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FACULTY OF BIOSCIENCE ENGINEERING

RNA interference technology as a potential control method for spotted wing drosophila (*Drosophila suzukii*)

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A dissertation submitted to Ghent University in partial fulfilment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences

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I have learned to use the word 'impossible' with the greatest caution

(Wernher von Braun)

Dutch translation of the title:

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Cover illustration

Top left: Female *Drosophila suzukii* and the projection of its ovipositor, **Top right**: Male *Drosophila suzukii* with spotted wings, **Lower left**: Two *Drosophila suzukii* larvae feeding within a strawberry (Photo credit: Hanna Burrack, NC State University), **Lower middle**: *Drosophila suzukii* adult emerging from a blueberry (Photo credit: Matteo Maspero and Andrea Tantardini, Centro MiRT - Fondazione Minoprio), **Lower right**: Damage caused to cherries before harvest by *Drosophila suzukii* (Photo credit: David Haviland, University of California)

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List of acronyms and abbreviations

A	
ACT	Actin
Adk3	Adenylate kinase-3
AeDNV	Aedes aegypti densovirus
Ago2	Argonaute 2
AM	Anterior midgut
αTub84B	Alpha-tubulin at 84B
B	
BLAST	Basic local alignment tool
BW	Body weight
βSe1	Beta integrin subunit 1
Bt	Bacillus thuringiensis
C	
ChS	Chitin synthase
CPB	Colorado potato beetle
CPP	Cell penetrating peptide
Ct (= Cq)	Threshold cycle (= quantification cycle)
Cry	Crystal
D	
Da6	Nicotine acetylcholine receptor subunit
Dcr2	Dicer 2
DCV	Drosophila C Virus
delta COP	Delta-Coatomer protein
DFV	Drosophila F virus
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPV	Drosophila P virus
ds	Double stranded
dsRNA	Double stranded Ribonucleic acid
DXV	Drosophila X virus

E	
eGFP	Enhanced green fluorescent protein
EPA	Environmental protection agency
EPPO	European and Mediterranean Plant Protection Organization
EU	European Union
F	
FHV	Flock House Virus
FNP	Fluorescent nanoparticle
G	
Gamma COP	Gamma-Coatomer protein
GAPDH1	Glyceraldehyde-3-phosphate dehydrogenase 1
GFP	Green fluorescent protein
GLM	Generalized linear model
γTub23C	Gamma-tubulin
I	
ILVO	Institute for Agricultural and Fisheries Research
iPS	in Plant System
l(2)NC136	lethal (2) NC136
L	
LB	Luria–Bertani
LDH	Layered double hydroxide
M	
Mad1	Mitotics arrest deficient-like 1
miRNA	Micro RNA
mRNA	Messenger RNA
MVBs	Multivesicular bodies
N	
NP2	Nitropin 2
P	
PCR	Polymerase chain reaction
PM	Posterior midgut
Q	
qPCR	Quantitative polymerase chain reaction

R

RdRP	RNA-dependent RNA polymerase
RH	Relative humidity
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
RNP	Ribonucleoprotein particle
Rpl32	Ribosomal protein L32
RPS13	Ribosomal protein S13
RT-qPCR	Reverse transcription quantitative real-time polymerase chain reaction

S

S2	Schneider 2
Shrb	Shrub
SID	Systemic RNA interference deficient
siRNA	Small interfering RNA
Snf7	Sucrose non-fermenting 7
SWD	Spotted wing drosophila

T

TMV	Tobacco Mosaic virus

U

US	United States

V

Vha26	Vacuolar H [+]-ATPase 26kD E subunit
Vha68	V/A-type ATP synthase catalytic subunit A
VIGS	Virus induced gene silencing

W

WCR	Western corn rootworm

Problem setting and thesis outline

Drosophila suzukii Matsumura (Diptera: Drosophilidae), also commonly known as spotted wing drosophila and native to Southeast Asia, is a rapidly emerging polyphagous invasive pest in America and Europe (Lee et al., 2011). Contrary to other closely related *Drosophila* species that normally infest mainly overripe and damaged fruits, and thus are not considered serious pests, *D. suzukii* has the ability to break the skin of maturing and undamaged healthy fruits and oviposits into them using its serrated ovipositor (Dreves et al., 2009; Hauser, 2011). The oviposition wounds caused by *D. suzukii* flies very often provide access points to secondary infection by both insects and pathogens, including fungi, yeasts and bacteria, hence, causing additional losses. These make *D. suzukii* a great concern as a pest of maturing and ripening fruits (Mitsui et al., 2006; Calabria et al., 2012). The damage caused by *D. suzukii* can reach up to 80% crop loss (Dreves et al., 2009; Goodhue et al., 2011; Walsh et al., 2011). Furthermore, the management of *D. suzukii* is primarily challenging because the fly can continuously infest a wide range of different fruits available throughout the year (Lee et al., 2011), it can survive in various climatic conditions in which their natural predators cannot keep up (Chabert et al., 2012) and it also has a short generation time (Kanzawa, 1939; Lee et al., 2011). Lack of adequate knowledge on how to effectively control *D. suzukii* and the zero tolerance for infested fruit bound for the fresh market or various export markets, has motivated the priority for more research into possible control options for this pest. The main goal of this thesis was to investigate the potential of RNA interference (RNAi) technology as a possible control method for *D. suzukii*.

In **Chapter 1**, a general introduction on *D. suzukii* and its threat to fruit production is presented; What *D. suzukii* looks like, why it is a threat to fruit production, its worldwide pest status and geographic spread, and its current state of management is further elaborated on. Additionally, an introduction on RNAi and its rising potential as a crop protection technology is presented.

Chapter 2, titled "RNAi evidence and screening of potential target genes in *D. suzukii*"**,** addresses two important questions: Is RNAi functional in *D. suzukii*? And which target genes can be exploited for RNAi-based pest control in *D. suzukii*? This chapter presents the findings from *in silico* transcriptome analysis, performed to verify whether core genes known to be present in the RNAi machinery are expressed in *D. suzukii*. Once confirmed, a microinjection protocol was set up, with which we could effectively deliver a known dose of double stranded RNA (dsRNA) to induce RNAi in *D. suzukii*. With the microinjection protocol in hand, we screened for potential target genes, based on the phenotype of mortality. In view of the possibility of applying RNAi to control *D. suzukii* in the field, we decided to evaluate and optimize a feeding delivery method for RNAi in **Chapter 3,** using selected target genes from **Chapter 2.**

Chapter 3, titled "RNAi induction by oral feeding in *D. suzukii*", presents different oral delivery methods tested and optimized for RNAi in *D. suzukii*. In this chapter, we first verify if RNAi can be induced by oral feeding of dsRNAs and then we present how bacteria can be engineered to become a micro-factory to produce *D. suzukii* target gene dsRNAs for oral delivery. Furthermore, we report on the use of lipid-based nanoparticles to improve the oral delivery of dsRNAs in *D. suzukii*. However, the key findings in Chapter 3 indicate that the cellular uptake of dsRNA from the gut of *D. suzukii* is the key bottleneck to the simple and cost effective oral application of naked dsRNA. A possible alternative method to deliver dsRNA is using a virus-based delivery system, since viruses have evolved to be able to penetrate into cells. Hence in **Chapter 4**, we describe the development of a novel virus-based RNAi delivery system.

Chapter 4 titled "Engineered Flock House Virus (FHV) for targeted gene suppression through RNA interference in fruit flies: A proof of concept in *D. melanogaster*", presents a novel FHV-based RNAi delivery system designed for improving RNAi in *Drosophila*. This chapter presents details on the engineering strategy of the virus. Furthermore, the functionality of the FHV-based

RNAi delivery system was confirmed *in vivo* and *in vitro* in the model insect, *D. melanogaster*, which is also closely related to *D. suzukii*.

In the last Chapter (**Chapter 5**) titled “General discussions and perspectives for future research”, the major research findings from this thesis are discussed together with perspectives for future research.

