these were either inactive or showed lethal phenotypes after dsRNA ingestion.

More than 50% of ds*lk*-fed GPAs died within two days on the plants whereas, for ds*capa* treatment, none of the fed GPAs survived beyond five days, the average longevity being three

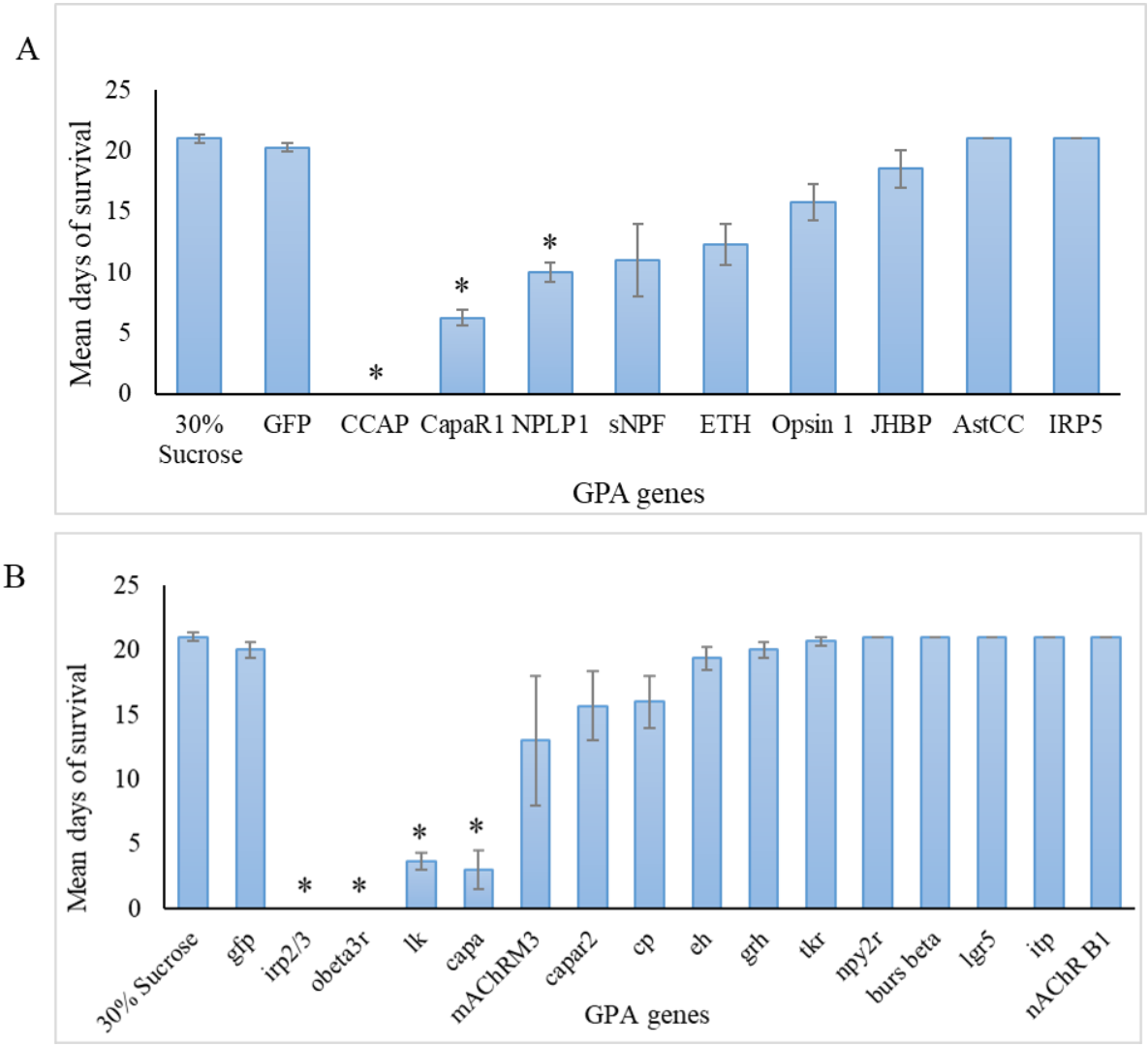


Figure 4.6 (A, B): Average days of survival of dsRNA-fed GPAs after transfer to tobacco plants. The significance is represented by * at $p<0.05$compared to no dsRNA control. Bars represent mean (n=3 to 4) ± SE of the mean. **(C)** showed the survival trend of dsRNA fed-GPAs for six target genes, showing a range of survival times (<10 days).

days. Another striking observation was noted for one BAR encoding gene, *mAChrM3*. After feeding with dsRNA, all treated nymphs showed obvious RNAi phenotypes (lethal and abnormal phenotypes) (Table 4.1) and later significantly low reproduction. However, their longer-term survival was not affected significantly (p=0.41). Although 40% of the fed GPAs died during the 24 hours period after dsRNA feeding, no further mortality was recorded over 21 days. Silencing of the *eth* gene, for which only 20% GPA mortality was recorded after 24 hours, did not affect longevity statistically, and no further mortality was observed.

Other than the genes described above, silencing of *nplp1* or *capar1* also reduced aphid longevity, although no phenotypic changes were seen for ds*nplp1* and ds*capar1* treated GPAs. Compared to the no dsRNA control, the mean survival duration was about half and one-third for ds*capar1*- and ds*nplp1*-fed GPAs, respectively. However, one *capar1* treated GPA survived for 12 days while the average GPA longevity was six days. In this case the aphid may have taken less dsRNA. The opposite scenario was documented for silencing of ds ds*jhbp* where dsRNA-fed GPAs showed uncoordinated movement after feeding treatment, but the reproduction and survival was not affected on plants.

4.4.6 Analysis of target gene expression

To assess whether expression of target genes was reduced after dsRNA feeding, total RNA was extracted from one or two sucrose-fed (as no dsRNA control), *gfp*-fed and dsRNA-fed GPAs. In total, six genes were analysed, of which five genes (*lgr5, irp5, jhbp, octβ3r* and *capar1*) were amplified from total RNA at 36 and 38 amplification cycles (Figure 4.7). Another gene, *ccap*, was amplified only from head-derived cDNA using the same PCR conditions (Figure

4.7). Two other genes, *mAChrM3* and *snpf*, were also initially selected for expression analysis, but could only be amplified until 41 cycles from control cDNA, and so were excluded from further analysis. Another gene, *lgr5* was included as control for which no distinct silencing effect was observed after the short-term and long-term duration.

Actin was used as an internal expression control: its expression was essentially the same in aphids after treatment with no-dsRNA and *gfp* controls. No detectable band for *ccap* from dsRNA fed cDNAs confirmed its successful knockdown and subsequent effects on GPA physiology. Faint bands from respected cDNA indicated the lower abundance of target mRNAs for *capar2, irp5* and *jhbp* where *capar1*-fed GPAs which correlate with longer-term impacts on the aphids. In contrast, *the lgr5* gene was up-regulated in ds*lgr5*-fed aphids. Depending on the band intensity, transcripts for *octβ3r* seemed to be unchanged, although RNAi phenotypes were recorded after dsRNA feeding (Figure 4.7).

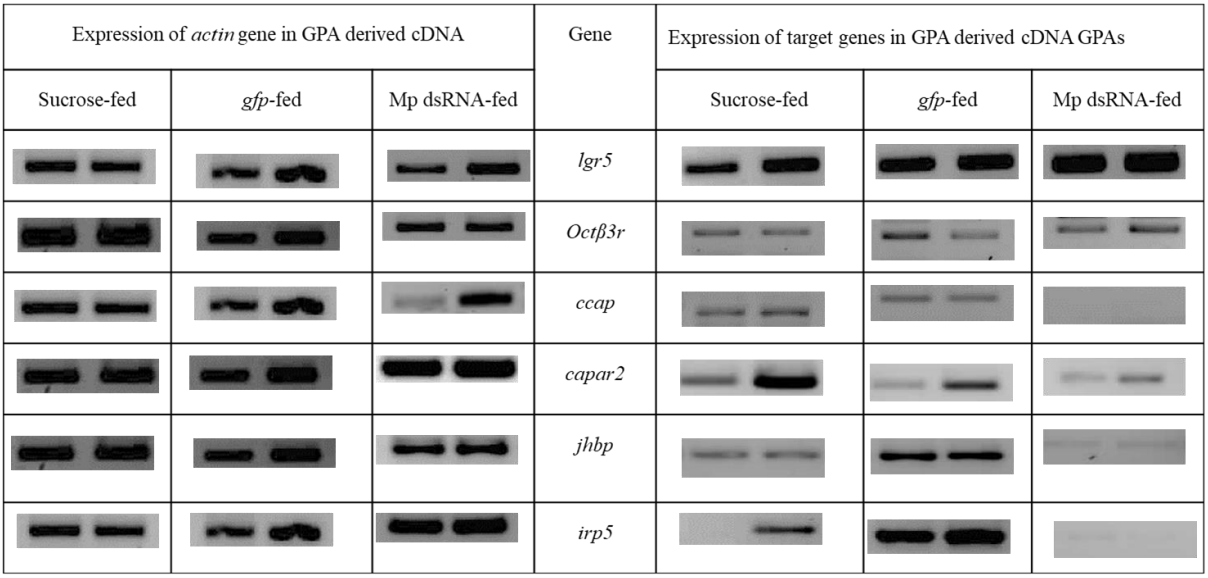


Figure 4.7: Semi-quantitative PCR for control and target GPA gene expression, using GSPs from control and dsRNA fed GPAs. In each pair of bands, the left band is from 36 cycles and the right band is from 38 cycles.

4.5 Discussion

This study was undertaken to identify neuronal genes of GPA for which silencing through artificial feeding with dsRNA affected behaviour, survival and reproduction. Silencing/down-regulation of nine of the 24 genes significantly affected GPA behaviour within 24 hours of ingestion of cognate dsRNA. Fecundity and survival was also affected for most of these aphids. Abnormal phenotypes of dsRNA-fed GPAs indicated successful knockdown of the target genes, and this was confirmed later by semi qPCR analysis. Similar RNAi phenotypes have also been reported for a few NSM-encoding genes in other insects. For example, knockdown of *ccap* and its receptors in *T. castaneum* and *R. prolixus* also resulted in higher mortality resulting from an interruption of the function of insect cardiac muscle contractions during ecdysis (Arakane et al., 2008; Lee et al., 2013). *Ccap* is reported to be critical for ecdysis in both holo- and hemi-metabolous insects: the gene regulates heartbeat rate and has a role in muscle contraction (Arakane et al., 2008; Dulcis et al., 2005; Estévez-Lao et al., 2013; Lee et al., 2013). Similarly, interrupting the *lk* gene function *via* delivery of kinin related peptides (LK) resulted in higher mortality in *A. pisum* (> 90%) (Smagghe et al., 2010) and a comparable effect was also found for ds*lk*-fed GPAs in this study. Since insects kinin have pleiotropic roles (e.g. gut motility or diuresis or release of digestive enzyme), LK or related NPs could be considered as promising targets for pest management (Nachman et al., 2009). In addition, silencing of JHBP gene *via* artificial feeding also caused higher mortality (up to 63%) in *A. gossypii* (Rebijith et al., 2009).

A considerable variation in reproduction was observed 12 days after dsRNA-treated aphids were transferred to tobacco plants. Reduced fecundity was recorded for aphids treated with dsRNAs of six genes: *ccap, capa, mAChrM3, oβ3r, lk* and *irp2/3*. For these aphids, dsRNA-fed GPAs also showed phenotypic changes 24 hours of treatments. It is possible that the

reduction in GPA reproduction after dsRNA ingestion results from direct or indirect disruption of functions of different muscles, including the heart and gut muscles. Contractions of such muscles are important for survival, feeding, locomotion and reproduction in many insects (Da Silva et al., 2011; Down et al., 2011; Estévez-Lao et al., 2013; Lahr et al., 2012; Lee et al., 2012; Lee et al., 2013; Ons et al., 2016; Wasielewski and Skonieczna, 2008), and this could also be the case for GPA.

The sNPF peptides are key regulators of insect feeding behaviour (Van Wielendaele et al., 2013). In *Schistocerca gregaria*, RNAi of the *snpf* receptor was also reported to stimulate food intake, while inhibitory feeding behaviour was observed following the overexpression of sNPF peptides (Dillen et al., 2013). In this study, ds*snpf*-fed GPAs had a swollen abdomen, indicating that their feeding behaviour may have been change, resulted in higher reproduction. Inhibitory feeding behaviour for RNAi of *snpf* or its receptors has also been studied in other insects, including *D. melanogaster*, *A. mellifera* and *P. americana* (Lee, et al., 2004; Mikani et al., 2012; Nagata et al., 2011). These results and others indicated that the gene silencing effect might be species-specific, and the same gene could have slightly different roles in different insect physiology systems, and so their silencing may not have the same consequences. It has also been suggested that insect nutritional status plays a vital role for some stages of reproduction like release of gametes and other metabolic products during copulation (Van Wielendaele, Badisco, et al., 2013) that might explain the increased fecundity recorded in this experiment.

After silencing different target genes, the longevity of DsRNA-fed GPAs varied. Nymphs treated with dsRNA of *irp2/3, capa, ccap, octβ3R* genes only survived for a short time after treatment (≤ five days), and they did not reproduce after transfer to tobacco plants. In contrast to ds*irp2/3*-fed GPAs, silencing of the *irp*5 gene did not alter GPA longevity, and this might

reflect a possible functional compensation by other insulin genes. For example, in *D. melanogaster*, lowering the transcripts of *dilp2* alone, or even deletion of *dilp1-dilp5* together, did not affect the lifespan or fecundity, and silencing of single *ilp* gene resulted in over-expression other insulin-related genes of that family (Broughton et al., 2008; Zhang et al., 2009). Therefore, the roles of *irp2/3* and *irp*5 in GPAs need to be studied to understand the variable effects of silencing this target gene in GPAs.

Silencing of genes that hinder insect metamorphosis directly, such as *octβ3R* or *eth* (Arakane et al., 2008; Ohhara et al., 2015) or indirectly such as *capa*, *ccap, lk* (Gäde and Hoffmann, 2005; Park et al., 2003) might lead to death of insects. This is because any deficiency during the ecdysis process may reduce ecdysone production, eventually causing insect mortality (Arakane et al., 2008). However, the underlying mechanisms of how the downregulation of *nplp1* and *mAChrM3* significantly reduced GPA fecundity and longevity are not clear from the available information and need further study. Limited studies have been done on the function of *mAChrM3,* and the roles of *nplp1* have not yet been characterised in any insect (Collin et al., 2013; Verleyen et al., 2009). In *D. melanogaster* overexpression of *nplp1* during ecdysis may offer an expected role in insect growth (Park et al., 2004).

Transient knockdown of gene expression has been found in such studies on other eukaryotes including nematodes and insects (Kimber et al., 2007; Luo et al., 2013; Turner et al., 2006). The observed transient RNAi in GPAs such as with ds*jhbp* treatment might be because of in adequate dsRNA uptake, which can be a limitation of oral delivery (Down et al., 2011; Li, et al., 2013; Matthews et al., 2008). It would be interesting to repeat the feeding experiment for all genes with more replications to provide more authentic data. However, to repeat a feeding experiment, at least 160 µg of target dsRNA was needed each time that was time consuming and expensive. Therefore, Because of time constraints and limited resources, artificial feeding

experiment was not repeated only for six genes showing lethal phenotypes, e.g *capa*, *ccap*, *octβ3r*, *irp2/3*, *mAChrM3* and *lk*. No substantial changes were recorded after experiments to silence the other 16 target genes, which were involved in insect water homeostasis (*itp*), growth and development (e.g. *astcc*, *bursβ, eh, cp, tkr*), reproduction (*grh*), or GPCRs (e.g. *capar2*, *lgr5*).