Chapter 1

Current status of *Drosophila suzukii* and the role of RNAi technology in insect pest management

1 *Drosophila suzukii* and its impact on fruit production

1.1 What does *D. suzukii* look like?

1.1.1 Identification

Drosophila suzukii Matsumura (Diptera: Drosophilidae), commonly referred to as the spotted wing drosophila (SWD), is an economic pest of stone and small fruit crops and is currently established in many production areas of these crops in Asia, Europe and America (Asplen et al., 2015). *D. suzukii* adults are drosophilid flies with a length of 2-3 mm, red eyes, a pale brown or yellowish brown thorax and black stripes on the abdomen. Sexual dimorphism is clearly evident in *D. suzukii* with males displaying a conspicuous dark spot on the leading top edge of each wing and females possessing a large serrated ovipositor (Fig. 1) (Kanzawa, 1939; Walsh et al., 2011).

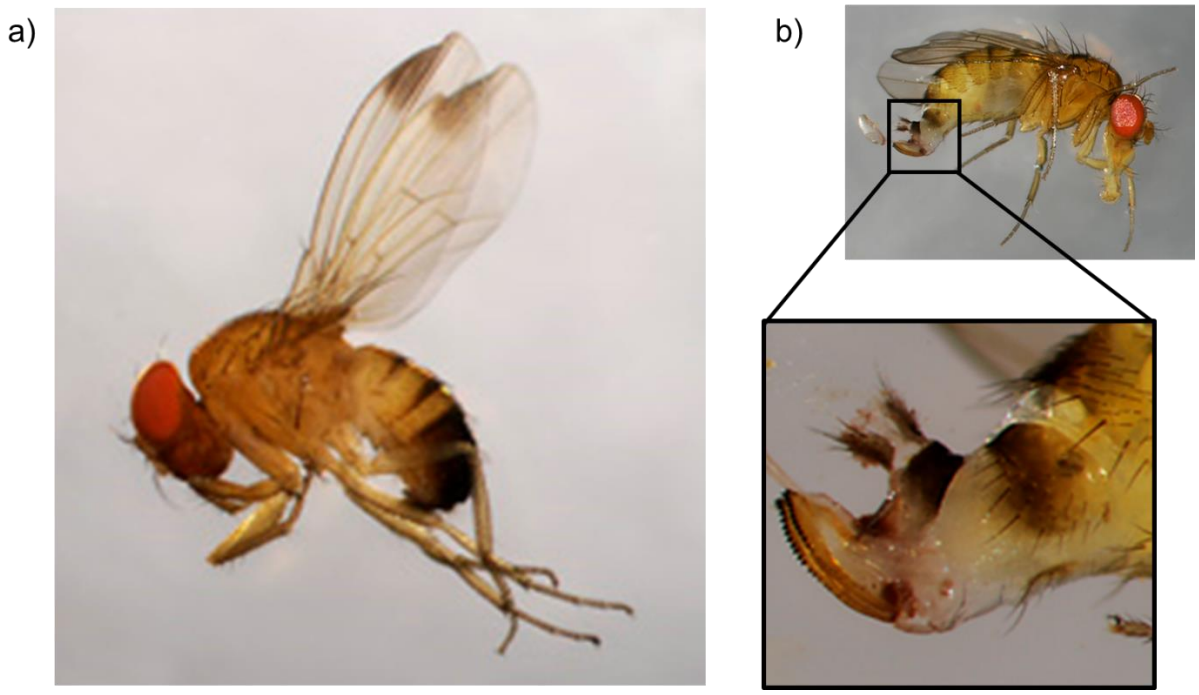


Figure 1: Adult *D. suzukii* **(a)** Adult male *D. suzukii* with spotted wings, **(b)** Close-up of serrated ovipositor on female *D. suzukii*. (Photo credit: Washington state University, Tree Fruit Research and Extension Center, SWD gallery)

However, the identification of *D. suzukii* still presents several challenges despite these evident features. *D. suzukii* adults can still be easily misidentified, as it happened in California, where it was at first erroneously identified as *Drosophila biarmipes* Malloch (Hauser, 2011). This is because the distinguishing characteristics of the two sexes (Males: black wing spots and Females: serrated ovipositor) are present in other *Drosophila* species, thus making species identification very difficult especially in areas where they are sympatric. For example, the black spots on *Drosophila subpulchrella* Takamori and Watabe males are very similar in position and shape to those of *D. suzukii* (Takamori et al., 2006). Furthermore, young or small *D. suzukii* males sometimes lack the black spot on their wings, which only becomes clearly visible around two days after emergence (Hauser, 2011) and could lead to misidentification with other *Drosophila* males. In such a situation, other characteristics could be used to guide identification, such as the sex combs on the fore tarsi. *D. suzukii* males have only one row of combs on the first and one row on the second tarsal segment (Parshad and Paika, 1965; Bock and Wheeler, 1972) (Fig. 2). In many cases, it is only by analysing a full set of features, also including the male genitalia (Parshad and Paika, 1965; Bock and Wheeler, 1972) that a reliable identification can be made.

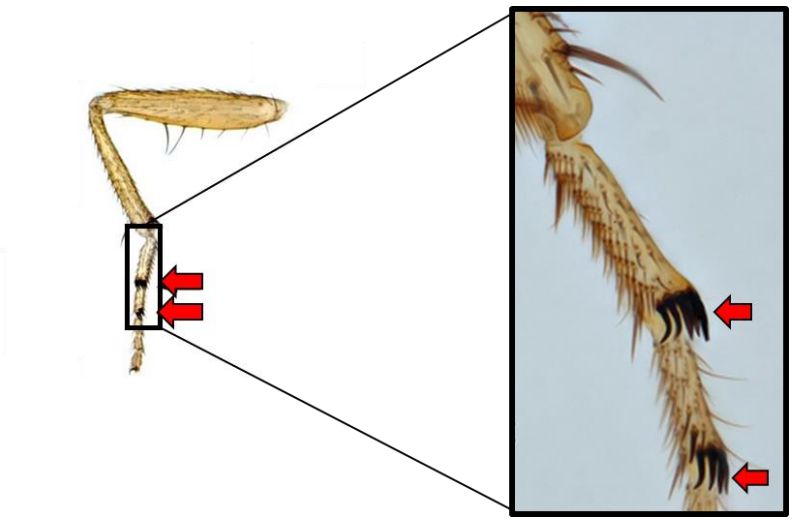


Figure 2: Sex combs on first and second tarsomere of *D. suzukii* male's forelegs. (Photo credit: Adapted from EPPO Bulletin and Martin Hauser, California Department of Agriculture)

Similar problems are encountered while trying to identify *D. suzukii* females. Based on the length and shape of the ovipositor, *D. suzukii* females can easily be differentiated from some related species, such as *D. biarmipes*, but not from others, such as *D. subpulchrella* (Takamori et al., 2006) and *Drosophila immigrans* Sturtevant, having very similar ovipositors (Hauser, 2011). In this situation, the final determination will rely on the relative size of the spermatheca compared to the ovipositor's size, meaning that it will only be feasible in the eyes of a trained taxonomist (Hauser, 2011). The identification of immature stages (eggs, larvae and pupae) of *D. suzukii* is equally complex, especially since no reliable morphological diagnostic features have been identified so far. The eggs of *D. suzukii* have two respiratory appendages but this character is not species-specific, leaving DNA barcoding as the only fully reliable identification tool. To facilitate the identification of *D. suzukii*, a comparative diagnostic account on *D. suzukii* has been published by Hauser (2011) and a good key for proper identification has also been provided by Vlach (2010).