There are several possible reasons dsDNA treatment of the latter genes did not affect behaviour of GPA nymphs. First, GPAs may not have taken up enough dsRNA to disturb gene function. This is a limitation of feeding bioassays (Surakasi et al., 2011), although the use of the marker dye to identify those aphids that had fed on the artificial diet should exclude those aphids which had not fed. Second, ingested dsRNA might be degraded quickly in the insect gut of hemolymph (Christiaens et al., 2014) or even in the diet during feeding (*npy2r* in the current study), leading to no silencing effects. However, degradation of ingested dsRNA in the aphid hemolymph is probably not the reason, because robust RNAi phenotypes were obtained for some of the dsRNA treatments. Third, RNAi of neuronal genes has been found to be refractory (that is, access of the dsRNA to neuronal tissues may be limited) in some organisms, such as *C. elegans* and *Locusta migratoria* (Kamath et al., 2003; Kamath et al., 2000; Luo et al., 2013). Additionally, there may be a compensatory feedback mechanism, similar to that of the miRNA pathway, which result in increased expression of the target mRNA after initial down-regulation (Bakhetia et al., 2005). This was observed for the ds*lgr5* treatment (Figure 4.7). Fourth, silencing the expression of one gene might be compensated increased expression of other members of a multigene family, as observed for the insulin gene family (Broughton et al., 2008). Finally, it is may be the case that, to interrupt essential physiological processes such as moulting or metamorphosis, silencing of more than one target gene (for instance, *eth* and *eh* together) instead of a single gene be needed to achieve a stronger RNAi response.

4.6 Conclusions

This study has demonstrated that RNAi can be used to target GPA NSM-encoding genes through oral delivery of dsRNA. Since there are no RNAi studies available for any Hemipteran species targeting most of the genes studied here, this work provides new information that RNAi targeting NSM genes in aphids can be achieved. It adds valuable additional evidence on the function and importance of these genes for control of GPAs, and possibly other Hemipterans. Since high mortality and reduced fecundity of GPAs was found after silencing some of the target genes, this indicates their potential value as targets for GPA control using the HIGS approach. Experiments using this approach are described in the next Chapter.

Chapter 5

The efficacy of host-mediated RNAi of nine neuronal genes of GPA

5.1 Introduction

Host Induced Gene Silencing (HIGS) offers a more natural means of delivering dsRNAs and siRNAs to sucking pests than does *in vitro* feeding. It provides a continuous supply of dsRNA/siRNAs, rather than the one-off treatment of feeding aphids dsRNA in an artificial diet. Although genetically modified plants expressing *Bt* toxin have been deployed successfully to control chewing insect pests, unfortunately, at present, sap-sucking insects such as whiteflies or aphids are not amenable to the *Bt* control strategy (Chougule and Bonning, 2012). In addition, populations of sucking pests like aphids have develop resistance to many current insecticides. There is therefore a real need to identify more suitable strategies for the control of sucking pests. HIGS is an alternative, genetic strategy which may confer host resistance against sap-sucking pests. Evidence to date shows that HIGS has the potential to provide a new strategy to confer plant resistance for crop pests in various insects, including Lepidoptera, Coleoptera and Hemiptera (Baum et al., 2007; Mao et al., 2007; Pitino et al., 2011; Xu et al., 2014).

Host-mediated RNAi for insect control was first reported in 2007 for *Diabrotica virgifera* in transgenic corn roots, and against *Helicoverpa armigera* using transgenic *Arabidopsis* and tobacco plants (Baum et al., 2007; Mao et al., 2007). These studies demonstrated proof-of-concept for successful delivery of gene-specific dsRNA and/or siRNA to insect pests. Since then, successful *in planta* RNAi studies have been reported for a few Hemipteran insects - *N. lugens*, *B. tabaci*, *S. avenae* and the GPA (Bhatia et al., 2012; Coleman et al., 2014; Guo et al., 2014; Mao and Zeng, 2014; Pitino et al., 2011; Thakur et al., 2014; Xu et al., 2014; Zha et al., 2011). For aphid species, particularly the GPA, several genes have been investigated through HIGS, but only one targeting a neuronal gene, *acetylcholinesterase 2* (*MpAChE2*) (Guo et al., 2014). Transgenic tobacco plants expressing *MpAChE2* hpRNA and artificial microRNA

(amiRNA) conferred aphid resistance. This work contributed to the decision to explore the potential of silencing other neuronal genes using HIGS for aphid crop protection.

5.2 Aim of this Chapter

The objective of this chapter was to investigate the effect of silencing neuronal genes of GPA using HIGS. In the previous Chapter a series of target genes were tested using oral delivery of dsRNA to each. Nine of these showed promises for aphid control, with detrimental effects on GPA fecundity and survival. These gene targets were selected for further investigation using HIGS. Hairpin constructs for nine gene fragments (*capar1, ccap, irp5, jhbp, nplp1, opsin, snpf, lk* and *oβ3R*) were used to generate transgenic tobacco plants, and three of the genes (*ccap, jhbp* and *nplp1*) were also used to generate transgenic *Arabidopsis* plants.

5.3 Experimental procedures

5.3.1 Development and screening of transgenic tobacco (*Nicotiana tabacum* cv. Wisconsin) plants using the 'leaf disc' method

Parent transgenic tobacco plants (T_0) were developed following the leaf disc method developed by Dr John Fosu-Nyarko (Nemgenix Pty Ltd.). A detailed description of the methods is provided in Section 2.6. In this project, seeds harvested from T_0 plants are referred to as T_1 seeds that produced T_1 plants. Seeds from T_1 plants are described as T_2 seeds and those T_2 seeds produce T_2 transgenic plants; T_3 seeds were harvested from T_2 plants. Seeds from different generations were carefully sterilised with sodium hypochlorite (NaOCl) following the modified protocol provided (Section 2.6). Transgenic seeds were selected with 150 mg/mL kanamycin on MS medium. Positive transgenic plantlets were carefully transferred to pasteurised soil for root establishment. Transferred plants were covered with plastic bags for four days to ensure adequate humidity and to facilitate root establishment. Plants were grown to flowering and

bagged to prevent pollen transfer. Well-dried seeds of T_1 and T_2 generation tobacco plants were collected.

T_1 transgenic plants (growing from the harvested seeds from T_0 plants) expressing the hp constructs for ten genes, including nine for neuronal and the *gfp* hp constructs were used for *in planta* RNAi study. For an individual hp construct, four events each with 10 replications were challenged with single GPA nymphs for 12 days. For T_2 plants (growing from the harvested seeds from T_1 plants) six GPA hp constructs were studied: *capar1, opsin, irp5, ccap, jhbp* and *nplp1*. Because of time constraints, T_2 transgenic plants expressing hp constructs of three target genes (*lk, octβ3r and snpf*) were not included in the bioassay. As controls, 15 replications of *gfp*-transgenic and wild type tobacco plants were challenged in all the bioassays.

5.3.2 Development of transgenic *Arabidopsis* plants

Arabidopsis plants were transformed with the hp constructs of *ccap, jhbp* and *nplp1* using a modified floral dip method (Bent, 2006). The detailed protocol is described in Section 2.7. To collect T_2 seeds, T_1 plants were kept in the PC2 glasshouse and enclosed with the plastic sheets and 'Aracones'. T_2 seeds were sterilised with the same procedure used for tobacco seeds and were screened with 50 mg/mL kanamycin. Selected T_2 plants were used for GPA bioassasy in the PC2 glasshouse. As for tobacco experiments, 15 replications of *gfp*-transgenic and wild type *Arabidopsis* plants (1 GPA/plant) were used in aphid challenges.

5.3.3 GPA challenges of RNAi transgenic tobacco and *Arabidopsis* plants

Once growth of T_1 and T_2 tobacco and T_2 *Arabidopsis* plants were established in soil, a single GPA nymph (3^{rd} to 4^{th} instar) was carefully transferred with a fine paint brush (size 02) onto individual plants. The plants with nymphs were then each covered with a plastic cup to prevent escape of nymphs (Figure 5.1 and 5.2). GPA nymphs on each plant were checked for the following two days, and a new nymph was placed on the plant if the original was not found. In

the case of T_1 tobacco plants, aphid fecundity data were recorded on the 12th day while for the T_2 generation for both tobacco and *Arabidopsis* plants, fecundity data were collected on the 4th, 8th and the 12th days after transfer. After recording the data, the test plants were sprayed with insecticide to kill the aphids, and the plants were grown to collect the next generation seeds. All the analyses undertaken for this chapter were done in the same manner as in Section 4.3.7.

5.3.4 Confirmation of T-DNA insertion in transgenic plants

Genomic DNA (gDNA) extractions were done using a quick DNA extraction protocol (Sika et al., 2015). PCRs were performed using 100-200 ng of the genomic DNA extracted from the different generations of transgenic tobacco and *Arabidopsis* plants to confirm that there was integration of T-DNA. About 100-200 ng of the genomic DNA was used with two separate sets of primers for the *nptII* gene. These primers were *nptII* F: 5′-AATATCACGGGTAGCCAACG-3′/ *nptII* R: 5′-AGCACGTACTCGGATGGAAG-3′ and *snptII* F: 5′-TGCTCCTGCCGAGAAAGTAT-3′/ *snptII* R: 5′-AATATCACGGGTAGCCAACG-3′ which produced *nptII* fragments of 256 bp and 364 bp, respectively. The PCR cycling profile was as follows: initial denaturation for three minutes at 95°C, followed by 25 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds and a final incubation step at 72°C for five minutes. Amplicons were later checked on 1% agarose gel. The expression of transgenes was confirmed using cDNA derived from randomly selected T1 tobacco plants. RNA extraction and cDNA synthesis procedures were followed as in Section 2.2.1.

5.4 Results

5.4.1 Analysis of transgenic tobacco and *Arabidopsis* plants by antibiotic selection and T-DNA integration

For tobacco, a high transformation efficiency (up to 92%) was obtained from leaf explants generating T_0 shoots for all RNAi constructs following antibiotic selection (Table 5.1). Plantlets excised from the tobacco leaf discs developed well-established, healthy root systems on MS-kanamycin medium, and these were selected as T_0 plants. Seeds from individual T_0 plants (referred as T_1 seeds) were collected for each of the ten dsRNA hairpin (hp) constructs including *gfp* (hp*gfp*). Non-transformed plants died within 10 days on kanamycin selection medium while transgenic plants developed roots with healthy green leaves after 3-4 weeks on the selection medium. These selected T_1 plants were challenged with GPA nymphs. From individual events expressing the hp constructs, the T_1 tobacco plant with the lowest aphid reproduction (out of 10 replicates) were selected for challenging in T_2 generation. Kanamycin resistant T_2 tobacco plants were transferred to the PC2 glasshouse for aphid challenges. The stages of production and *in planta* experiments, from screening and selection of transgenic seeds to aphid bioassays are illustrated in Figures 5.1 and 5.2.

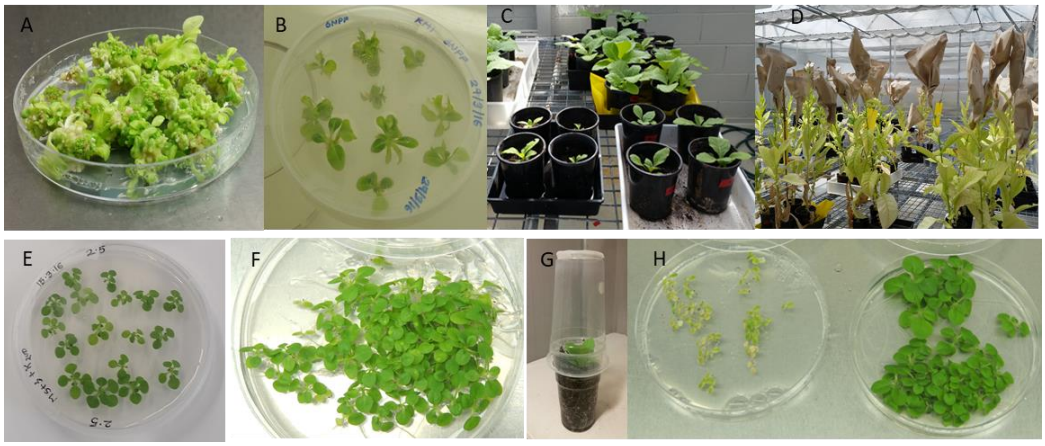


Figure 5.1: Selection of transgenic tobacco plants used for GPA bioassay. **(A)** T_0 callus on callus induction medium; **(B)** T_0 tobacco events on rooting medium; **(C-D)** T_0 screened transgenic plants in PC2 glasshouse for T_1 seed collection; **(E, F)** T_1 and T_2 transgenic tobacco events on kanamycin medium after 3-4 weeks, respectively; **(G)** *In planta* bioassay of T_2 transgenic plants with GPA; **(H)** Wild tobacco started to die on kanamycin medium, but survived on sucrose plates after 2 weeks.

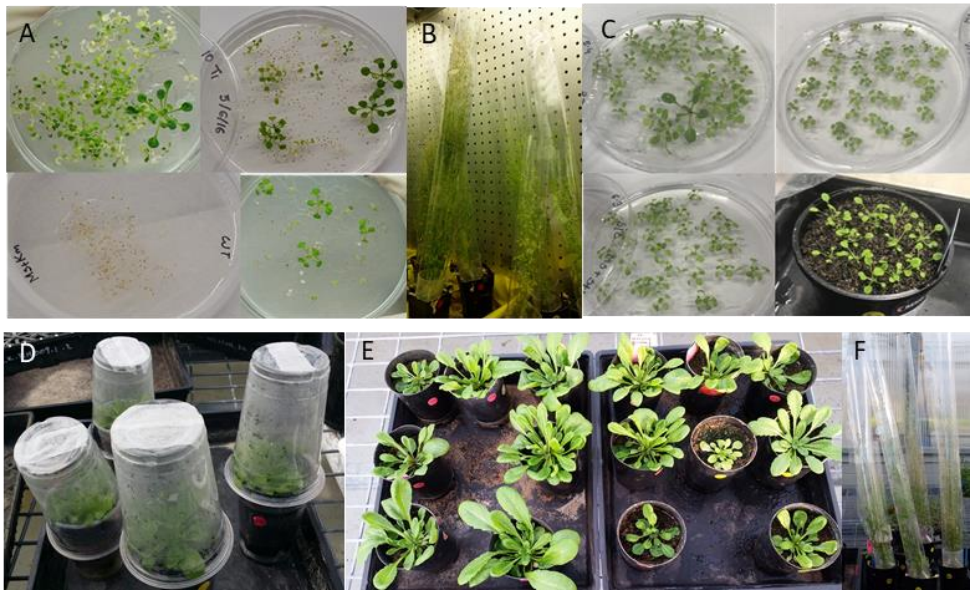


Figure 5.2: Analysis of transgenic *Arabidopsis* plants and GPA bioassay. **(A)** T_1 transgenic events on kanamycin medium for more than 3 weeks; **(B)** T_1 *Arabidopsis* seeds collection using 'Aracones'; **(C)** Kanamycin screening of T_2 transgenic events **(D)** *In planta* bioassay with T_2 plants; **(E)** Transgenic plants sprayed with insecticide to kill aphids at the end of experiment for collecting next generation seeds; **(F)** T_2 *Arabidopsis* seed collection.

Table 5.1: Number of T_0 tobacco events obtained expressing 10 different hairpin constructs of target genes

Hairpin constructs	No. of explants (leaf discs)	No. of explants resistant to kanamycin	No. of T_0 transgenic plantlets in rooting medium with kanamycin	No. of T_0 events well-established in soil
CapaR1	25	23	20	16
Irp5	25	22	18	15
Opsin	25	21	18	15
Ccap	20	14	10	07
Jhbp	20	15	11	08
Nplp1	20	15	12	08
sNPF	20	18	17	16
Lk	20	16	13	10
Octβ3r	20	15	11	07
GFP	25	24	22	20

Unlike tobacco, the transformation efficiency of *Arabidopsis* was not as high and varied for different constructs. Five events were obtained for the hp*ccap* construct, while three and two events were obtained for *jhbp* (hp*jhbp*) and *nplp1* (hp*nplp1*), respectively. T_2 seeds of *gfp*-transformed *Arabidopsis* were used as a control: these had been developed previously in the lab (Herath, 2016; Iqbal, 2015).