1.1.2 Taxonomy

Taxonomically, *D. suzukii* belongs to the subgenus *Sophophora*, which is divided into different species groups. Among these species groups, *melanogaster* is the species group that contains *Drosophila melanogaster* Meigen, which is a famous model insect in experimental biology and genetics (Powell, 1997). The *melanogaster* group further divides into several species subgroups, one of which is the *suzukii* subgroup. The *suzukii* subgroup together with 6 other subgroups compose the "oriental lineage" (Kopp and True, 2002; Van Der Linde et al., 2010). The relationships within and between these subgroups are complex and still far from being resolved. The *suzukii* subgroup itself is commonly regarded as polyphyletic (Kopp and True, 2002). In fact, *D. biarmipes* was suggested as a sister species of *D. suzukii* (Yang et al., 2012), in accordance with previous findings (Kopp and True, 2002; Barmina and Kopp, 2007), but in contrast with others (Prud'Homme et al., 2006; van der Linde and Houle, 2008), which rather

supported *D. subpulchrella* as a sister species of *D. suzukii*. These conflicting results are probably linked to inadequate sampling of species from different taxa and characters.

1.2 Why is *D. suzukii* a threat to fruit production?

Unlike most other Drosophilidae, possibly exempting *D. subpulchrella*, *D. suzukii* is able to lay eggs in healthy, unwounded fruit thanks to the serrated female ovipositor (Fig. 3) and not only on damaged or overripe fruits (Cini et al., 2012; Bellamy et al., 2013). In fact, ripening fruits are even preferred over overripe ones (Mitsui et al., 2006). Furthermore, although it is unlikely for *D. suzukii* to oviposit into fruits with thick and hard skin or fuzzy surfaces such as apples, cranberries, oranges, peaches, and pomegranates, it can complete its larval development when these 'suboptimal' hosts are damaged, rotted, or overripe (Steffan et al., 2013). Therefore, numerous cultivated and uncultivated fruits can be utilized by *D. suzukii* (Lee et al., 2015; Stacconi et al., 2015).

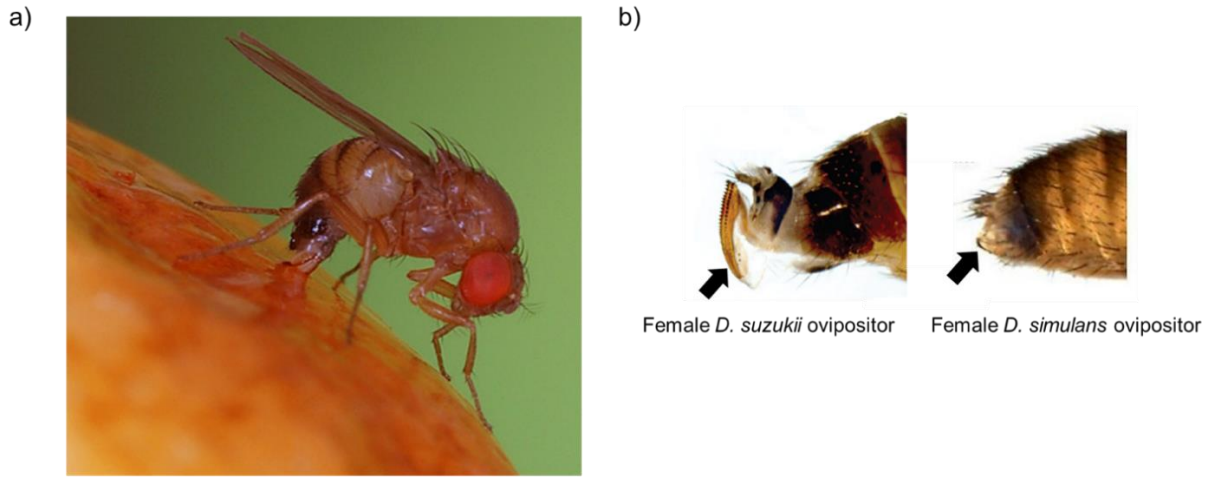


Figure 3: female *D. suzukii* **(a)** female *D. suzukii* piercing through the skin of a healthy fruit using its hook-like ovipositor, **(b)** Right: *D. suzukii* hook-like ovipositor. Left: *D. simulans* ovipositor. (Photo credit: (a) Washington state University, Tree Fruit Research and Extension Center, SWD gallery, (b) Adapted from Martin Hauser, California Department of Agriculture)

Although most of the damage caused by *D. suzukii* is due to larvae feeding on fruit flesh, the insertion of the prominent ovipositor into the skin of the fruit can cause physical damage to the fruit. This in turn provides access to secondary infections of pathogens such as, yeasts, filamentous fungi and bacteria that may cause faster deterioration and further losses (Hamby et al., 2012; Ioriatti et al., 2015) (Fig. 4).

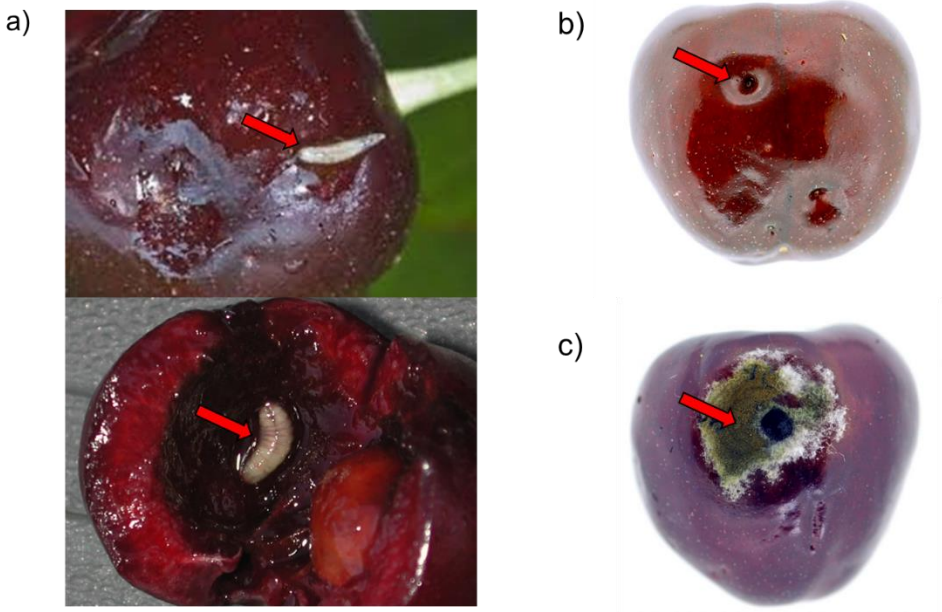


Figure 4: Direct and indirect damage caused by *D. suzukii* to fruits **(a)** Sweet cherry infested with *D. suzukii* larvae, **(b)** Wound on the oviposition site, **(c)** Fungi infection around the oviposition site. (Photo credit: (a) Mike Bush, Washington State University Extension, (b) and (c) Martin Hauser, California Department of Agriculture)

Additional costs are mostly related to increased production costs (monitoring and chemical input costs, increased labour and fruit selection, reduction of the fruit shelf life, storage costs) and to the decrease of market appeal for fruits from contaminated areas (Goodhue et al., 2011). Nevertheless, the oviposition habit itself is not enough to explain the dramatic impact of *D. suzukii* on fruit production. In the next sections the main features making *D. suzukii* a threat of high concern for the fruit production sector are discussed.

1.2.1 Extreme fecundity

Mating in *D. suzukii* optimally occurs from the first days after emergence and females start to lay eggs already from the second day. Depending on the temperature, females typically lay 1-3 eggs per fruit in up to 7-16 fruits per day (Kinjo et al., 2014). Since they are able to oviposit for 10-59 days, they can lay up to a total of 600 eggs during their lifetime (around 400 eggs on average) and once eggs are laid in the fruit, it can no longer be controlled with pesticides. Eggs hatch within 2 to 72 hours after being laid inside the fruits, and larvae mature (inside the fruit) in 3 to 13 days. Pupae reside for 3 to 15 days either inside or less frequently outside the fruit. Depending on the temperature, a minimum of 10 days is required from oviposition to adult emergence (Fig. 5). This very short generation time has a huge impact on fruit production. It implies that *D. suzukii* can complete several generations in a single cropping cycle and up to 7 to 15 generations in a year, according to the specific climatic conditions, thus allowing an explosive population growth. The life-cycle details can be found in several other studies (Kanzawa, 1939; Mitsui et al., 2006; Walsh et al., 2011; Tochen et al., 2014; Wiman et al., 2014).