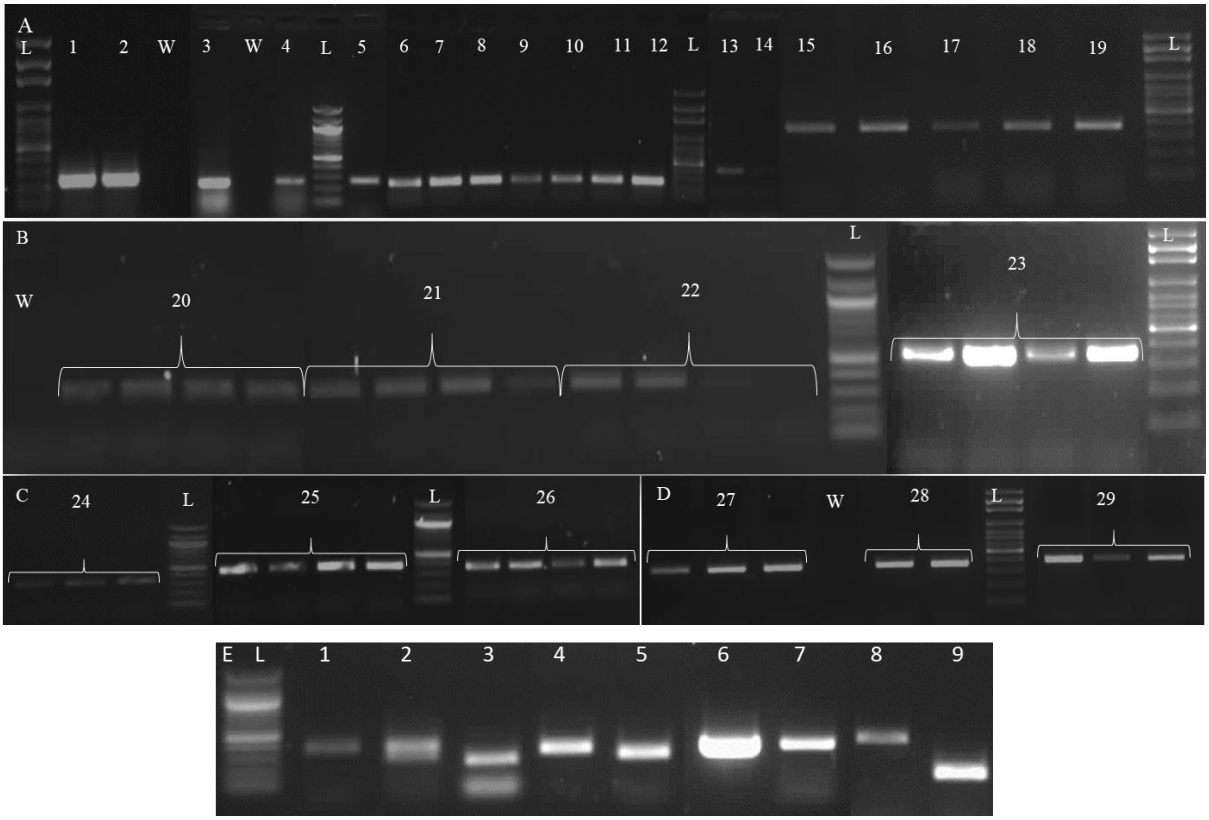


Figure 5.3: PCR analysis of *nptII* gene fragment in different generations of transgenic tobacco and *Arabidopsis* plants confirming the successful transfer of target T-DNA **(A-D)**. **(A)** T_0 tobacco transgenic events; **(B-C)** T_1 transgenic tobacco events; **(D)** T_2 *Arabidopsis* transgenic events. In the figure, L: 100 bp ladder; **1-2, 20**: hp*opsin* events; **3, 4, 21**: hp*irp5* events; **5, 6, 22**: hp*capaR1* events; **7-8**, **23, 27**: hp*ccap* events; **9-10, 24, 29**: hp*jhbp* events; **11-12, 25, 28**: hp*nplp1* events; **13-14, 26**: hp*gfp* events; **15-16**: *lk*; **17-18**: hp*octβ3r* events; **19**: hp*snpf* events and **w**: wild type tobacco or wild type *Arabidopsis*. **(E)** Amplification of target gene fragments from cDNA of T_1 transgenic tobacco plants for individual hairpin construct. In the figure, L: 100 bp ladder; **1-**: hp*irp5* event; **2**: hp*ccap* event; **3**: hp*jhbp* events; **4**: hp*snpf* event; **5**: hp*opsin* event; **6**: hp*capar1* event; **7**: hp*lk* event; **8**: hp*nplp1* event; **9**: hp*octβ3r* event.

For further confirmation of T-DNA integration, *nptII* fragments from the extracted gDNA of tobacco and *Arabidopsis* transgenic plants were amplified by PCR. For tobacco, selected T_0 plants (parent transgenic plants developed from leaf discs) were checked for the integration of T-DNA (Figure 5.3 A). Only T_1 tobacco plants amplifying *nptII* fragments were used for GPA bioassays (Figure 5.3B and 5.3C), and advanced to the next generation for collecting T_2 seeds.

Amplifications of *nptII* fragments from gDNA of different transgenic lines confirmed the transgenic nature of screened lines and successful transfer of target T-DNA. No *nptII* amplicon was found for wild type plants. For *Arabidopsis*, T_2 events showing good resistance to GPA infestation were confirmed by molecular analysis using pooled gDNA extracted from randomly selected T_2 *Arabidopsis* leaves (Figure 5.3D). Amplification of target genes from plant cDNA also confirmed the presence of target aphid genes in T_1 transgenic tobacco plants (Figure 5.3E).

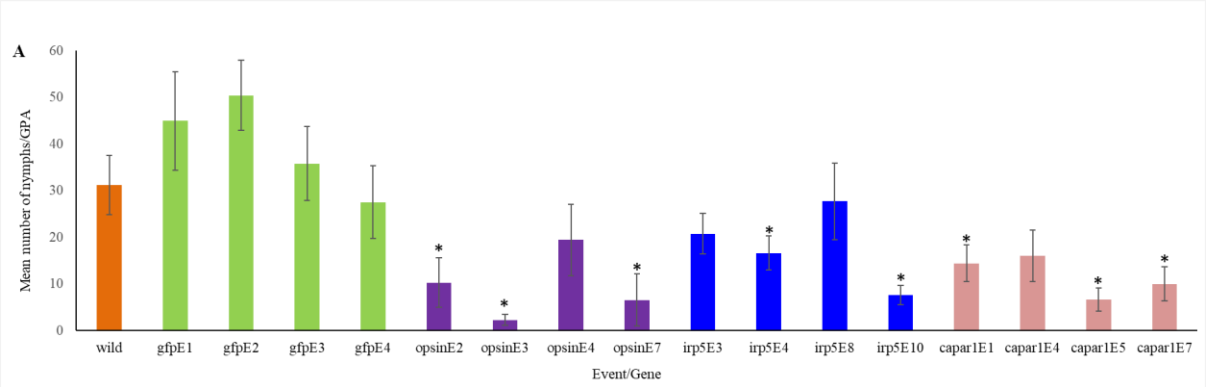
5.4.2 Evaluation of T_1 transgenic tobacco plants for their effects on GPA fecundity after feeding for 12 days

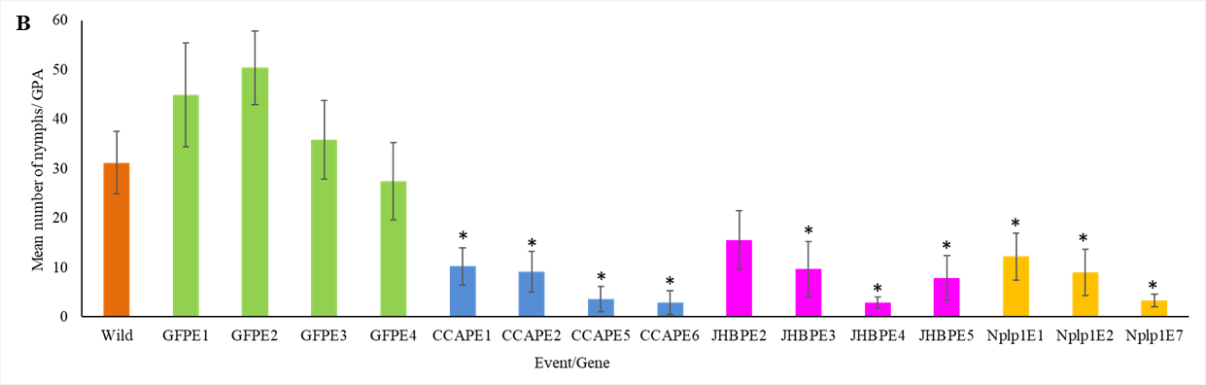
T_1 tobacco plants for nine hp constructs and hp*gfp* were challenged with a single 3rd to 4th instar GPA nymph, and the number of nymphs produced by the original GPA was counted at the end of the assay on the 12th day after transfer. For the T_1 tobacco plants, four events with 10 replications of each hp construct (altogether, 380 T_1 transgenic tobacco for nine hp constructs and 15 wild type plants) were used in these aphid bioassays. GPA fecundity was reduced significantly (p<0.05) for most of the events of the T_1 generation tobacco plants, and different events of each construct showed different levels of resistance to GPA reproduction (Figure 5.4).

The fecundity of GPAs on the events of hp*gfp* was not statistically significant compared with that of the wild-type tobacco. It is also important to note that the variation in controls for T_1 tobacco (wild type and hp*gfp* events) was due to the time when the experiments were conducted. Of the nine target genes, *in planta* study for six genes was done in April' 2016, while challenges for the other three genes (*lk*, *octβ3r* and *snpf*) was done in December' 2016.

The mean reproduction of GPAs on transgenic lines expressing hps of GPA genes ranged from 3% to 89% of controls, with the lowest fecundity for two hp*octβ3r* events (event 2 and event 5) (Figure 5.4C). A reduction of 97% was recorded for the two hp*octβ3r* events in the T_1

generation. Nearly, 80% or more reduction in nymph reproduction was found for all the hp*lk* events, with the maximum decrease of 95% for the event 4. Interestingly, all the lines expressing hp*ccap,* hp*nplp1* and hp*lk* had significantly lower GPA populations (p<0.05), with the maximum 39% for the hp*nplp1* event 1. For all hp*ccap* events, mean numbers of nymphs ranged between 9% to 32%, and for hp*nplp1*-expressing lines, it was from 10% to 39%.





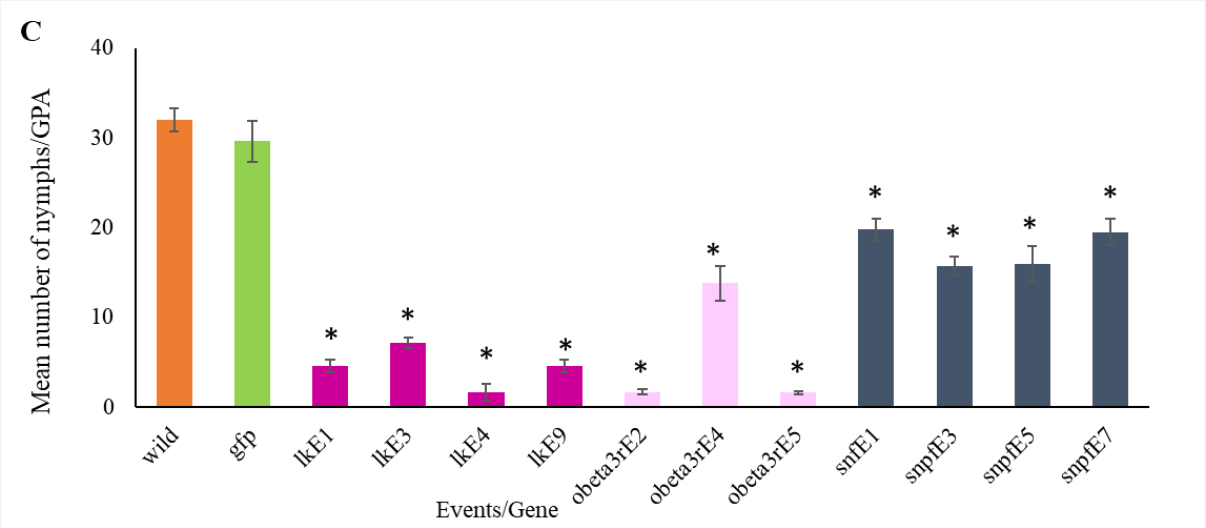


Figure 5.4 (A-C): GPA reproduction on T_1 transgenic tobacco plants for nine constructs of GPA target genes compared to controls after 12 days. The average number of GPA nymphs of target hp plants can be compared to those on hp*gfp* and control non-transgenic plants (n = 15). An asterisk (*) indicates the significant differences in transgenic lines and controls (p<0.05). Reproduction data are presented as mean ± SE of the mean. Here, data for **A** and **B** were collected in April'2016 while **C** were collected in December'2016.

A noticeable dissimilarity in aphid resistance was observed among different events of the same hp constructs of hp*opsin*, hp*jhbp* and hp*oβ3R*, although most of those events had few aphid nymphs (Figure 5.4A and 5.4B). For instance, 7% to 62% reproduction was recorded for all four transgenic events expressing hp*opsin*. Hp*irp5*-expressing transgenic lines also allowed higher GPA reproduction, and for two of these events (event 3 and event 8) there was similar reproduction to that of wild type control, which was statistically non-significant (p≥0.05). Similarly, all the hp*snpf* events showed comparatively lower resistance to GPA reproduction, and a more nymphs were recorded over the period of 12 days. Although these hp*snpf* events were statistically significant (p<0.05) compared to wild-type control, three of the four events had about half as many nymphs as did on wild-type plants.

5.4.3 Challenge of T_2 tobacco plants to GPAs fecundity after feeding for 12 days

T_2 transgenic tobacco plants expressing hp constructs correspondent to six GPA genes were screened, grown and challenged with GPAs. Fecundity data were taken at different time points: the 4th, 8th and the 12th day after one nymph was placed on each test plant. In the T_2 generation GPA challenges, four events of six hp constructs each with 10 replications (i.e. 230 T_2 lines), and 15 replications of two controls (hp*gfp*-expressing lines and wild-type tobacco plants) were used. As for GPA challenges on T_1 plants, there was significantly lower GPA reproduction after 12 days ($p<0.05$) on most of the T_2 transgenic events (Figure 5.5). In most cases, a significant reduction in aphid fecundity was recorded from day 8, while for four different events, hp*opsin* event 3 and event 4, hp*irp5* event 8 and hp*capar1* event 5 showed a reduction in nymphs only after 12 days.

There was a significant reduction in GPA fecundity on most of the T_2 events over the period of the experiment ranging from 3% to 69%. The lowest fecundity for any of the events was recorded for the hp*irp5* event 4 (1.00±1.60) and hp*opsin* event 7 (1.30±0.76). All the T_2 events of hp*ccap*, hp*jhbp* and hp*nplp*1 showed significantly better resistance with limited numbers of aphid progeny as found in the T_1 generation challenges, except for hp*jhbp*. Amongst these three genes, GPAs feeding on hp*ccap* lines produced fewest nymphs, followed by hp*nplp1*- and hp*jhbp* -expressing lines. Compared to wild type controls, the average number of aphid nymphs were reduced by 90% or more for hp*ccap* events and hp*nplp1* events, while for the hp*jhbp* events there was 80% less reproduction after 12 days. Higher GPA mortality was also observed among the replications of hp*ccap*-expressing events: event 1 and event 5.