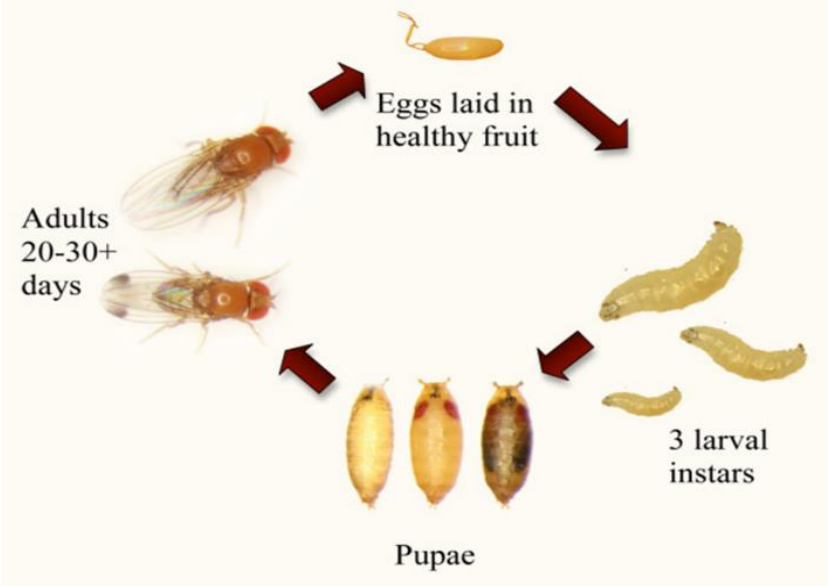


Figure 5: Life cycle of *D. suzukii*. (Photo credit: UMass Extension Fruit program, the center for Agriculture, Food and the Environment)

1.2.2 Tolerance of a wide range of climatic conditions

The ability to reproduce and survive in a wide range of climatic conditions is obviously a relevant factor for insects and especially for invasive pest insects like *D. suzukii*. Limiting temperatures recorded for *D. suzukii* reproduction are between 10 and 32 °C for oviposition and up to 30 °C for male fertility (Sakai, 2005). The peak of activity and development is around 20 to 25 °C (Kanzawa, 1939; Tochen et al., 2014). *D. suzukii* can thus be considered a species with high thermal tolerance, being both heat tolerant (viable *D. suzukii* populations can resist the hot summers in Spain) and cold tolerant (*D. suzukii* is present in cold areas, such as mountain regions in Japan and Alpine areas). Adult *D. suzukii* are particularly more tolerant to cold compared to other drosophilids (Sasaki and Sato, 1995; Mitsui et al., 2010) and mated females in reproductive diapause are considered to be the overwintering stage of *D. suzukii* (Kanzawa, 1939; Mitsui et al., 2010; Walsh et al., 2011). Whether this tolerance is physiological or mediated by behavioural adaptation is still unclear and several authors suggest that *D. suzukii* survival under harsh conditions might be increased by altitudinal migration (Mitsui et al., 2010), acclimatization (Walsh et al., 2011) and/or overwintering in manmade habitats or other sheltered sites (Kimura, 2004).

1.2.3 Wide host range

D. suzukii is able to develop on a very wide range of both cultivated and wild soft-skinned fruits in the native and invaded areas, with berries being the preferred hosts (Table 1). Although laboratory experiments showed a significantly lower oviposition susceptibility and developmental rate of *D. suzukii* on grapes than on berries and cherry (Lee et al., 2011), observations in vineyards in Northern Italy indicated that *Vitis vinifera* can become a field host (with soft skinned varieties being more impacted) (Griffo et al., 2012). This suggest that host preference is heavily dependent upon the local abundance of hosts. The host-choice flexibility of *D. suzukii* is also demonstrated by the ability to develop on tomato under controlled laboratory conditions. However, tomato has not been recorded so far as a host in the field,

even though *D. suzukii* adults have been trapped in tomato crops in France (EPPO website). In addition to cultivated fruits, many wild, ornamental, and uncultivated plants can serve as potentially important hosts (Lee et al., 2015; Klick et al., 2016).

The wide host range *of D. suzukii* represents a pest management constraint in many affected regions. This is not only because *D. suzukii* can cause damage to many species, but also because populations can survive almost everywhere, alternating hosts with different ripening times through the year, both cultivated and wild. Crop plants usually cultivated in high density monoculture, allow rapid and impressive population growth, while wild hosts and ornamental plants may serve as refuges from management treatments, and provide later re-infestation sources and overwintering habitats observed (Klick et al., 2016). Furthermore, the ability to damage thick ripening fruits and the wide host range, allows *D. suzukii* to occupy a wide but at the same time specialized ecological niche. Nevertheless, overlapping niches and possible competition with other drosophilids needs to be investigated.

Table 1: List of *D. suzukii* host plants grouped based on botanical family

Family name	Host plants*	References
Rosaceae	*Fragaria ananassa* (strawberry), *Rubus idaeus* (raspberry), *Rubus fruticosus*, *Rubus laciniatus*, *Rubus armeniacus* and other *Rubus* species and hybrids of the blackberry group, *Rubus ursinus* (marionberry), *Prunus avium* (sweet cherry), *Prunus armeniaca* (apricot), *Prunus persica* (peach), *Prunus domestica* (plum), *Eriobotrya japonica* (loquat)	(Kanzawa, 1939; Bolda et al., 2010; Grassi et al., 2011), (Seljak, 2011; Walsh et al., 2011; Klick et al., 2016; Kenis et al., 2016; Mazzi et al., 2017)
Ericaceae	*Vaccinium* species and hybrids of the blueberry group	(Hampton et al., 2014)
Grossulariaceae	*Ribes* species including the cultivated currants	(Cini et al., 2012)
Moraceae	*Ficus carica* (fig), *Morus* spp. (mulberry)	(Lee et al., 2011; Cini et al., 2012)
Rhamnaceae	*Rhamnus alpina* ssp. fallax, *Rhamnus frangula* (buckthorn)	(Asplen et al., 2015; Kenis et al., 2016)
Cornaceae	*Cornus* spp. (dogwood)	(Kenis et al., 2016; Pelton et al., 2016)
Actinidiaceae	*Actinidia arguta* (hardy kiwi)	(Kinjo et al., 2014)
Ebenaceae	*Diospyros kaki* (persimmon)	(Kanzawa, 1939; Hamby et al., 2014)
Myrtaceae	*Eugenia uniflora* (Surinam cherry)	(Cini et al., 2012; Lee et al., 2015)
Rutaceae	*Murraya paniculata* (orange jasmine)	(Mann et al., 2011; Lee et al., 2015)

Myricaceae	*Myrica rubra* (Chinese bayberry)	(Cini et al., 2012; Asplen et al., 2015)
Caprifoliaceae	*Lonicera spp.* (honeysuckle)	(Lee et al., 2011; Cini et al., 2012)
Elaeagnaceae	*Elaeagnus* spp. (silverberry or oleaster)	(Cini et al., 2012; Kinjo et al., 2012; Asplen et al., 2015),
Adoxaceae	*Sambucus nigra* (black elder)	(Lee et al., 2011; Cini et al., 2012; Lee et al., 2015)
Vitaceae	*Vitis vinifera* (common Grape Vine), *Vitis labrusca* (fox Grape)	(Cini et al., 2012; Van Timmeren et al., 2013)

* Non-exhaustive and tentative host list, since some information is not well documented

1.2.4 Economic impact

Despite its relatively recent detection in Europe, *D. suzukii* has already caused severe yield losses in several small fruit crops grown across southern Europe, such as sweet cherries, strawberries, raspberries, blackberries, and blueberries. Extreme damage has been reported for locations in Northern Italy (Trentino) and in France, with up to 100 % damage reported on caneberries, strawberries, and sweet cherries (Cini et al., 2012; Warlop et al., 2013). The first evaluation of the economic impact in Europe was presented by De Ros et al. (2013), although the study only focused on Trento Province, Italy. It was estimated in the study that 400 ha of soft fruit production areas faced losses of around 500,000 € in 2010, and 3 million € in 2011. While the magnitude of these economic impacts in Trentino can be ascribed to high levels of blueberry production, this estimate is also somewhat conservative in that it did not consider the costs of control strategies and other societal consequences resulting from increased chemical inputs. In France, *D. suzukii* has also been reported on apples and peaches, although without economically significant damage (Warlop et al., 2013). Besides crop loss in the fields, additional economic impact is attributed to the cost-intensive secondary selection of fruits in the storage facilities after harvest, as well as to losses due to the shorter shelf life of fruit containing the eggs of *D. suzukii*. In fact, an expert working group of the European and Mediterranean Plant Protection Organization (EPPO) for *D. suzukii* in 2010 concluded that the potential for economic consequences due to *D. suzukii* incursions in Europe were ‘high’. Nevertheless, the estimated economic impact of *D. suzukii* varies between regions, depending on the climate, vegetation and cropping strategies.

1.2.5 High dispersal potential

D. suzukii has a high dispersal potential (Hauser, 2011; Calabria et al., 2012), which could be confirmed by its rapid spread in invaded countries and its presence on several continents, as well as remote islands (e.g. Hawaii; (Kaneshiro, 1983)). Passive diffusion due to global trade is likely the main cause of the spread of *D. suzukii*, as for many other invasive species

(Westphal et al., 2008). The apparently intact and healthy status (before larval activity) of the fruits infested with *D. suzukii* is likely to compound the problem, as it increases the risk that infestation will remain undetected and thus increases the risk of passive dissemination of *D. suzukii* (Calabria et al., 2012).

1.3 Management strategies for *D. suzukii*

The effective management of *D. suzukii* is a challenge, owing to the wide host range and short generation time. Pesticide applications have been the primary control tactic against *D. suzukii* (Beers et al., 2011; Bruck et al., 2011). Four classes of registered pesticides, spinosyns, organophosphates, pyrethroids and diamides have demonstrated good contact or residual activity, while there is a pressing need to identify more organic alternatives, as there are few effective products available (Beers et al., 2011; Bruck et al., 2011; Andreazza et al., 2017). However, the efficacy of insecticide-based programs could be limited by the abundant non-crop host plants that may act as reservoirs for *D. suzukii*'s reinvasion into the treated commercial crops, resulting in multiple applications per crop season (Van Timmeren and Isaacs, 2013; Pelton et al., 2016). *D. suzukii* is a highly mobile pest, even migrating from low to high altitudes between winter and summer seasons in Japan, likely seeking better host sources or climatic conditions (Mitsui et al., 2010). Immigration from unmanaged hosts may support the persistence of *D. suzukii* in commercial orchards, which would otherwise be eliminated by recurrent insecticide applications (Klick et al., 2016). Host plants in unmanaged habitats could then act as sinks or sources of *D. suzukii* populations in commercial crops (Briem et al., 2016). It is therefore critical to understand the pest's seasonal phenology and factors triggering dispersal and persistence of this pest's populations on a landscape-scale in order to develop reduced risk strategies for highly mobile and polyphagous pests such as *D. suzukii*.

While pesticides have been the focus to provide growers with immediate chemical options, other management practices are critical to developing a sustainable *D. suzukii* integrated pest management (IPM) program. A summary of knowledge and practices used for the IPM of *D. suzukii* around the world, including chemical, cultural, and biological control is provided by Haye et al. (2016). Furthermore, longer-term research is under way on the use of other methods, including mass trapping, semiochemicals, sanitation, landscape management, biological control, post-harvest treatment and biotechnological tools such as RNAi technology for the management of *D. suzukii*.

1.4 Worldwide pest status and geographic spread of *D. suzukii*

D. suzukii was initially described for the first time in Japan in 1916, where it was found to attack cherries, but it is still uncertain whether it is native to this region or possibly introduced (Kanzawa, 1939). *D. suzukii* is also present in the eastern part of China (Peng, 1937), Taiwan (Lin et al., 1977), North and South Korea (Chung, 1955; Kang and Moon, 1968), Pakistan (Muhammad et al., 2005), Myanmar (Toda, 1991), Thailand (Okada, 1976), the Russian Far East (Sidorenko, 1992) and India (Kashmir region, (Parshad and Duggal, 1965)), where it was described as the *D. suzukii* subspecies indicus (Parshad and Paika, 1964). *D. suzukii* is currently spreading in many areas, such as the USA (West and East coast), Canada, Brazil (Deprá et al., 2014), Mexico and Europe (a history of the introduction in North America is reviewed by Hauser (2011)) (Fig. 6). A key feature of its rapid spread was the initial lack of regulation over the spread of any *Drosophila* species.

Figure 6: Worldwide *D. suzukii* distribution map. (Asplen et al., 2015).

D. suzukii is rapidly spreading across Europe. It was first reported in autumn 2008 in Spain (Rasquera Province) (Calabria et al., 2012), despite the recent proposal that southern France was the first propagation center (Cini et al., 2014). Malaise traps deployed in Tuscany (San Giuliano Terme, Pisa, Italy) in 2008 caught *D. suzukii* adults contemporaneously with those deployed in Spain (Raspi et al., 2011). By 2009, *D. suzukii* adults were recorded in traps in other regions of Spain (Bellaterra, near Barcelona), France (Montpellier and Maritimes Alpes) and Italy (Trentino) (Grassi et al., 2009; Calabria et al., 2012). In Trentino, both first oviposition on wild hosts (*Vaccinium, Fragaria* and *Rubus spp*.) and economically important damage on several species of cultivated berries were reported (Grassi et al., 2009; Sarto and Royo, 2011). By 2010-2011, the range of *D. suzukii* was further enlarged. In Italy it was reported in several other regions: Piedmont, Aosta Valley, Lombardy, Veneto, Emilia Romagna, Liguria, Marche and Campania (Süss and Costanzi, 2010; Franchi and Barani, 2011; Pansa et al., 2011; Griffo et al., 2012; Baser et al., 2015; Mazzetto et al., 2015) and in France it was found from Corsica up to Ile de France. Then, many other European countries made their first record: Switzerland (Baroffio and Fischer, 2011; Baroffio et al., 2014), Slovenia (Seljak, 2011), Croatia (Milek et

al., 2011), Portugal (Rota-Stabelli et al., 2013), Austria (Lethmayer, 2011), Germany (Vogt et al., 2012; Vogt, 2014; Briem et al., 2015), Belgium (Mortelmans et al., 2012; Belien et al., 2014), The Netherlands (Helsen et al., 2013), United Kingdom (Eppo, 2012), Hungary (Kiss et al., 2014; Kiss et al., 2016), Poland (Łabanowska and Piotrowski, 2015), Greece (Papachristos et al., 2013), Romania (Chireceanu et al., 2015), Bulgaria (EPPO, 2015), Serbia (Toševski et al., 2014), Bosnia and Herzegovina (Ostojić et al., 2014) and Czech Republic (Březíková et al., 2014). This reflects the current known distribution of *D. suzukii* in Europe.