In total, RNAi transgenic lines for six hp constructs exhibited significantly lower GPA reproduction in both the T_1 and T_2 generations compared to the respective controls (Figure 5.4

and 5.5). Interestingly, 14 transgenic tobacco lines expressing different hp constructs showed consistently better resistance to GPA over the two generations and supported lower GPA populations. These included hp*ccap* events: event 1, event 2, event 5 and event 6; hp*nplp1* events: event 1, event 2 and event 7; hp*opsin* event 3 and event 7, hp*irp5* event 4, hp*capar1* event 1 and event 7, three hp*jhbp* events: event 3, event 4 and event 5. In contrast, higher fecundity was also recorded on three transgenic lines over the two transgenic generations studied, namely hp*opsin* event 4, hp*irp5* event 8 and hp*capar1* event 4. These consistent results for transgenic tobacco lines emphasise the successful delivery of target dsRNA and/or siRNA to GPA during feeding from transgenic host plants.

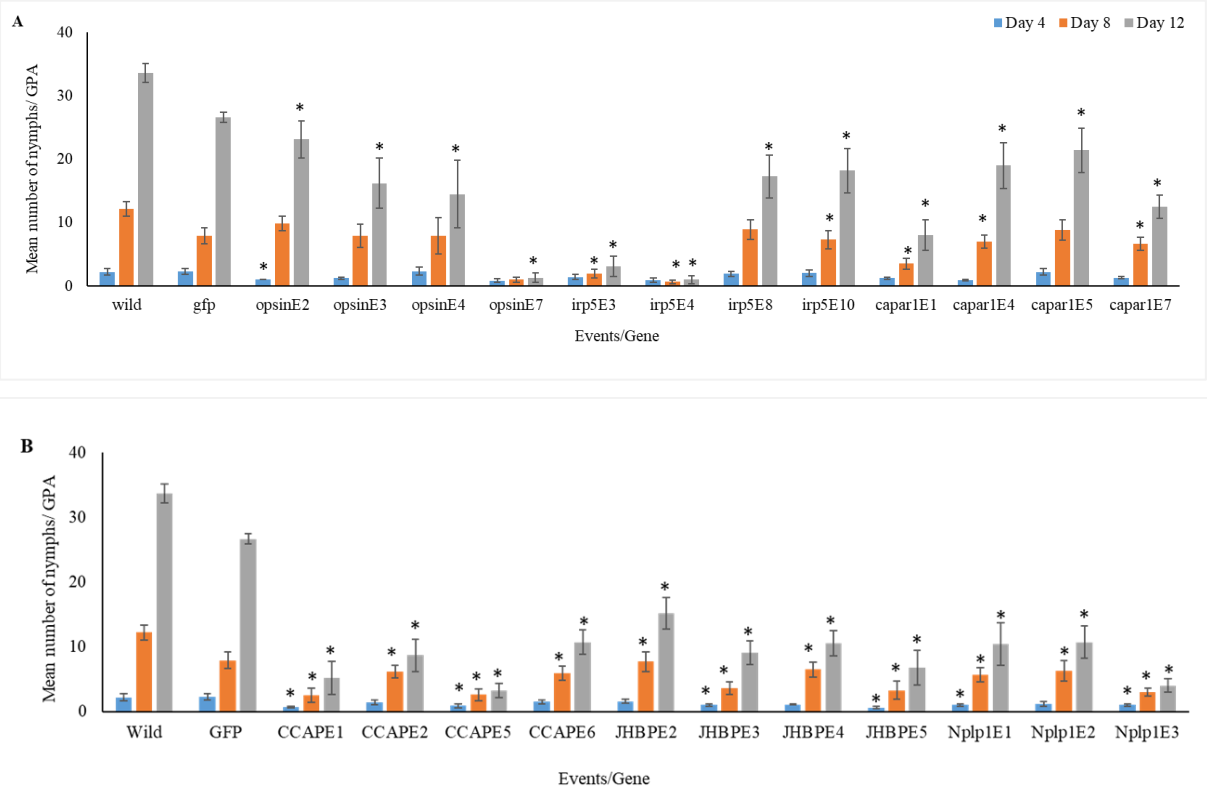


Figure 5.5: GPA mean reproduction on T_2 tobacco plants for the six GPA hp constructs of target genes compared to controls after 12 days expressing (A) hp*opsin* events, hp *irp5* events, hp *capar1* events; and (B) hp*ccap* events, hp*jhbp* events and hp*nplp1* events. The average number of nymphs on experimental treatments can be compared with numbers on wild type

tobacco (n = 15). An asterisk (*) indicates significant differences between transgenic lines and controls (p<0.05). Numbers of nymphs are presented as mean ± SE of the mean.

5.4.4 GPA fecundity on T_2 transgenic *Arabidopsis* plants

As for tobacco, transgenic *Arabidopsis* plants expressing three hp constructs (hp*ccap*, hp*jhbp* and hp*nplp1*) also showed better resistance when challenged with a single GPA nymph. For five hp*ccap*-expressing events, five to 13 replications were used for the study, while for two other hp constructs, hp*jhbp* and hp*nplp1*, 10 to 17 replications were challenged with GPAs. Wild type *Arabidopsis* and hp*gfp*-expressing *Arabidopsis* lines were used as controls (n = 15 replications). Although nymph production on hp*gfp*-expressing lines was significantly reduced after eight days compared to wild plants (p=0.0004), it became non-significant (p=0.1) at the end of the experimental period.

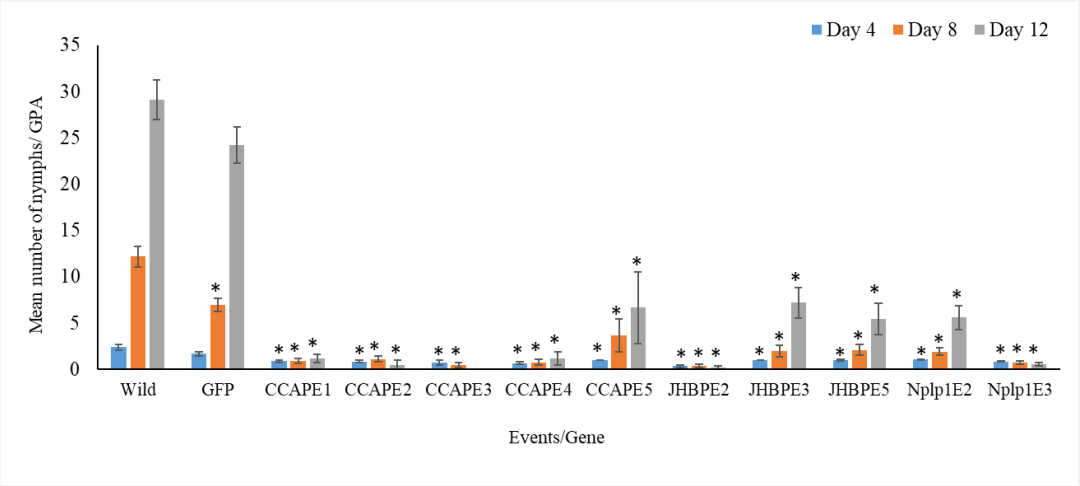


Figure 5.6: Average GPA reproduction on T_2 *Arabidopsis* plants for hp*ccap* events, hp*jhbp* events and hp*nplp1* events after12 days. The average number of GPA nymphs present can be compared with wild *Arabidopsis* plants (n=15). An asterisk (*) indicates significant differences between transgenic lines and controls (p<0.05). Numbers of nymphs are presented as mean ± SE of the mean.

A highly significant reduction in aphid fecundity, ranging from 80% to 100% of the wild type controls, was found for T_2 transgenic *Arabidopsis* plants during the period of aphid bioassay

(Figure 5.6). Lethality was observed for one hp*ccap* expressing event 3, where all aphids died within twelve days. The lowest reproduction was for the hp*jhbp* event 2 (only 1%) followed by hp*ccap* event 2 (2%). In contrast, the highest reproduction (25%) was recorded on hp*jhbp* - expressing lines for event 3, followed by 19% on hp*jhbp* event 5 and hp*nplp1* event 2. These results clearly suggest that delivery of selected target hp constructs *via* transgenic *Arabidopsis* plants (i.e. HIGS) can significantly reduce GPA fecundity and survival.

5.5 Discussion

In this chapter, HIGS of nine GPA ESTs putatively encoding NSMs were evaluated *via* plant-mediated RNAi study in two model plants, tobacco and *Arabidopsis*. These plants were selected because they are good hosts of GPA and transgenic materials from those plants can be developed reasonably quickly (Pitino et al., 2011). These plants were modified to express hpRNAs to silence the expression of target GPA genes after aphid feeding. In general, a significant reduction in GPA fecundity was observed during *in planta* assessment after the silencing of the GPA genes studied. Silencing of three genes namely, *ccap, jhbp* and *nplp1* caused a significant reduction in GPA reproduction in tobacco (T_1 and T_2 generations) as well as in *Arabidopsis* (T_2 generation). Additionally, silencing of *lk* also caused a severe reduction in GPA fecundity in T_1 tobacco plants. However, three of the GPA genes (*lk*, *snpf* and *oβ3R*) were studied only in T_1 tobacco plants, due to time constraints.

There was a range of reduction in fecundity of GPAs, which depended on which of the nine neuronal genes was targeted for silencing by HIGS. The results from transgenic plants essentially supported and mirrored results from *in vitro* feeding reported in Chapter 4. As is often the case, the impact of silencing target genes on GPA reproduction varied both with the target gene, and for different events of the same transgene. Similar results for host-mediated RNAi have been reported not only for insects, but for other species such as plant parasitic

nematodes (De Souza Júnior et al., 2013; Sindhu et al., 2008). Factors which might contribute to the observed variation include different sites of integration of target T-DNA in different transgenic events. The sequences flanking the hp insert can influence the level of expression of a transgene. After plant transformation, one or more copies of the transgene can be integrated into the plant genome in different events (Tinland, 1996). Although *Agrobacterium*-mediated transformation mostly delivers single copy insertions, multiple copies (two, three, four, or more copies) may be present. For example, in transgenic barley plants at least 10 copies of a transgene were inserted (Gelvin, 2003; Tingay et al., 1997). Analysis of the copy number of target inserts would confirm the number of integrated transgenes in different events. The variation in PCR band intensities for the *nptII* gene for different events may reflect differences in copy number. Other factors which affect the efficacy of HIGS include the length of dsRNA used, expression of sufficient dsRNA processing to siRNA and the availability of dsRNA and siRNA at feeding sites. The actual sequence of the target gene chosen can also affect efficiency of target gene knockdown.

Although the minimum length of dsRNA for greatest silencing efficiency has not yet been studied extensively for aphid species, it is evident from different reports that aphids can take up longer dsRNA (> 500bp) *via* oral feeding (Mao and Zeng, 2012). This is in agreement with the results for GPA in the *in vitro* feeding assay (Chapter 4), where silencing effects were evident after ingestion of 530 bp long ds*nplp1*. The presence of long dsRNAs and processed siRNAs in the plant phloem sap has been confirmed in transgenic tobacco and *Arabidopsis* plants generated using the constitutive promoter (CaMV35S) to deliver the expression of hp cassettes (Bhatia et al., 2012; Thakur et al., 2014; Zha et al., 2011). It therefore can be assumed that the availability of silencing molecules at GPA feeding sites was not an issue in this study.

Another key factor affecting the RNAi response is the amount of dsRNA or siRNA ingested by the target organisms. For host-delivered RNAi, there is no assurance of sufficient uptake of dsRNA/siRNA to produce a strong RNAi response, and so the observed variation in GPA fecundity and mortality with different events for the same target gene could be related to the amount of dsRNA and siRNA ingested by individual aphids amongst the replicates (n =10). Christiaens and Smagghe (2014) suggested that sap-sucking insects mainly ingest siRNA (processed by the plant's RNAi machinery) rather than long dsRNA from transgenic plants, while insect RNAi machinery 'prefers' to process longer dsRNAs. As a result, partial knockdown effects may result, reducing the effect on insect physiology. A similar observation was also made in this project - for instance, *in vitro* feeding of ds*ccap*, ds*lk* or ds*octβ3r* caused up to 40% GPA mortality within 24 hours and 100% within first six days on plants for the artificial dsRNA treatments. In contrast, for dsRNA ingestion *via in vitro* feeding, GPA mortality was lower for the same target gene in the HIGS study. The difference in mortality between *in vitro* and *in planta* studies reflect the different forms and amounts of dsRNA ingested by the GPAs.

A similar inconsistency was reported for the closely related aphid species, *A. pisum* for the silencing of a salivary gland gene, C002 depending whether dsRNA was delivered by injection of dsRNA or the HIGS approach (Mutti et al., 2006; Pitino et al., 2011). *A. pisum* survival was reduced by silencing of *C002* if siRNA was injected into aphids, but not when delivered from plants stably or transiently expressing hp*C002*. Unlike survival, aphid fecundity was decreased in both studies for *A. pisum*. In agreement with those results, reduced GPA fecundity was also found here for *in vitro* as well as *in planta* RNAi studies. In contrast, using triple dicer-mutant *Arabidopsis* plants expressing longer dsCYP6AE14, strong RNAi effects were obtained for *H. Armigera* due to high level and intact expression of long target dsRNAs (Mao et al., 2007).

The availability of longer dsRNA in transgenic lines has only been studied in transgenic rice challenged with *N. lugens* (Zha et al., 2011). However, consistent aphid resistance was displayed by two plant species for two generations, which reinforces the efficacy of HIGS approach targeting aphid NSM-encoding genes for controlling sap-sucking insects.

5.6 Conclusions

This study shows the enormous potential of using transgenic plants to control GPA infestation by silencing expression of vital neuronal genes, and that preliminary screening to identify the best target genes can be achieved by starting with *in vitro* studies. The information provided here could well be applied to commercial crops, for example to develop GPA-resistant transgenic canola or other crop species.

Chapter 6

The effects of different length sequences of *jhbp* dsRNA provided to GPAs by *in vitro* feeding

6.1 Introduction

As discussed in Chapter 5, although transgenic plants engineered to express target dsRNA (*in planta* RNAi, HIGS) offer potential for controlling crop pests by silencing vital genes, there are a number of factors that need to be investigated. One such factor that needs further study is how the length of the dsRNA used for silencing affects the application of gene silencing. This is of interest in developing 'non-GM' to pest control, for example by spray or 'ectopic' delivery of dsRNAs to plants, using dsRNAs as agrochemicals. For this type of approach, it is also necessary to understand whether short dsRNAs could be as effective as longer dsRNAs, because to develop cost-effective RNAi pesticides it would be cheaper to synthesise them in bulk for spray-delivery strategies of pest control. In other words, can shorter dsRNAs deliver the same level of target gene silencing as longer dsRNAs?

For insects, spraying dsRNA of target gene sequences has been effective for *A. aegypti,* and spraying of ds*AaeIAP1* (an inhibitor of the apoptosis protein-one gene), successfully caused mortality of female *A. aegypti* (Pridgeon et al., 2008). A similar effect was also found for *O. furnacalis* by topical application of dsRNA of two larval stage specific genes, *Ds10* and *Ds28* (Wang et al., 2011). For practical field application, the agricultural company Monsanto has been working to develop dsRNA spray-delivered insecticides (*via* a subsidiary, 'BioDirect').

However, there is evidence that the length of dsRNA is also a crucial parameter to achieve better RNAi responses in some organisms (Baum et al., 2007; Huvenne and Smagghe, 2010; Whyard et al., 2009). Regarding the prefered length of dsRNA length, several experiments show that size selection mechanisms exists at the level of cellular uptake, and that relatively shorter lengths of dsRNA or siRNA are not as effective in gene silencing in insects (Bolognesi et al., 2012; Li et al., 2015; Miller et al., 2012; Miyata et al., 2014).

More work has been undertaken on this subject for *C. elegans*, for example the length dependence of RNAi efficiency for this organism is that shorter dsRNA is less effective - dsRNA of *unc-22* resulted in reduced RNAi phenotypes in F1 progeny compared to longer dsRNA (Parrish et al., 2000). The length of dsRNA used was between 26 bp to 81 bp, and no 'twitching' phenotype was observed for the 26 bp dsRNA treatment but it was for the 81 bp dsRNA treatment. In insects, the size-activity relationship for effective RNAi has been investigated in the Coleopterans: *T. castaneum* and *D. virgifera* (Bolognesi et al., 2012; Miller et al., 2012). Visible silencing effects and better knockdown of the target gene was obtained for longer dsRNA treatments but not for the shorter dsRNAs. However, at present there appears to be no available information on the effects of length of dsRNAs related to their effectiveness in RNAi responses for Hemipteran insects.

6.2 Aim of this Chapter

The aim of work in this chapter was to evaluate the effects of feeding different lengths of dsRNA of the *jhbp* gene on subsequent GPA phenotype, reproduction, survival, and movement.

6.3 Experimental procedure

6.3.1 Primer design, target gene amplification

jhbp gene was chosen as the target for this study because *in vitro* silencing caused uncoordinated movement of treated GPAs and host-induced silencing also resulted in a reduction of fecundity of the aphids. Primers were designed to amplify five different lengths of the GPA EST ES224026 having a conserved domain, jhbp (pfam 06585) (Table 6.1). The expected amplicons were designated as *jhbp* (284 bp), $jhbp_1$ (70 bp), $jhbp_2$ (86 bp), $jhbp_3$ (158 bp) and $jhbp_4$ (166 bp) - the dsRNA generated are respectively referred to as *dsjhbp*, $dsjhbp_1$,

dsjhbp$_2$, *dsjhbp*$_3$ and *dsjhbp*$_4$. Amplification of the fragments from cDNA of a single GPA is described in Section 4.3.6.

Table 6.1: Primers used to amplify fragments of different lengths based on the GPA EST encoding the JHBP protein

Amplicon	Primer pairs for PCR of fragments (5´ to 3´)	Primer position in GPA EST, ES224026	Amplicon size (bp)
jhbp$_1$	F: CTGTGCAGCTCGAAGAAATTG	75-145	70
	R: ACGATAACTCTTCCACGC		
jhbp$_2$	F: GATTTTAGTTACTTACTG	273-359	86
	R: GTTGTCATAAGGCCAAAT		
jhbp$_3$	F: CTGTGCAGCTCGAAGAAATTG	201-359	158
	R: GTAAAAGCTGAAGCAACA		
jhbp$_4$	F: GTGAAACCACTTATAAAC	75-241	166
	R: GTTGTCATAAGGCCAAAT		
Jhbp	F: CTGTGCAGCTCGAAGAAATTG	75-359	284
	R: GTTGTCATAAGGCCAAAT		

6.3.2 DsRNA synthesis, *in vitro* feeding and assessment of silencing effects on GPAs

Synthesis of the five dsRNAs for the jhbp gene and *dsgfp* were undertaken as described in Section 2.4. In addition, siRNAs produced from the long dsRNA *dsjhbp*, using ShortCut RNase III (NEB), which dices long dsRNA into a heterologous mixture of 18-25 bp siRNAs, was used in one experiment. The siRNAs were generated following the manufacturer's protocol. Briefly, 10 µg of dsRNA of *dsjhbp* was digested with 5U of ShortCut RNAseIII in a 100 µL reaction at 37 °C for 30 minutes. The reaction was stopped by adding 10 µL of 50 mM of EDTA and the small RNAs were precipitated with 3M sodium acetate as described in Section 2.1.1. Purified siRNAs were resuspended in 30 µL of nuclease-free water. The integrity of the generated siRNAs was not assessed by the gel electrophoresis.

Delivery of dsRNAs to aphids was done as described in Section 2.4. For each of the five long dsRNAs and siRNAs treatments, as well as the *dsgfp* and no-dsRNA controls, 10 GPA nymphs of 3rd to 4th instar were used as replicates. Phenotypes of dsRNA-treated GPA nymphs and control treatments were examined under the microscope 24 hours after treatment. The effects

of silencing treatments on aphid locomotion was assessed using isoelectric focussing trays (described in Section 4.3.5). Silencing effects on aphid fecundity was also assessed after transfer aphids to tobacco plants. The number of offspring of treated aphids was recorded over a period of 12 days, with data taken on the 4^{th}, 8^{th} and the 12^{th} days.

6.3.3 Semi quantitative PCR and data analysis

Transcript abundance of the target genes in dsRNA-fed GPAs was analysed by semi-qPCR as described in Section 4.3.6. Total RNA from a single dsRNA-fed GPA was used for cDNA synthesis, and one microliter of cDNA was used as template for gene expression analysis. *Actin* was used as internal reference for the semi-qPCRs. Differences of means were calculated using student *t*-test as described in Section 4.3.7. An online RNA webserver tool was used for predicting the secondary structures of different dsRNA sequences, GC content as well as minimum free energy (MFE) (Hofacker, 2003). The R statistics, employing the Pearson correlation test, was used to study the relationship between dsRNA lengths and thermodynamics properties of the dsRNAs and silencing effects of the dsRNAs on GPAs (Core, 2016).

6.4 Results

6.4.1 Phenotypic changes of GPAs 24 hours after ingesting dsRNA

Abnormal phenotypes and mortality of dsRNA-treated aphids were recorded 24 hours after exposure to dsRNA. Complete or partial paralysis was evident for aphids treated with the longest of the dsRNAs ds*jhbp* (284 bp) and ds*jhbp* $_2$ (86 bp); for both of these treatments, the behaviour of all the aphids was affected and included uncoordinated movement (Figure 6.1).

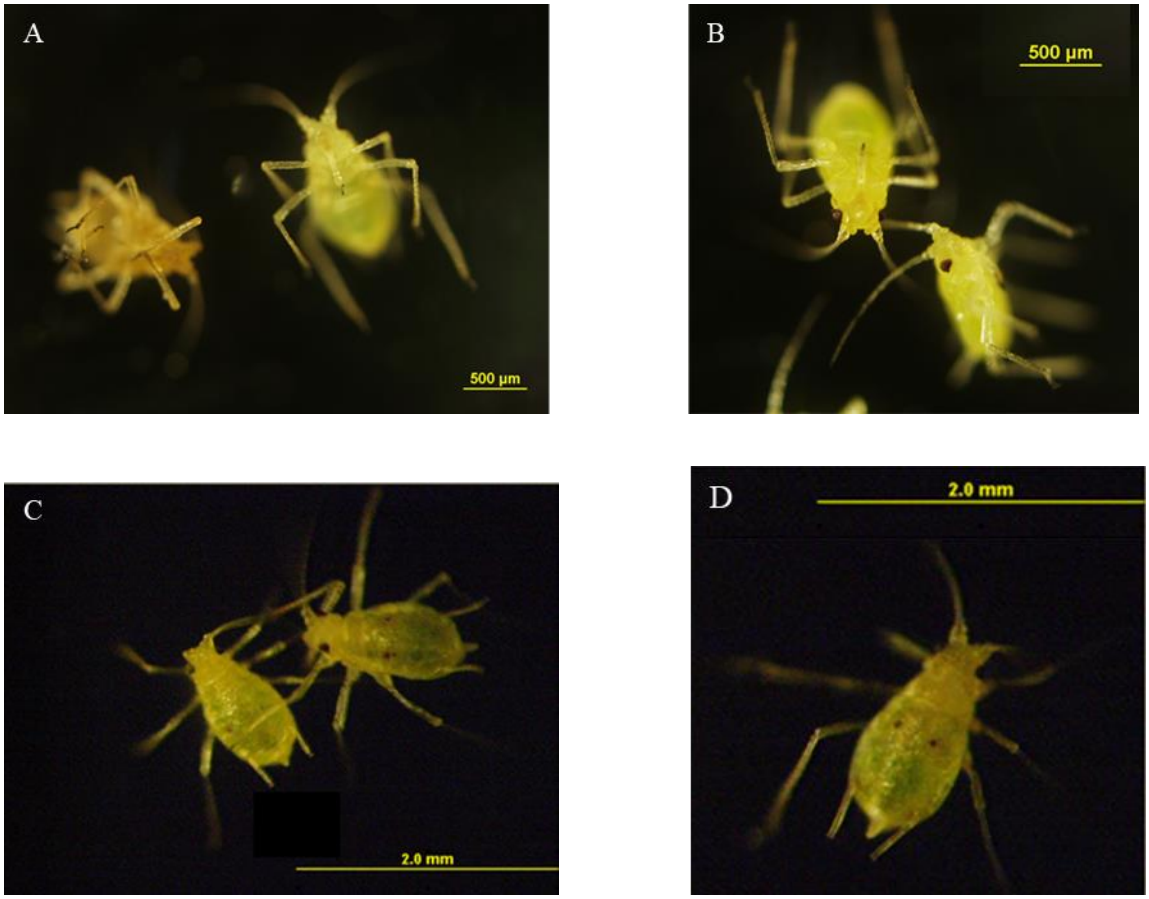


Figure 6.1: RNAi phenotypes of dsRNA-fed GPAs after 24 hours of artificial feeding. Ds*jhbp*$_2$- and ds*jhbp*-treated GPAs showed **(A)** lethal phenotypes and **(B)** uncoordinated movement; **(C)** No behavioural changes in GPAs treated with ds*jhbp*$_1$, ds*jhbp*$_3$ or si*jhbp*) and **(D)** ds*gfp* and no-dsRNA controls.

In addition to abnormal movement, 50% or more of ds*jhbp* -fed aphids exhibited lethal phenotypes for the ds*jhbp*$_2$ and ds*jhbp* treatments. In contrast, only 10% mortality occurred for ds*jhbp*$_3$-fed nymphs 24 hours after dsRNA ingestion (Table 6.2). There were no visible changes for aphids treated with the other dsRNAs, or for the shortcut siRNAs and all aphids were active and moved normally, as did the aphids treated with ds*gfp* and those not treated with dsRNA.

Table 6.2: RNAi Phenotypes of GPAs exposed to dsRNA for 24 hours

DsRNA	RNAi phenotypes of 3rd to 4th instar GPA nymphs
dsjhbp (384 nt)	100% GPAs affected, demonstrating impaired movement or paralysis, and 70% of the GPAs died
dsjhbp$_1$ (70 nt)	No visible changes and normal movement
dsjhbp$_2$ (86 nt)	100% GPAs with uncoordinated movement and 50% of those died-with 24 hours of dsRNA feeding
dsjhbp$_3$ (158 nt)	No visible changes, 10% of GPAs died
dsjhbp (166 nt)	No visible changes and normal movement
Shortcut siRNA	No visible changes and normal movement
Controls	No visible changes and normal movement

From Figure 6.2 it is evident that the dsRNAs in the artificial diet with 30% sucrose plus 0.02% NR dye were essentially intact 24 hours after the aphids fed on them. This result shows that the dsRNAs were not degraded over the course of the experiment, and that their integrity was not affected by aphid secretions or mixing with the sucrose.

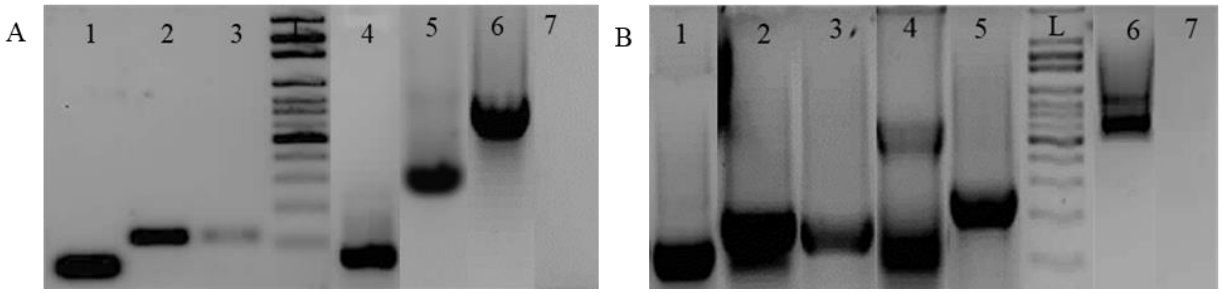


Figure 6.2: DsRNA integrity in artificial sucrose diets. (A) DsRNA before addition to 30% sucrose diet (B) DsRNA in 30% sucrose diet 24 hr after aphid feeding. Lane 1: ds*jhbp*$_1$; Lane 2: ds*jhbp*$_3$, Lane 3: ds*jhbp*$_4$; Lane 4: ds*jhbp*$_2$; Lane 5: ds*jhbp*; Lane 6: ds*gfp* and Lane 7: no dsRNA control (30% sucrose), L= 100 bp ladder.

6.4.2 Ingestion of different lengths of dsRNA affected GPA locomotion differently

The ability of GPAs to move towards a feed attractant (sucrose with 0.02% NR dye) 24 hours after exposure was affected by the length of the dsRNA used (Figure 6.3). On average, GPAs exposed to ds*jhbp*, ds*jhbp*$_1$ and ds*jhbp*$_2$ migrated significantly shorter distances in the lanes towards the feed over the 60 minutes of the assay (p = 6E-05, 0.01 and 10E-05 for ds*jhbp*,

ds*jhbp*$_1$ and ds*jhbp*$_2$, respectively) compared to those exposed to diet without dsRNA. Within the hour assay period, the control aphids exposed to only the sucrose or to *dsgfp* had moved about 63% and 59% respectively, along their lanes. In contrast, ds*jhbp* -fed GPAs moved shorter distances (the mean distance was 0.27 cm), that is only about 2% of the length of the lane, followed by ds*jhbp*$_2$-fed GPAs (mean distance travelled was 0.43 cm, approximately 3% of the length of the tray). No significant difference in aphid movement was observed for the other dsRNA treatments compared to the controls.

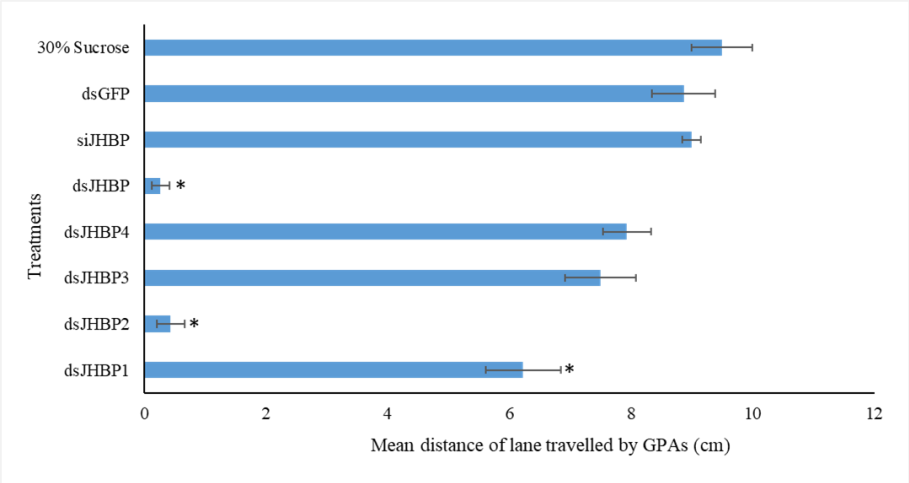


Figure 6.3: Migration of GPAs after feeding on different lengths of dsRNA. Mean distance (n=3) travelled by nymphs was compared with that of nymphs fed only on sucrose (control). An asterisk (*) indicates significant difference between treatment and control (p<0.05). Data is represented as mean ± SE of the means.