D. suzikii appears to be spreading rapidly and all of continental Europe is at risk for invasion. It is worthwhile to note that the lack of reports from several areas is probably due to a lack of monitoring rather than to an actual absence of *D. suzukii*. Thus, the history of reports might reflect differences in the sampling effort and/or problems of awareness rather than the true *D. suzukii* distribution. Considering the reports together with the outputs of available degree-day phenological models (Damus, 2009; Coop, 2010) and analysis of *D. suzukii* host plants distribution (EPPO website), it is very likely that *D. suzukii* will spread all over Europe. Ecological simulations indicate that the northern humid areas are more suitable ecosystems compared to the Mediterranean drier environments, especially because desiccation seems to be a limiting factor for drosophilids (Walsh et al., 2011). If the current climate changes are taken into account, even Scandinavian countries cannot be considered exempt from these risks of invasion. On a wider geographic perspective, according to *D. suzukii* biology, the global expansion in regions with climatic conditions spanning from subtropical to continental is highly likely to happen (Walsh et al., 2011). Furthermore, niche shifts, as occurred for other pests (e.g. *Zaprionus indianus* Gupta, (Da Mata et al., 2010)), should not be excluded (Calabria et al., 2012). This suggests that *D. suzukii* could become a global problem for fruit production.

2 RNA interference in insects

RNA interference (RNAi) is a post-transcriptional gene silencing pathway based on sequence-specific suppression of gene expression, which is triggered by double stranded RNA (dsRNA). This dsRNA trigger can be of endogenous origin, in the form of microRNA (miRNA) or it can be exogenous dsRNA, in the form of viruses, transposons or introduced into a cell for research purposes. Since its discovery in the nematode *Caenorhabditis elegans* (Fire et al., 1998), it has rapidly developed as a widely used molecular research tool in a variety of insects including Diptera (Taning et al., 2016b; Abul Khair et al., 2018), Lepidoptera (Vatanparast and Kim, 2017; Yoon et al., 2017), Coleoptera (Knorr et al., 2018; Li et al., 2018), Hemiptera (Taning et al., 2016a; Singh et al., 2018) and Hymenoptera (Costa et al., 2016; Li et al., 2016). RNAi has transformed insect science research because it enables the researcher to suppress a gene of interest and thereby link a phenotype to gene function. For basic research purposes, RNAi offers a route to functional genetics in all insects, including those for which transgene resources do not exist (Bellés, 2010). Additionally, RNAi has enormous potential for applied entomology (Price and Gatehouse, 2008; Xue et al., 2012; Zotti et al., 2017). For example, RNAi can be used for insect pest control by suppressing essential genes leading to reduced fitness and/or mortality (Zotti et al., 2017). Furthermore, by priming the antiviral RNAi response with innocuous viral sequences, beneficial insect species, such as the honey bee (*Apis mellifera*), can be protected from highly pathogenic viral infections (Maori et al., 2009; Hunter et al., 2010). However, the reality does not yet match the envisioned potential of RNAi. Practitioners are increasingly aware that RNAi in insects can be capricious; efficacy varies across insect taxa, among genes, with mode of delivery, and even between different laboratories (Terenius et al., 2011; Christiaens et al., 2014). This implies that the parameters required for a successful RNAi will have to be validated independently for each insect species.

2.1 Molecular mechanism and cellular uptake in insects

2.1.1 Molecular mechanism

In insects, including *Drosophila melanogaster*, three RNA silencing pathways are known to exist which are mediated by small interfering RNA (siRNA), micro RNA (miRNA) and Piwi-interacting RNA (piRNA), respectively (Tomari et al., 2007). The miRNA pathway primarily uses endogenous products transcribed from the cell's genome with a dsRNA structure to regulate developmental processes, while the piRNA pathway is involved in epigenetic and post-transcriptional silencing of retrotransposons in germ line cells. Hence, these two pathways will not be further discussed in detail here. The siRNA pathway is involved to primarily function as a defense response against exogenous dsRNAs and is exploited for RNAi-related studies in insects. The application of RNAi technology for insect pest control is based on the introduction of dsRNA into the insect body to silence a gene of interest, thereby activating the siRNA pathway. Briefly, upon entry into the cell, the exogenous dsRNA is processed into siRNAs, by a ribonuclease III enzyme called Dicer-2. These 21–24 nucleotide duplexes are subsequently incorporated into a multiprotein silencing complex called RNA-induced silencing complex (RISC), where the duplex is unwound. Next, the guide strand of the siRNA guides the RISC and allows Watson-Crick base pairing of the complex to the complementary target mRNA for endonucleolytic degradation of the homologous target mRNA by the Argonaute 2 (AGO2) protein. This then leads to specific post-transcriptional gene silencing (Agrawal et al., 2003) (Fig. 7).

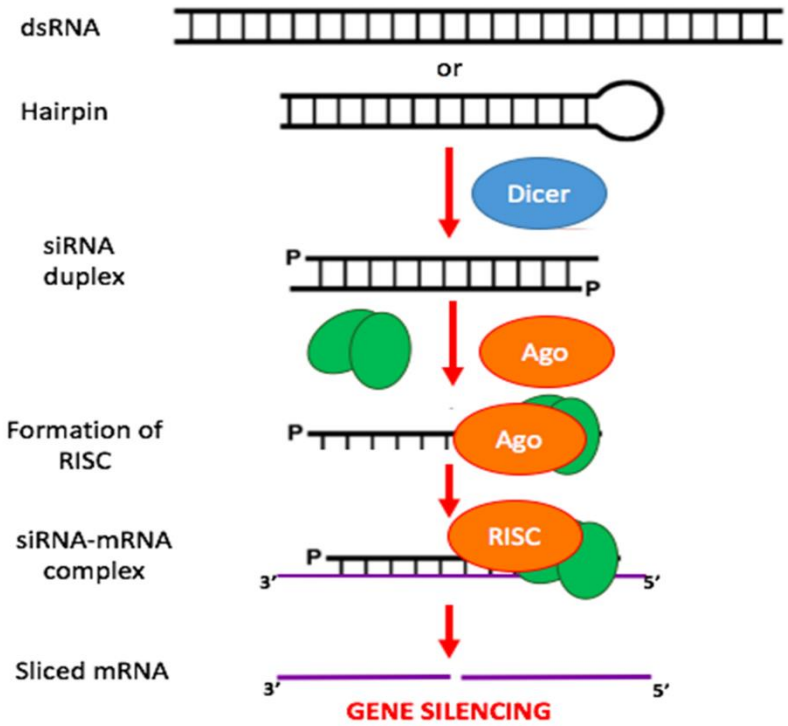


Figure 7: Schematic representation of the siRNA pathway. DsRNA introduced into the cell is recognized and cleaved into smaller pieces (siRNAs) by an RNase III endoribonuclease (Dicer 2). The siRNAs then associate with the RISC complex, unwind and guide the endonucleolytic degradation of the homologous mRNA by an RNase H enzyme (AGO2), which is also part of the RISC complex, leading to gene silencing.

2.1.2 Cellular uptake of dsRNA in insects

The introduction of dsRNA in a single cell and the resulting RNAi silencing effect in that cell is termed cell autonomous RNAi. In this scenario, the site of introduction or production of dsRNAs and its resulting RNAi effects is the same, namely the cytoplasm of the cell. Conversely, in non-cell autonomous RNAi, the site of the RNAi effect is different from the site of dsRNA introduction or production. Non-cell autonomous RNAi can either be environmental RNAi, when the cell takes up dsRNA molecules from the environment, leading to a silencing response in the cells in contact with this environment, or it can be systemic RNAi, when the dsRNA molecules or silencing signal is originating from other cells or tissues (Whangbo and Hunter,

2008; Huvenne and Smagghe, 2010; Baum and Roberts, 2014). In a number of insect species, a robust silencing response has been observed for both environmental and systemic RNAi (Baum et al., 2007; Tomoyasu et al., 2008; Zhu et al., 2011; Bolognesi et al., 2012; Cappelle et al., 2016; Prentice et al., 2017). So far, two pathways have been proposed to explain cellular uptake of dsRNA in insects; trans-membrane channel-mediated uptake mechanism and an alternative endocytosis-mediated uptake mechanism (Saleh et al., 2006; Ulvila et al., 2006; Huvenne and Smagghe, 2010; Xue et al., 2012; Cappelle et al., 2016; Joga et al., 2016).