6.4.3 Fecundity of GPAs after feeding on different lengths of dsRNA

The reproduction of GPAs treated with the different lengths of dsRNA was also affected when they were transferred to tobacco plants for 12 days (Figure 6.4). One day after the transfer, all aphids previously fed on the longest dsRNA, ds*jhbp,* died. Compared with aphids not exposed to dsRNA, significantly fewer progeny were recorded for ds*jhbp*$_2$-treated GPAs (p<0.05). At the end of the 12 days, the mean number of nymphs produced by aphids which had fed on ds*jhbp*$_2$ was significantly lower than the controls. However, after eight days there was also a

significant reduction in reproduction for the GPAs treated with three dsRNAs; ds*jhbp*$_{1,}$ ds*jhbp*$_{3,}$ ds*jhbp*$_{4}$ and ds*gfp* (p<0.05).

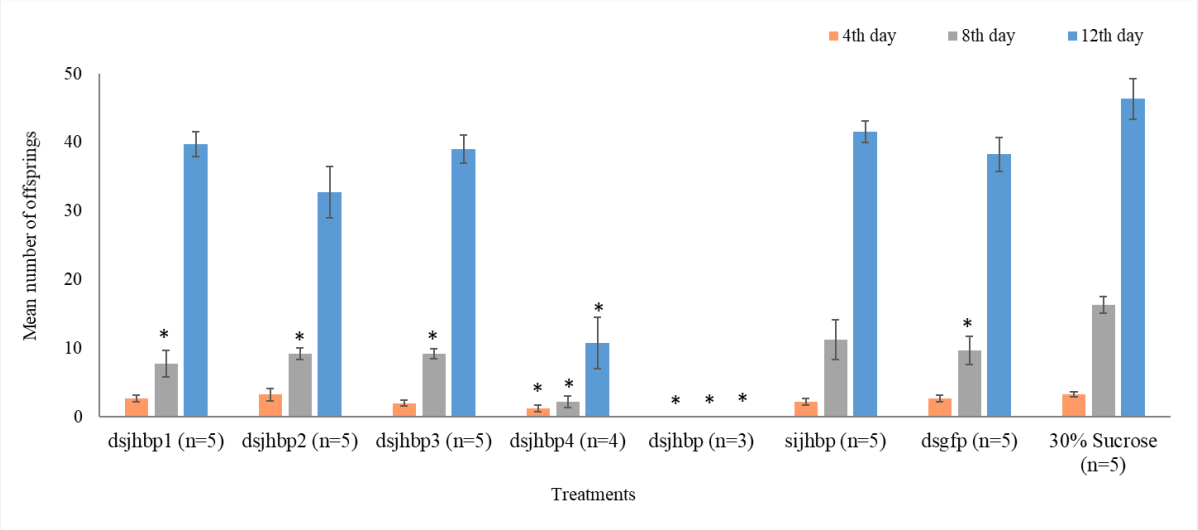


Figure 6.4: Effects of feeding aphids with different lengths of dsRNA on GPA reproduction. The average number of GPA nymphs on tobacco plants after feeding were compared with 30% sucrose -fed GPAs (n = 3 to 5). A significance difference in the means of treatments and the 30% sucrose control is represented by an asterisk '*' at p<0.05. Data are represented as mean ± SE of the means.

6.4.4 Transcript abundance of the *jhbp* gene in dsRNA-fed GPAs exposed to dsRNAs of different lengths

Semi-qPCR was used to assess transcript abundance of target gene expression in dsRNA-fed aphids of different treatments: ds*jhbp*$_{1,}$ ds*jhbp*$_{2,}$ ds*jhbp*$_{,}$ ds*gfp* and no dsRNA-fed GPAs. However, transcript abundance was not assessed for the treatments of si*jhbp*, ds*jhbp*$_{2}$ and ds*jhbp*$_{3}$ because no silencing effect was found after dsRNA ingestion. Total cDNA, synthesised from the same amount of RNA derived from single aphids which had ingested dsRNA, and controls, was used. The expression of *actin*, was used as an internal control, and was similar in all treated aphids (Figure 6.5A). There were no visible bands from the cDNA of ds*jhbp*-fed GPAs, whereas faint bands were amplified from the cDNA derived from the ds*jhbp*$_{2}$-fed GPAs

reflecting a slightly reduced knockdown of the target gene. Aphids fed on ds*jhbp* and ds$jhbp_2$ were severely affected compared to the controls, confirming that these sequences down-regulated *jhbp* gene expression and that this adversely affected aphid survival, movement and behaviour. For these two treatments, 50% to 70% lethal phenotypes were recorded. In contrast, no reduction in *jhbp* expression was found from the cDNA of ds*gfp*- and no dsRNA controls, while bright bands were amplified from the cDNA of ds$jhbp_1$-fed GPAs after 38 cycles - no mortality or reduced fecundity was observed for this treatment, although aphid movement was slightly affected (Figure 6.3).

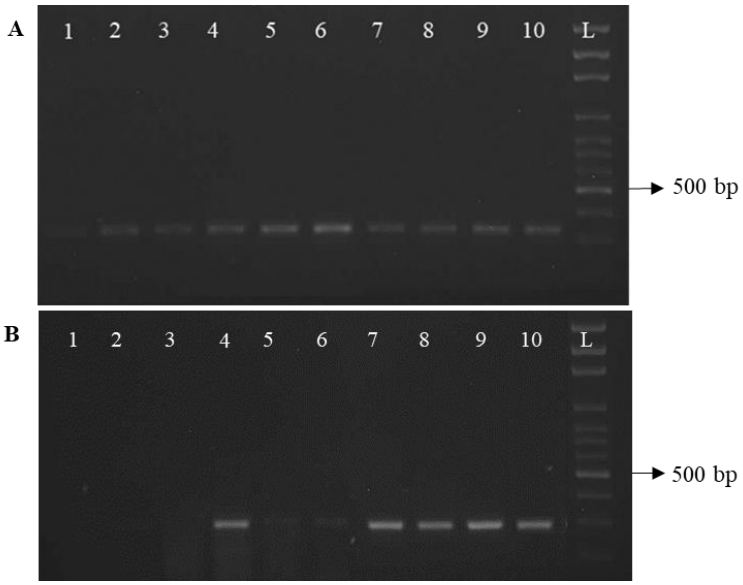


Figure 6.5: Transcript abundance of **(A)** A*ctin* and **(B)** *Jhbp* in GPAs after dsRNA feeding after semi qPCR analysis. Lane 1 and 2: expression in ds*jhbp*-fed GPAs; Lane 3 and 4: expression in ds$jhbp_1$-fed GPAs; Lane 5 and 6: expression in ds$jhbp_2$-fed GPAs, Lane 7 and 8: expression in ds*gfp*-fed GPAs and Lane 10 and 11: expression in no dsRNA-fed GPAs and L: 100 bp ladder. Lane 1, 3, 5, 7 and 9 showed the amplification from 36 cycles and 2, 4, 6, 8, and 10 showed the amplification from 38 cycles.

6.4.5 Prediction of RNA structure of the different dsRNAs of the *jhbp* gene

To provide possible explanations for the different levels of silencing induced by the different dsRNA lengths of the *jhbp* gene, the GC content and predicted secondary structures of the five

dsRNAs were analysed (Figure 6.6). Relatively lower GC contents were calculated for the dsRNAs ds*jhbp*$_2$ (29%) and ds*jhbp*$_4$ (30%) (Figure 6.6A). The longest dsRNA, ds*jhbp*, also had a comparatively lower GC content (34%) with ds*jhbp*$_1$ and ds*jhbp*$_3$ having the highest GC content among the dsRNAs (40%). It appears the dsRNAs designed from the 5´ region (*dsjhbp*$_1$ and *dsjhbp*$_3$) of the EST had a higher GC content than those designed from the 3´ region (*dsjhbp*$_2$ and *dsjhbp*$_4$) (Figure 6.6A). However, the positions of the dsRNAs with respect to the EST used and the differences in the GC contents do not appear significant enough to explain the differences in the RNAi phenotypes they induced in the GPA nymphs.

Also, predicted secondary structures of the target dsRNA regions were analysed to identify if the complexity of folding may have affected silencing efficacy. The predicted structures and their minimum free energy (MFE), predicted using the online RNAfold tool (Hofacker, 2003) are shown in Figure 6.6B. The longest of the dsRNAs, ds*jhbp*, has the highest number (18) of loops including hairpin loops and internal loops followed by the shortest of the dsRNAs, ds*jhbp*$_4$, with 12 loops. On the contrary, the lowest number of loops was predicted for ds*jhbp*$_1$ and ds*jhbp*$_2$ (only 4) although the lengths for these two dsRNAs were significantly different; respectively 86and 158 bp long. The MFEs for the dsRNAs ranged from -5.40 Kcal/mol for ds*jhbp*$_2$, to -48.40 Kcal/mol for the longest dsRNA (Figure 6.6B).

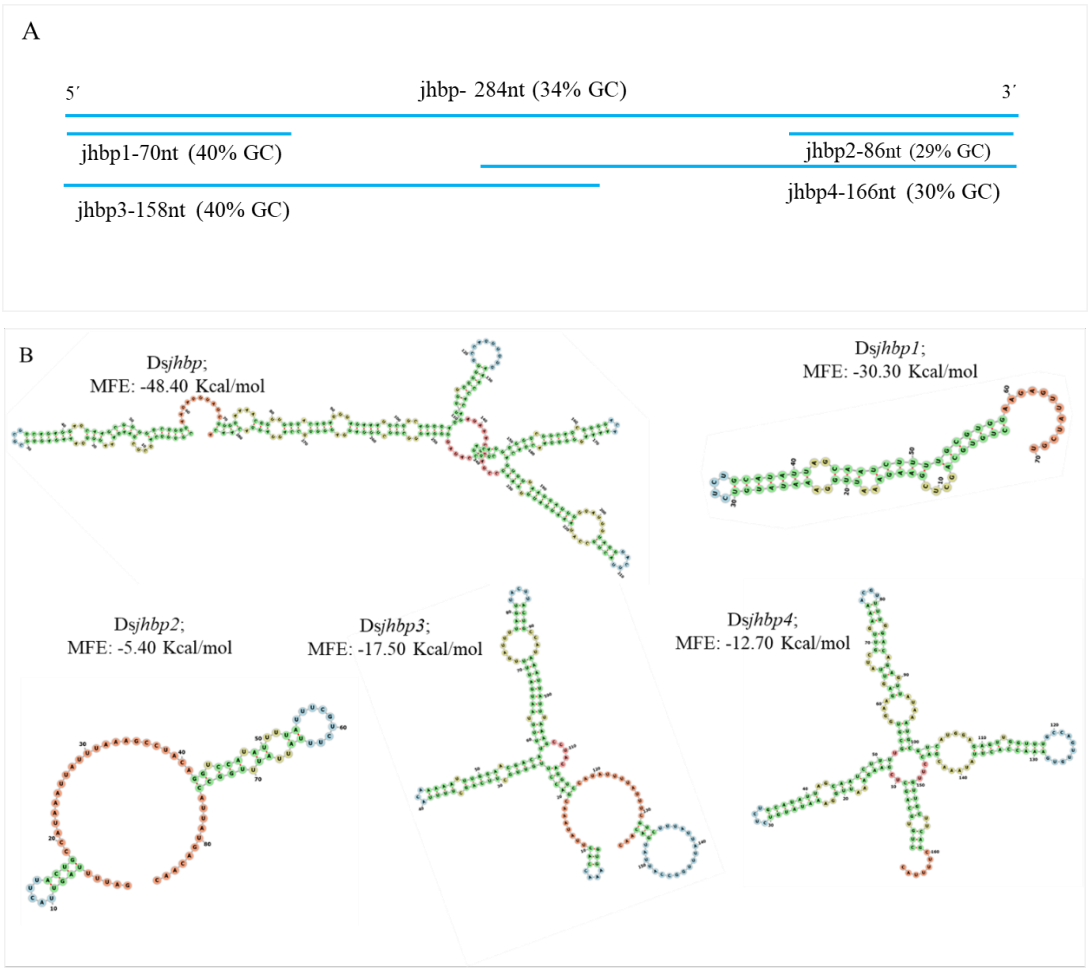


Figure 6.6: (A) Position and GC contents of different lengths of dsRNAs based on the GPA EST encoding the *jhbp* gene. **(B)** Predicted secondary structures of the dsRNAs using the RNAfold webserver (Hofacker, 2003), Green: Stems (canonical helices), red: Multiloops (junctions), yellow: Interior Loops, blue: Hairpin loops, Orange: 5′ and 3′ unpaired regions.

6.4.6 Correlation between the lengths of dsRNA lengths, their predicted structures and induced RNAi phenotypes

The Pearson correlation test was carried out to determine whether there was an association between the different lengths of the dsRNA and the predicted secondary structures, their thermodynamics properties and the RNAi phenotypes induced. The specific parameters tested were GC content, MFEs, a number of loops present in the predicted RNA structures of the dsRNAs, and the ability of aphids treated with these dsRNAs to move towards an attractive food source, their mortality, and reproduction on a host plant after treatment. A very strong

negative correlation between the length of dsRNAs and the predicted MFE (r = -0.93) indicates that MFE is dependent on the length of dsRNAs (Figure 6.7). A moderately positive relationship (r = 0.50 to 0.70) existed between GPA mortality and the number of loops in a predicted secondary structure of dsRNA. There is a weak negative correlation between aphid reproduction and the lengths of dsRNAs (r = -0.55). However, there was no association between the lengths of dsRNAs and the GC content or aphid movement after dsRNA treatment.

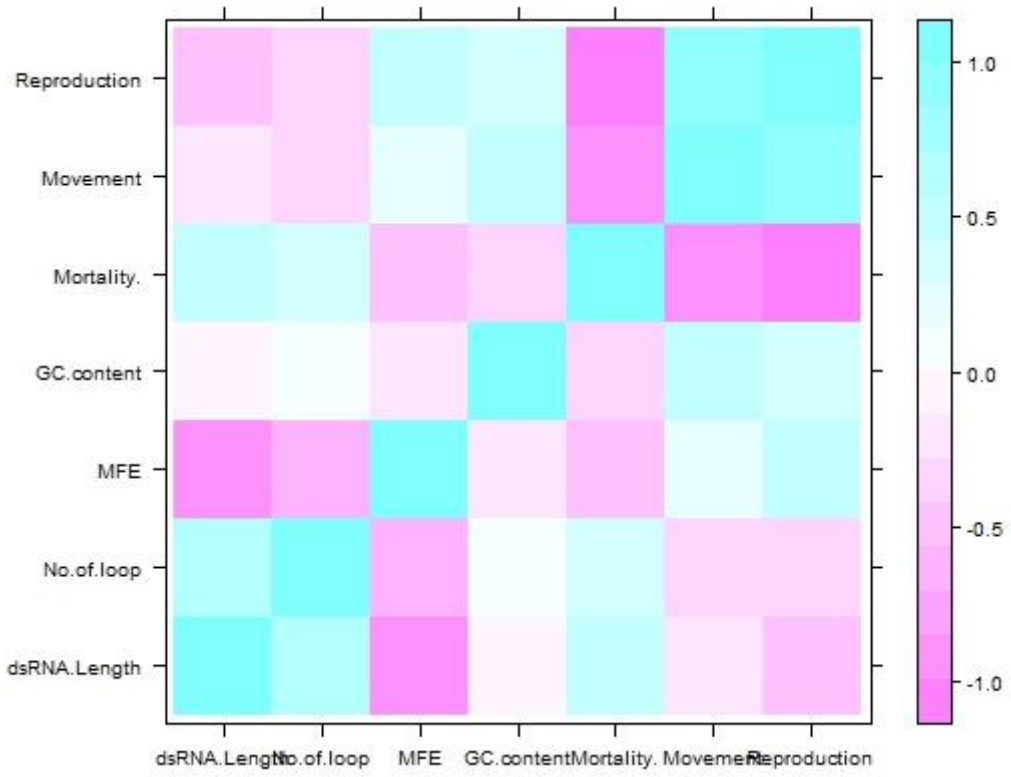


Figure 6.7: Correlation matrix between dsRNA lengths and structural properties of the dsRNA and the RNAi phenotypes they induced. The colour scale indicates the correlation coefficient value (r).

Table 6.3: **Correlation coefficient value (r) value of pairwise comparison between the parameters used in the study**

Parameters used for analysis	DsRNA length	No. of loops	MFE	GC content	Mortality	Movement	Reproduction
DsRNA length							
No. of loops	0.61						
MFE	-0.93*	-0.63					
GC content	-0.10	-0.26	-0.26				
Mortality	0.54	0.40	-0.46	-0.40			
Movement	-0.28	-0.34	0.20	0.44	-0.95*		
Reproduction	-0.55	-0.38	0.46	0.40	-1.00*	0.95*	

In the Table, '*' indicates significance at 95% confidence level.

From the correlation matrix, it appeared that GPA mortality had a strongly inverse relationship with reproduction (r = -1.00) and movement (r = -0.95) which was obvious as reproduction or movement was not possible for the lethal phenotypes. In contrast, a significant positive relationship was found between the aphid reproduction and movement. Although a weak to moderate relationship was present between few parameters, such as GC content with GPA RNAi phenotypes or the number of loops with the GPA mortality, no significant relationship (p >0.05) was determined from the correlation matrix (Table 6.3).

6.5 Discussion

This study analyses one of many factors that influence the effectiveness of gene silencing - the length of dsRNA employed. It demonstrates that different lengths of dsRNA targeting the myosuppressin gene resulted in different degrees of silencing effects on GPAs, affecting locomotion, fecundity and longevity to different extents. Ingestion of the longest of the dsRNAs, ds*jhbp* (284 bp), and a shorter (not the shortest) dsRNA, ds*jhbp*$_2$ (86 bp) appears to have affected the treated aphids the most, resulting in similar observable (lethal) effects including complete paralysis and aphid fecundity. Investigations into the features of the

dsRNAs suggest that the minimum free energy, the GC content and folding of the dsRNA do not influence its effectiveness as a silencing trigger.

The effects of different lengths of dsRNA as triggers of gene silencing have been studied in other organisms including nematodes and insects (Bolognesi et al., 2012; Miller et al., 2012; Parrish et al., 2000). The results of most of these studies do not provide conclusive evidence on what size (length) of dsRNA is optimum to induce the most effective silencing. It appears this effectiveness is dependent on other factors, including the target gene, whether there are multi-gene families or compensatory mechanisms, and the organism itself. However, as found in this study and for most reported experiments, it does appear feeding on longer dsRNA usually results in more and effective silencing, judging from the observable effects and transcript abundance. For example, for the Coleopteran insect *Diabrotica virgifera*, shorter dsRNA, such as 60 bp dsRNA of *v-ATPase C* and *Snf7* has been reported to induce lethal phenotypes, whereas 15 bp or 25 bp dsRNAs or siRNAs did not (Bolognesi et al., 2012; Li et al., 2015). Similarly, ds*lac2* and ds*ebony*, targeting two pigmentation genes of *D. virgifera*, induced noticeable pigmentation defects only for dsRNAs longer than 100 bp dsRNA and not the 30 bp or 50 bp long dsRNAs (Miyata et al., 2014). One possible reason for this observation is that processing of longer dsRNAs may have generated more effective siRNAs than shorter dsRNAs, so accounting for the more pronounced silencing effects observed. It has also been postulated that in the case of *D. virgifera*, the variation in the silencing was due to the inability of the insect cells to take up dsRNAs shorter than 60 bp (Bolognesi et al., 2012).

However, shorter dsRNAs in the form of siRNAs synthesised *in vitro* have been used successfully to silence target genes of *A. pisum*, termites (Isoptera) and *Tetranychus urticae* (Khila and Grbić, 2007; Mutti et al., 2006; Zhou et al., 2006). In the case of the *A. pisum*, there was an anecdotal suggestion that cells of the embryo could have taken up injected siRNAs

readily and that could explain the lethality of nymphs produced from adult aphids injected with siRNA of the C002 gene (Mutti et al., 2006). In contrast, 16 synthetically generated 21nt siRNAs of *vATPaseC* of *D. virgifera* did not produce any silencing effects compared to the 184 bp long dsRNA. Similar results have led to a somewhat premature conclusion that synthetic siRNAs are not effective triggers of gene silencing in insects. This has led to speculations that where siRNAs may have silenced target genes in *A. pisum* and *T. castaneum,* the siRNAs may have been contaminated with long dsRNAs as the latter were derived from the former *in vitro* using Dicer enzymes (Miller et al., 2012; Tomoyasu and Denell, 2004). It is tempting to suggest that the lack of any visible signs of silencing on nymphs treated with *in vitro*-generated siRNAs of the *jhbp* gene in this study imply the generated siRNAs were ineffective. However, it is worth mentioning that the observations may not fully explain silencing or lack of it by the *sijhbp* as it was not possible to assess the quality and integrity after preparation.

Thermodynamic properties are important criteria for generating high-quality siRNAs used for gene silencing in many mammalian systems (Reynolds et al., 2004; Schubert et al., 2005). Among these factors, siRNAs with lower GC content and free energy are considered more effective. Application of similar analysis to the predicted hairpin structures of the *jhbp* dsRNAs of different lengths indicated that there was a correlation between the length of dsRNA and MFE, but this parameter and none of the others could predict the effect/extent of gene silencing induced by any of the dsRNAs. Since, only one of two dsRNAs of the same lengths (60 bp) synthesised from different regions of the *v-ATPase* gene of *D. virgifera* caused larval mortality (Li et al., 2015), it can be suggested that other factors may be involved in determining the efficacy of different dsRNAs. One of these factors could be the number and type of effective siRNAs generated from the different dsRNAs. In this case, the authors suggested that the observed variation could be a result of the position of the dsRNA in relation to the target gene.

6.6 Conclusions

This study is the first to assess the variation of RNAi response in any Hemipteran insect using different lengths of dsRNA for the same gene. The results demonstrate that longer dsRNA was more effective in inducing RNAi of the *jhbp* gene of GPA. However, one of the shorter dsRNAs was also capable of producing gene silencing impacts to an extent, similar to that of the longest dsRNA. The length of the dsRNAs appears to be associated with the free energy but does not fully predict the effectiveness of dsRNAs in inducing silencing. As for many reports, it suggests the longer the dsRNA, the more effective gene silencing but beyond this, unknown factors appear to contribute to the efficacy of dsRNAs as triggers of *in vitro* gene silencing. These factors need more in-depth study to predict optimum lengths of dsRNAs, with minimum GC contents, MFEs and secondary structures of hairpins for silencing essential genes in GPA and in Hemipterans. Such analyses could lead to accurate design of silencing triggers for essential genes of insect pests that could potentially be synthesised precisely and commercially for use as economically viable next generation RNAi insecticides for insect pest management.

Chapter 7

General Discussion

This project was undertaken to assess the potential use of genes expressed in the GPA nervous system as targets for its control using RNAi. The initial aims of the research were to:

(i) identify potential target GPA ESTs encoding insect NPs and GPCRs from transcriptomic and genomic data *in silico*,

(ii) investigate the effects of silencing a subset of these (24 target NSM) genes using an *in vitro* feeding system to deliver dsRNA of these genes to GPAs

(iii) assess the efficiency of silencing promising target genes, based on *in vitro* feeding results, using the HIGS approach, in which dsRNA of target genes is delivered *via* transgenic plants, and

(iv) compare the effectiveness of different lengths of dsRNA of the same NP gene on gene silencing in GPAs *via* artificial feeding

The research outcomes from this PhD project have been described in detail in the previous Chapters. Here, some of the main findings and conclusions are discussed in a broader context.

GPA is one of the major sap-sucking insect pest with a broad range of plants hosts. Although the application of chemical insecticides is still used extensively in agriculture to control aphids, their ability to develop insecticide resistance points to a need to develop new and safer control measures. The recent commercial application of RNAi technology for other traits indicates that this tool could well provide an alternative approach to control insects and other pathogens by silencing specific vital genes. The identification of suitable target genes is crucial for successful development and application of RNAi. The nervous system of insects has been an effective target for control using chemical insecticides, but when this study was started there was only one report targeting GPA *acetylcholinesterase 2* (*MpAChE2*) (Guo et al., 2014). Therefore, this

group of NSMs genes were chosen for study as targets for RNAi, using GPA as the experimental aphid.

7.1 *In silico* identification of target transcripts encoding NSMs in GPA and five important aphid species

The first aim of this project was to identify GPA transcripts which encoded appropriate target NSMs. Recent developments in high throughput sequencing technology have played a major role in enabling *in silico* identification of target genes both in model and non-model insects (Ansorge, 2009). NP and GPCR sequence data identified in other insect species were used to identify more than 30 NPs, 24 NPRs and 9 BARS *in silico* from the available GPA database, and the transcriptome data generated in our lab. Sequences for most of the previously identified *A. pisum* NSMs were also present in GPA sequences, with some exceptions such as Allatostatin B, FMRFamide, short NPF receptor, bursicon receptor or glycoprotein receptor (Huybrechts et al., 2010). However, lower homologies between GPA sequences and those of the reference were observed for four transcripts, namely corazonin, sulfakinin, arginine vasopressin receptor and trissin receptor (bit score 51.6 - 53.6) which was assumed to be absent in *A. pisum* and/or hemimetabolous insects (Amare and Sweedler, 2007; Huybrechts et al., 2010; Li et al., 2013). Whether a particular gene is considered to be present or not, can, to some extent, depend on the stringency used for the BLAST searches.

To further understand NSMs of insects in general, a comparative analysis was also undertaken among sequences of five different aphid species (*A. glycines*, *A gossypii*, *D. noxia*, *S. avaneae* and *M. euphoribae*). Some NSM transcripts were identified, but for some of these species, there was limited information available for NSMs. The most NP transcripts were found in *A. gossypii* (30 NPs), whereas *D. noxia* and *A. glycine* had the most NPR homologous sequences (27

NPRs). From the analyses undertaken, it appeared that GPA sequences shared more homology with those of *A. pisum:* both belong to the tribe Macrosiphini of the family Aphididae.

The NSM genes selected for further study have vital roles in GPA physiology, in metamorphosis, reproduction, muscle contraction, water balance and feeding: to the best of my knowledge, none of these genes have been studied previously *via* RNAi in GPA (reviewed by Zhang et al., (2013).

Overall, the information generated in the bioinformatics study here could also be applied to improve understanding of brain physiology in GPA and other aphid species.

7.2 Functional assessment of GPA targets using *in vitro* RNAi

Once a set of target NSM genes had been identified *in silico*, the next steps involved isolating them using PCR, sequencing them to check that the correct sequence had been obtained, and cloning them to produce dsRNA which could be used to determine whether their knockdown reduced viabilty or reproduction of GPA. This process was followed for a subset of 24 target GPA ESTs from which synthetic dsRNAs mixed with an artificial diet of 30% sucrose were delivered to nymphs. Out of this set of target genes, silencing of nine by *in vitro* feeding for 24 hours resulted in immediate severe and abnormal phenotypes, including incomplete metamorphosis, impaired movement, paralysis or lethal phenotypes. The silenced genes associated with these effects were: *eth, irp2/3, ccap, capa, lk, octβ3r, jhbp, mAChrM3* and *snpf*, and six of these also caused longer term effects of reduced fecundity and/or longevity (*ccap, capa, irp2/3, lk, octβ3r* and *mAChrM3)*. There was up to 40% aphid mortality after dsRNA treatments, leading to 100% lethality within six days for surviving aphids transferred to tobacco plants (ds*ccap*-, ds*capa*-, ds*lk*- and ds*octβ3r*-fed aphids).

These results agree with those reported for *T. castaneum* and *R. prolixus*, in which high levels of mortality (up to 100%) were found after silencing of the genes *ccap* or *ccapr*, as a result of interruption of ecdysis (Arakane et al., 2008; Lee et al., 2013). In contrast, knockdown of *bursβ* did not cause visible changes in GPAs, whereas abnormal wing formation was reported for *T. castaneum*. This observation probably reflects the fact that *bursicon* is involved in insect wing formation, but in this project treated GPAs were wingless. It is noteworthy that silencing of two *capar* genes showed different results; silencing of one *capar* gene, referred as *capar1* (from the database), severely affected GPA reproduction and longevity, while silencing of *capar*2 (identified from transcriptome) caused no visible change. Although it is difficult to explain the basis of these variations, possible explanations are that the amount of dsRNA ingested was not sufficient to induce silencing of *capar2* or that spatial expression of the two genes were different at the time of the experiment. This type of variation is not uncommon, and has also been recorded for RNAi of two insulin-related genes: *irp2/3* (40% mortality within 24 hours of feeding and 100% on plants) and *irp5* (slower movement but no mortality or reduced fecundity was observed).

Not surprisingly, silencing of genes which caused immediate mortality or reduced reproduction were mostly those involved either in insect muscle contraction or metamorphosis or ecdysis (Da Silva et al., 2011; Down et al., 2011; Estévez-Lao et al., 2013; Lahr et al., 2012; Lee et al., 2013; Lee et al., 2012; Rabijith et al., 2015; Wasielewski and Skonieczna, 2008). Some of the genes targeted could also have had pleiotropic activities, such as *lk, ccap, capa, capar,* and so knocking down expression of these genes could have affected more than one pathway, and so be more likely to lead to aphid death. To assess the potential for the practical application by HIGS, nine of target genes were chosen for further study.

7.3 *In planta* RNAi to assess the efficacy of GPA NSMs as a control strategy

To achieve the third objective, following cloning of constructs which would generate dsRNA into an *Agrobacterium* binary vector, transgenic tobacco and *Arabidopsis* plants were generated for the nine most promising GPA NSM target genes. Transgenic tobacco plants were successfully produced which expressed hairpin constructs for nine (*ccap, jhbp, nplp1, capar1, lk, opsin, irp5, octβ3r* and *snpf*) genes, and *Arabidopsis* plants expressing target hairpin constructs for three NSM genes (*ccap, jhbp* and *nplp1*) were also generated. The reason why transgenic plants were not generated expressing dsRNA of *mAChrM3* and *capar1* was because although the constructs were made, there was not enough time to generate and test transgenic plants.

In general, the results from *in vitro* treatments were mirrored by challenges of transgenic plants with GPAs. Aphids feeding on most of the transgenic events of tobacco and *Arabidopsis* plants expressing target dsRNA and siRNA were clearly affected. Significantly lower reproduction was found for all the events expressing hp*ccap*, hp*nplp1* and hp*lk* in T_1 tobacco whereas in T_2 tobacco, hp*jhbp*, hp*ccap* and hp*nplp1* had significantly reduced aphid reproduction (less than 10%) on all events. Likewise, a substantial reduction in GPA nymphs (reduced by 80% to 100%) also occurred for GPAs fed on T_2 *Arabidopsis* plants for the three targets compared to controls, and there was complete mortality for one of the hp*ccap* expressing events (event 3).

Although there was good agreement between results for *in vitro* RNAi and *in planta* treatments, there are differences in the form and timing of the dsRNA provided. For *in vitro* feeding experiments, 2 µg/µL of long target dsRNA was provided for 24 hours, whereas the transgenic plants probably processed all the long dsRNA before ingestion by aphids, so that their diet consisted of siRNAs of unknown concentration over a much longer time period. However, the

exact amount of dsRNAs ingested by GPAs could not be determined for either the *in vitro* or *in planta* studies.

In some systems, it appears that long dsRNAs may be more effective in silencing target genes than siRNAs (e.g. for nematodes and some insects), because the pest RNAi pathway may process long dsRNA slightly differently from plant cell processing pathways. If this is the case, that is, that the insect RNAi machinery is more effective if provided with long dsRNA, this could reduce the efficacy and impact of *in planta* RNAi (Christiaens and Smagghe, 2014).