Trans-membrane channel-mediated uptake mechanism

In the nematode *C. elegans*, environmental and systemic RNAi involves several membrane-associated proteins, labeled SID (**s**ystemic RNA **i**nterference **d**eficient) proteins (Winston et al., 2002; Winston et al., 2007; Jose et al., 2009; McEwan et al., 2012). Two of these proteins, SID-1 and SID-2, are transmembrane proteins. SID-1 is a multispan trans-membrane protein, found in all non-neuronal cells (Winston et al., 2002). It has been reported to be involved in the passive transport of dsRNA among cells in *C. elegans* (Fig. 8a) (Jose et al., 2009). The other transmembrane protein, SID-2 is directly involved in the uptake of ingested dsRNAs from the intestinal lumen and has been reported to be expressed in the intestinal cells of the nematode, *C. elegans* (Winston et al., 2007; McEwan et al., 2012). Therefore, systemic RNAi involves the sequential function of SID-1 and SID-2, where SID-2 mediates the initial uptake of dsRNA directly from the intestinal lumen or environment, while SID-1 functions at secondary step, by exporting the silencing RNAs to other neighbouring cells through SID-1 channels by passive movement and also the import in non-intestinal cells (Whangbo and Hunter, 2008; Jose et al., 2009; Cappelle et al., 2016). In most insects, a homolog of *sid-1* has been discovered, but so far no *sid-2* genes have been found in insect species whose genomes have been sequenced (Tomoyasu et al., 2008; Zha et al., 2011; Cappelle et al., 2016). Moreover, a phylogenetic analysis suggested that *sid-1* like genes in *Tribolium* may not be orthologous to *sid-1*, but rather to the *C. elegans tag-130* gene, which is not associated in systemic RNAi in nematodes

(Tomoyasu et al., 2008). This implies that SID-1 might not be imperative for the uptake of the silencing signal in insects. An alternative dsRNA uptake mechanism might exist in insects, since robust systemic RNAi response was observed in some insects such as *T. castaneum* and mosquitoes even in the absence or upon knockdown of *sid* homologs (Boisson et al., 2006; Tomoyasu et al., 2008).

Another major difference between insects and nematodes is found in the amplification of the RNAi system. In *C. elegans*, secondary siRNAs are created via an RNA-dependent RNA polymerase (RdRp) system, which amplifies and prolongs the silencing effect. In insects, no clear homologs for this RdRp have been discovered yet. However, this does not necessarily mean that insects do not have a similar amplification system, as it can be based on a different enzyme with a similar working mechanism as RdRp, or a completely distinct mechanism that still remains to be unraveled (Joga et al., 2016). In some species, for example some coleopterans, the RNAi effect is so strong and can last so long that it would indeed be likely that such a system is present in these insects. On the other hand, many other insects do require large amounts of dsRNA to elicit a moderate effect, which is often short lived.

Endocytosis-mediated uptake mechanism

The endocytosis-mediated silencing signal uptake mechanism is based on the receptor-mediated endocytosis (Saleh et al., 2006; Ulvila et al., 2006). According to this model, insect cells take up the silencing signal from the environment by receptor-mediated endocytosis and then actively spread the silencing signal through vesicle-mediated intracellular trafficking (Fig. 8b) (Saleh et al., 2006). Previous studies on uptake mechanisms in *D. melanogaster* S2 cells found out that more than 90% of dsRNA uptake depended on SR-CI and Eater receptors. *D. melanogaster* SR–CI shares high similarity with the mammalian class A scavenger receptors, suggesting their possible involvement through receptor-mediated endocytosis dsRNA uptake in *D. melanogaster* (Saleh et al., 2006; Ulvila et al., 2006). The endocytosis-mediated silencing

signal uptake mechanism is considered to be more energy consuming than the trans-membrane channel-mediated uptake mechanism. This could explain why RNAi efficiency is relatively lower in Dipterans such as *Drosophila* (known to possess only the endocytosis-mediated silencing signal uptake mechanism) compared to Coleopterans such as *Leptinotarsa decemlineata* that possess both mechanisms (Saleh et al., 2006; Cappelle et al., 2016). However, the mechanisms by which dsRNAs are transported into the cell are still not clearly understood.

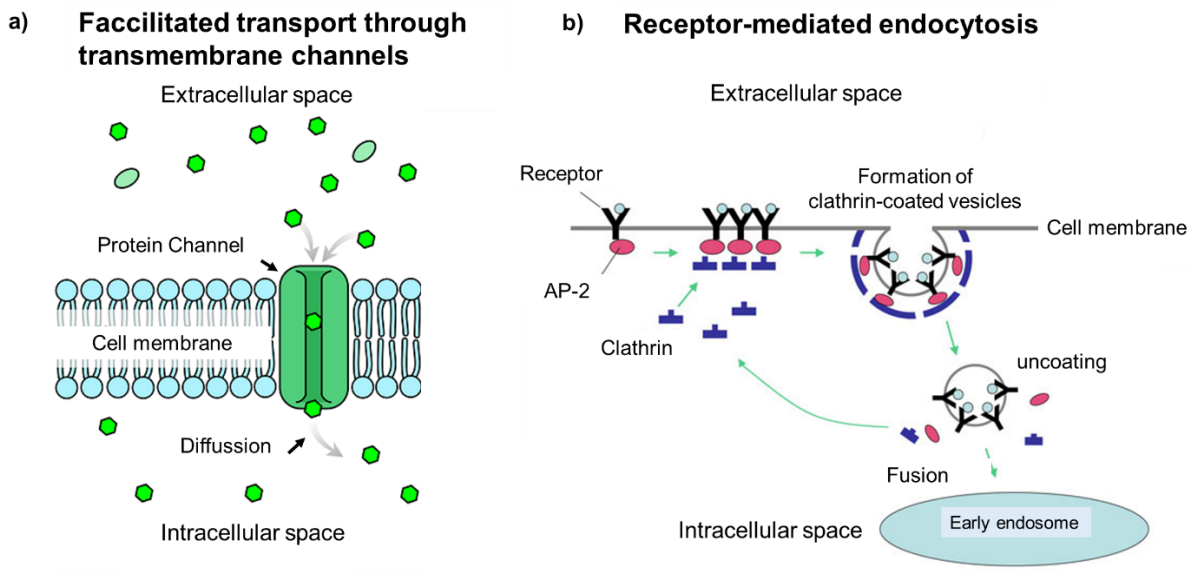


Figure 8: Schematic overview of the two cellular dsRNA uptake mechanisms discussed in this thesis **(a)** The SID-1(-like) proteins form transmembrane channels through the cell membrane through which dsRNA can be taken up passively, **(b)** dsRNA is recognized by a receptor which recruits clathrin to form an invaginated vesicle coated by clathrin molecules. This vesicle is then released into the cytoplasm (Grant and Sato, 2006).

2.2 dsRNA delivery into insects

The overall success of RNAi is dependent on the mode of delivery of dsRNA. Therefore, it remains a major consideration for planning any strategy to use RNAi in insect control. Various

delivery systems have been studied in different groups of organisms. As gene silencing is only limited to cells that take up and process dsRNAs, the main challenge is the selection of the delivery system (Terenius et al., 2011). The main dsRNA delivery methods tested so far include soaking, injection, feeding, and transgenic plants expressing dsRNA.