Differences in RNAi response have been reported *in planta* and *in vitro* treatments for other Hemipteran insects. For instance, in *A. pisum* 100% lethal phenotypes were observed after injecting ds*C002*, whereas no mortality, but reduced fecundity, resulted when aphids fed on hp*C002*-expressing tobacco plants (Mutti et al., 2006; Pitino et al., 2011).

Another factor which can cause differences in responses to different transgenic events is the site of insertion and the copy number of the transgenes. This phenomenon is well documented for other transgenic studies (Fosu-Nyarko and Jones, 2015, 2016; Jones and Fosu-Nyarko, 2014). In addition, for T_2 transgenic plants used in bioassays, some could still be heterozygous and the population still segregating. Therefore, if time were available it would be better to undertake bioassays using single copy number advanced homozygous generations; these would probably provide more reliable information on the efficacy of the HIGS approach.

For this project aphid challenge experiments were carried out in a glasshouse with controlled conditions. Further testing should be undertaken in natural field environments, so that interactions with other biotic and abiotic stresses could be tested.

7.4 RNAi efficiency differs with the lengths of dsRNA in GPA

The fourth goal of this research was to investigate how different lengths of dsRNA influenced the RNAi response in GPAs, by silencing Juvenile hormone Binding Protein (*jhbp*) gene. *In vitro* RNAi of *jhbp* resulted in extremely abnormal GPA phenotypes - impaired movement as an immediate effect, and reduced reproduction from feeding on *in planta* hp*jhbp* -expressing tobacco and *Arabidopsis* plants in the generations tested. This study showed that the effect of its silencing on GPA was influenced by the length of dsRNA used – a result that agrees with previous reports for nematodes and insects, in that longer dsRNA was more effective than shorter dsRNA. However, the results obtained were not so straightforward, because it was not simply the length of the dsRNA in itself which was important – rather the position in the gene (or the specific sequences chosen) also influenced the extent of the RNAi effect. For the shorter dsRNA treatments, significant effects were recorded only for the dsRNA designed from 3´ region (ds*jhbp*$_2$), but not for similar lengths of dsRNA from the 5´ regions. A similar effect was reported for silencing of the *v-ATPase* gene of *D. virgifera* (Li et al., 2015), and the authors suggested that the position in the genes from which the dsRNA originated (i.e. dsRNA from 3´ region) influenced the efficiency of silencing but depending on the target gene. These results again confirm that the efficiency of gene silencing efficacy is influenced by a range of factors, such as the potential that some sequences generate 'hostspots' of siRNAs from dsRNAs, which can increase the efficacy of target gene silencing.

7.5 Ranking the effectiveness of target genes

After evaluating the effects of down-regulating the expression of the set of target genes chosen, both direct feeding on dsRNA and HIGS, it is concluded that at least eight NSM genes could be studied further as potential RNAi targets to control GPAs. Combining the results obtained from *in vitro* (Chapter 4) and *in planta* (Chapter 5), provides a ranking of these target genes for

their potential to control GPAs (Table 7.1). Three other target genes namely, *capa*, *irp2/3* and *mAchrM3*, were not investigated *in planta* because of time limitations, although they also showed potentially effective RNAi phenotypes.

Table 7.1: Ranking of nine potential GPA genes for their potential for GPA control

Target gene	Behavioural changes after 24 hours feeding	Mortality of dsRNA-fed within 10 days on wild-type plants	GPAs on T_1 events on tobacco	GPAs on T_2 events on tobacco	GPAs on T_2 events on *Arabidopsis*	Ranking of target gene
Wild type	No	No	31.2±6.3	33.6±1.46	29.13±2.11	
GFP	No	No	27.5±7.8	26.64±0.78	24.23±1.97	
CCAP	Yes	Yes	2.9±2.37	3.20±1.08	0	1
JHBP	Yes	No	2.9±1.15	6.80±2.66	0.23±0.17	2
Nplp1	No	Yes	3.3±1.30	4.00±1.04	0.59±0.19	3
Lk	Yes	Yes	1.7±0.93			4
Octβ3r	Yes	Yes	1.10±0.4			5
CapaR1	No	Yes	6.7±2.47	8.00±2.41		6
Irp5	Yes	No	7.6±2.06	1.00±0.63		7
Opsin	No	No	2.3±7.37	1.30±0.76		8
sNPF	Yes	No	15.7±1.01			9

These results indicate that GPA neuroactive genes can be effective targets for development of an RNAi-mediated pest management strategy to control GPAs and perhaps other closely related species. Further studies at a field level would be needed to determine whether the results from glasshouse challenges held up in the field, and whether such experiments would change the rankings shown in Table 7.1. This process would determine which are the best candidates to take forward to commercial application, if this course is followed. One aspect of RNAi experiments is that the RNAi trait alone does not normally result in 100% control of a pest, for example when used to confer plant resistance to nematodes (Jones and Fosu-Nyarko, 2014, Jones et al., 2016) and so a two (or more) gene resistance strategies may be needed.

Bt-based insect resistance has been very successful to control Lepidopteran insect pests in cotton and maize. The current trend appears to be not to rely on a single resistance gene, for example Bollgard 3 insect resistance (http://bollgard3.com.au/) in cotton now includes three different resistance genes (Vip3A, Cry1Ac and Cry1Ab), to increase the durability. Moreover, RNAi-based resistance is now being considered as a complement to *Bt* transgenic crops as an alternative approach to counter the development of insect resistance to *Bt* crops. For example, RNAi plus *Bt* pyramided cotton is effective in controlling *H. armigera,* and computational modelling predicts that development of resistance would be delayed for the pyramided cotton line compared to *Bt* cotton alone (Ni et al., 2017).

7.6 Other approaches to delivering dsRNA for insect control

There are two major issues which confront, delay and increase the expense of commercialisation of RNAi-based pest resistance. These are the regulations that surround commercialisation and growing of GM crops, and issues of the public acceptance, now often called the 'social licence'. One possible approach to reduce or side-step these issues is known as 'Spray Induced Gene Silencing' (SIGS), in which dsRNA to target genes is delivered ectopically by spraying. There are a number of technical problems which need to be overcome to develop this area. These include the stability of dsRNA in field application, uptake and systemic transport in plants. In this case dsRNA is used as an agrochemical, and the plants are not genetically modified. An advance in the SIGS approach has been published by Mitter et al., (2017), in which the dsRNA is adsorbed onto nanoparticles ("bioclay"), and this protects the dsRNA for at least 20 days, enables some dsRNA to be taken up by the plant, and can give protection against virus replication. It remains to be seen whether ectopically-delivered dsRNA can reach the sieve elements in the phloem, and so be effective in controlling sucking insect pests like aphids.

7.7 Future Research Directions

The main goal of this project was to identify potential GPA NSM genes as targets to develop next-generation insect-resistant plants using RNAi technology. The research findings are encouraging, but further research will be required for practical application of RNAi-mediated transgenic crops. As indicted above, public acceptance of GM foods and the strict regulatory policies make the commercialisation of RNAi-based insect resistant crops challenging. Nevertheless, there are some examples of successful commercialisation of GM crops with RNAi traits. These include non-browning 'Arctic' apples for sale in the USA and Canada, tomatoes with high carotenoids, high-amylose and low-gluten wheat, and oranges with elevated levels of beta-carotene (Nehra and Taylor, 2015). In June' 2017, the US EPA (Environmental Protection Agency) approved the first RNAi mediated insect-resistant corn line (SmartStax Pro) to control *D. virgifera via* silencing the Dv*snf7* gene, which was developed by Monsanto and Dow, and is expected to be released commercially soon (Zhang, 2017). Thus, RNAi can be a stable trait, and will enter the commercial world as a potential pest control strategy.

Also, as indicated above, RNAi-based traits do not normally cause 100% silencing of target genes. One strategy to achieve the highest levels of silencing in transgenic plants, is to express dsRNA plant chloroplasts. The advantages here are that chloroplasts originated as prokaryotic cells, and so do lack the RNAi machinery, there are many copies of the chloroplast genome in each chloroplast, and there are many copies of chloroplasts in green cells. This means that they can deliver high levels of long hpRNAs instead of pre-processed siRNAs. Available reports indicate that dsRNA expressed in chloroplasts resulted in better silencing responses for the insects, *Leptinotarsa decemlineata* and *H. armigera* (Bally et al., 2016; Jin et al., 2015; Zhang et al., 2015), although this approach has yet to be studied for sap-sucking insects.

Alternatively, tissue-specific transgene expression is another option to control sap-sucking insect pests including aphids. Using different phloem-specific promoters isolated from Banana bunchy top virus (BBTV) (Javaid et al., 2016) enabled the silencing of two target genes, and this resulted in nearly 100% mortality of three Hemipteran insects including GPA. Silencing of the potential GPA NSM genes identified in this project could well provide better knockdown impacts using phloem specific promoters to drive dsRNA production, and this aspect should be investigated further for GPA control. However, it remains to be seen how siRNAs reach the phloem, since sieve elements lack nuclei and possibly RNAi machinery. It is generally assumed that phloem companion cells support the activities of associated sieve elements, and there are complex plasmodesmatal connections between companion cells and sieve elements. Thus, siRNAs generated by RNAi may be supplied to sieve elements *via* companion cells, so enabling a phloem-targeted RNAi strategy for aphid control. The silencing of two or more target genes using RNAi has been studied, and this could also be applied to control insect pests. However, data from other insects and nematodes indicates that competition can occur between multiple RNAi treatments, and this does not necessarily result in better pest control (Jones and Fosu-Nyarko, 2014, Miller et al., 2012).

There have also been recent advances in new breeding technologies, in particular 'Genome-Editing', which is a novel technology to generate targeted mutations at precise sites. In the most common approach it uses a double stranded DNAse (Cas9) and an RNA guide sequence to generate a double stranded break in a specific sequence, and errors in repair result in mutations at that site. At present, it is difficult to see how genome editing could displace RNAi as a method of aphid control, unless host genes vital for aphid susceptibility are identified and mutated, without affecting the performance of the plant. So, an RNAi-mediated pest management strategy still appears to be the best choice for aphid control.

7.8 Conclusion

The overall aims of this thesis were achieved successfully. A set of vital GPA NSM genes were identified, isolated and characterised, and their potential as targets for aphid control using RNAi were assessed by *in vitro* and *in planta* RNAi studies. The research has generated new knowledge for this field and provided a subset of promising new target genes for GPA control. The principles developed in this project can also be applied to control other sap-sucking pests.

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Appendix

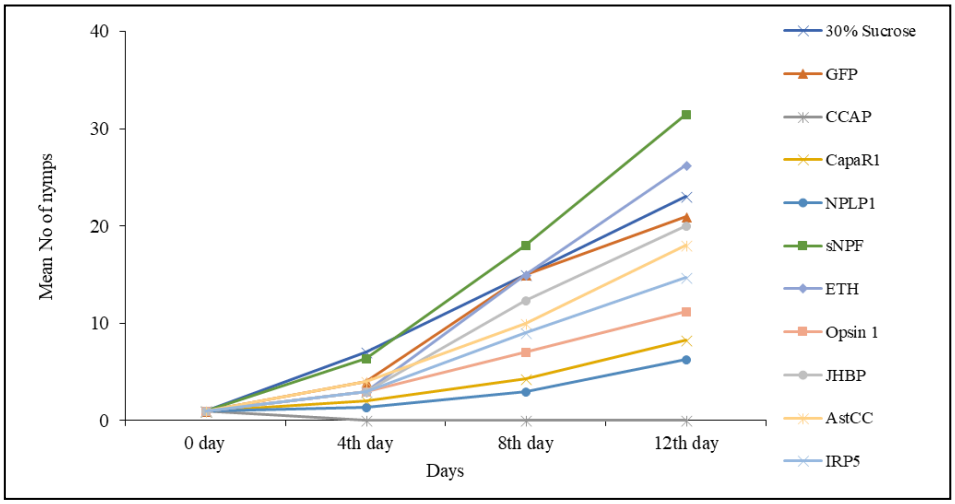

Figure S1: Average number of GPA nymphs produced by aphids fed dsRNA of nine genes (set 1) at different times after transfer to plants.

Table S1: Average number of aphid nymphs (± SE) produced by dsRNA-fed GPAs (set 1) at different times after transfer to plants.

Gene/treatment	**4 days**		**8 days**		**12 days**	
	Mean	**SE**	**Mean**	**SE**	**Mean**	**SE**
30% Sucrose	7	1	15	2.08	23	0.63
GFP	4	0.57	15	3.21	21	0.41
CCAP	0	0	0	0	0	0
CapaR1	2	0.57	4.33	1.33	8.25	0.75
NPLP1	1.33	0.33	3	1.52	6.25	1.60
sNPF	6.33	1.2	18	2.08	31.5	1.55
ETH	3	0.57	15	1.52	26.25	4.05
Opsin 1	3	1.15	7	2.3	11.25	5.31
JHBP	3	0	12.33	0.66	20.03	2.96
AstCC	4	1.15	10	1.73	17.99	0.88
IRP5	3	0.57	9	1.15	14.75	4.91

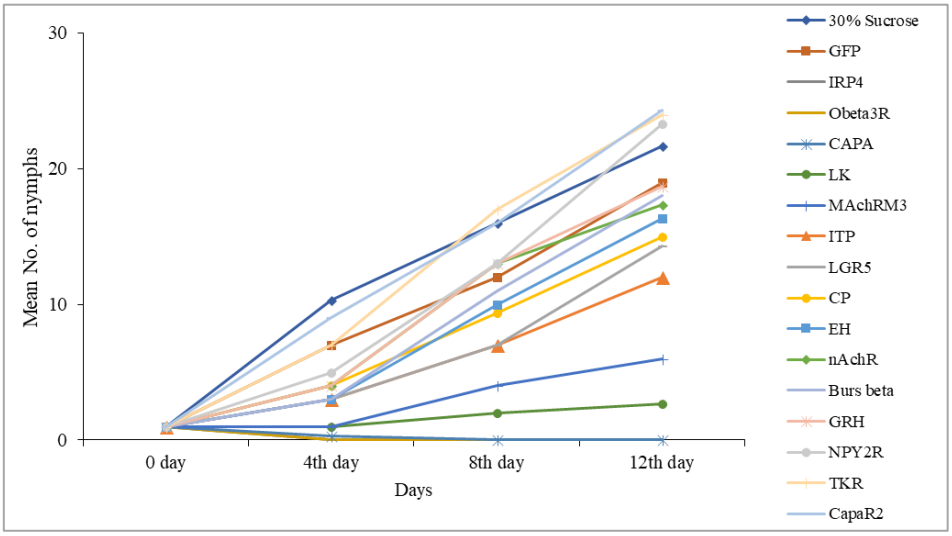

Figure S2: Average number of GPA nymphs produced by aphids fed dsRNA of fifteen genes (set 2) at different times after transfer to plants.

Table S2: Average number of aphid nymphs (± SE) produced by dsRNA-fed GPAs (set 2) at different times after transfer to plants.

Gene/treatment	4 days		8 days		12 days	
	Mean	SE	Mean	SE	Mean	SE
30% Sucrose	10.33	1.2	16	2.08	21.67	1.76
GFP	7	0.57	12	1	19	1
IRP4	0	0	0	0	0	0
Octβ3R	0	0	0	0	0	0
CAPA	0.33	0.33	0	0	0	0
LK	1	0	2	0.57	2.67	0.33
MAchRM3	1	0	4	1.527	6	1.15
ITP	3	0.57	7	1	24.33	1.15
LGR5	3	0.57	7	0.57	15	2.33
CP	4	0.57	9.33	0.88	14.33	1.15
EH	3	0.57	10	2.52	16.33	0.67
nAchR	4	0.57	13	2.89	12	3.93
Burs beta	3	1	11	2.08	23.33	4.52
GRH	4	1	13	1.73	18.67	1.86
NPY2R	5	0.57	13	2.89	18.67	6.12
TKR	7	1	17	3.61	24	2.29
CapaR2	9	1.15	16	3.61	18	1.76