2.2.1 Delivery through soaking

Soaking of insects in a dsRNA solution is a convenient method for triggering an RNAi response in the insect. However, delivery of dsRNA by this method is applicable only for certain insect cell lines and tissues, as well as for specific insects of developmental stages that readily absorb dsRNA from the solution. The first cell line used for a soaking experiment was the S2 cell line derived from *D. melanogaster* embryos (Clemens et al., 2000). By direct addition of specific dsRNA to the cell growth medium, suppression of specific gene expression was reported (Caplen et al., 2000). Subsequently, soaking became the most commonly used method to induce an RNAi response in several other insect cell lines (Levashina et al., 2001; Sivakumar et al., 2007; Kolliopoulou and Swevers, 2013). However, it was soon found that simply adding dsRNA to the culture medium did not induce RNAi in every insect cell line. For example, no RNAi response was observed when dsRNA was directly added to Hi5 cells (derived from *Trichoplusia ni*) in culture, whereas by adding the dsRNA together with the help of a transfection reagent to improve cellular uptake, a downregulation of the target gene was observed (Beck and Strand, 2005). Transfection-mediated gene silencing has also been reported in Sf21 cells (derived from ovaries of *Spodoptera frugiperda*) (Valdes et al., 2003). This implies that transfection causes a more efficient RNAi response compared to simply soaking, most likely because it facilitates a more efficient uptake of dsRNAs into the cell.

Despite extra barriers such as the insect cuticle, uptake of the dsRNA by whole insect bodies is possible. Direct spray of dsRNA on newly hatched *Ostrinia furnalalis* larvae resulted in considerable mortalities ranging from 40% to even 100% after treatment and this effect

correlated with the downregulation of the target gene expression as verified by qPCR (Wang et al., 2011). It is worthwhile to mention that although spraying dsRNA might be useful to control some pest population in the field, it will not be effective in the control of all insect pests. For example, using a spray method may not affect piercing-sucking pests feeding on phloem sap, or stem borer pests feeding in the plant stems (Li et al., 2015a).

In the context of insect pest control in the field, the US-EPA anticipates four likely categories of dsRNA active ingredients that could be present in sprayable RNAi-based products: (1) “direct control agents”, defined as a dsRNA active ingredient that has direct toxic effects upon the metabolism of the pest resulting in mortality, (2) “resistance repressors”, defined as a dsRNA active ingredient that suppresses genetic resistance to a traditional chemical control, (3) “developmental disruptors”, defined as a dsRNA active ingredient that interferes with the normal development or growth of the target pest such that the target pest or its progeny either die (indirect mortality), are less competitive, or are sterile, and (4) “growth enhancers”, defined here as a dsRNA active ingredient that stimulates, inhibits, or otherwise mimics the activity of a naturally-occurring plant hormone (EPA, 2014).

2.2.2 Delivery through injection

During the initial stages of research on RNAi technology, the direct injection of dsRNA into target tissues and life stages of insects was used as a promising approach for initiating RNAi effects (Dzitoyeva et al., 2001; Bettencourt et al., 2002; Quan et al., 2002). The dsRNA is synthesized according to the target gene in the insect *in vitro* and then injected into the insect haemocoel (Dzitoyeva et al., 2001; Taning et al., 2016b; Prentice et al., 2017). Soon after the genome of *D. melanogaster* was sequenced and published, a study the following year reported the knockdown of the *lacZ* transgene and *GM06434*, the *D. melanogaster* homologue of the *C. elegans nrf* (nose resistant to fluoxetine) gene, by injection of dsRNA targeting these genes in *D. melanogaster* (Dzitoyeva et al., 2001). This was the first case of RNAi successfully

applied in insects through injection. Since then, RNAi has been successfully induced in several other insect species through injection (Gatehouse et al., 2004; Martin et al., 2006; Moriyama et al., 2008; Prentice et al., 2017).

The direct injection of dsRNA into insects has both advantages and disadvantages compared to other methods of dsRNA delivery (Yu et al., 2013). One important advantage is that it allows researchers to get the dsRNA immediately to the tissue of choice or into the haemolymph and thus avoid possible barriers such as the integument or the gut epithelium which could be a problem in feeding or soaking experiments. Another advantage is that the exact amount of dsRNA brought into the insect is known, in contrast to delivery by soaking or in some cases by feeding. The disadvantages of delivery by injection are that the work itself is more delicate and time-consuming than the alternatives, and it also requires quite some optimization. Factors such as optimal volume, needle choice and place of injection are very important and differ greatly between different insect species. Therefore, these factors have to be carefully optimized before starting any experiment. Although microinjection is a good delivery method in functional genomic studies, it is not a suitable method to control insect pests in the field.

2.2.3 Delivery through ingestion

The delivery of dsRNA by oral feeding is less invasive and comparatively simple as compared to injection. Following the first demonstration of oral delivery of dsRNA in *C. elegans* (Timmons and Fire, 1998), this delivery method has been tested in several insect species (Zhu et al., 2011; Xiong et al., 2013; Taning et al., 2016a; Taning et al., 2016b; Prentice et al., 2017). This dsRNA delivery method is comparatively attractive as it is convenient, causes less damage to the insect, requires easier manipulations, and is a more natural method of introducing dsRNA into insect body (Chen et al., 2010). However, it has some limitations. The efficiency of RNAi by ingestion of dsRNA varies between different species possibly due to a different gut environment. Furthermore, it is hard to determine the amount of dsRNA brought inside the

insect through ingestion (Surakasi et al., 2011), which could compromise many investigations. Therefore, optimization of the concentration of dsRNA used to trigger RNAi and the method of feeding is important (Turner et al., 2006). Different dsRNA delivery methods through the feeding approach include: feeding of dsRNA expressed in micro-organisms (Gu et al., 2011; Murphy et al., 2016; Ganbaatar et al., 2017), direct feeding of enzymatically synthesized dsRNA (Cappelle et al., 2016), nanoparticle/liposome-mediated dsRNA feeding (Whyard et al., 2009; Zhang et al., 2015b; Taning et al., 2016b) and feeding of dsRNA expressed in transgenic plants (Xue et al., 2012; Joga et al., 2016; Zotti et al., 2017).

Feeding of enzymatically synthesized dsRNA

DsRNAs can also be synthesized *in vitro* and the dsRNA solution can be easily mixed with food for oral delivery to insects. DsRNAs of a species-specific E-subunit of the *vATPase* gene of *Tribolium castaneum, Acyrthosiphon pisum*, and *Manduca sexta* were synthesized in cell-free condition, dissolved in liquid artificial food or overlaid on the surface of solid foods for these insects. The ingestion of the vATPase dsRNAs led to 50–75% mortality for all three insects (Whyard et al., 2009). In another RNAi study, the expression of β1 integrin subunit (*βSe*1) in the gut epithelium of fourth instar *Spodotera exigua* was transiently suppressed when pieces of cabbage soaked in a dsRNA solution, targeting *βSe*1, was fed to the larvae (Surakasi et al., 2011).

Nanoparticle or liposome-mediated dsRNA feeding

Nanoparticles can be used to reduce dsRNA degradation in the insect gut and to increase the cellular uptake of intact dsRNA. For example, dsRNA encapsulated in chitosan nanoparticles and mixed with diet was used to achieve RNAi in mosquitoes through oral feeding (Zhang et al., 2010; Zhang et al., 2015b). Chitosan nanoparticles are designed by self-accumulation of polycations with dsRNA via electrostatic forces between positive charges of the amino groups in the chitosan and negative charges of the phosphate groups on the backbone of the nucleic

acid. Additionally, chitosan polymers are nontoxic and easily biodegradable (Dass and Choong, 2008). When newly hatched larvae of the RNAi recalcitrant lepidopteran pest, Asian corn borer (*Ostrinia furnacalis*), were fed on four different treatments (diet containing the mixture of fluorescent nanoparticle (FNP) and CHT10-dsRNA; naked CHT10-dsRNA; FNP and GFP-dsRNA; and GFP-dsRNA treatments), only the larvae fed on the diet containing the mixture of FNP and CHT10-dsRNA showed clear RNAi gene silencing (He et al., 2013). The study clearly indicated that the use of nanoparticles to protect dsRNA can be vital in eliciting a strong enough gene knockdown in an RNAi recalcitrant pest.

Liposomes are composed of natural lipids and they are non-toxic and easily biodegradable (Van Rooijen and van Nieuwmegen, 1980). They are already used in drug formulations, where the drugs are enclosed in the liposome and these liposomes are then transferred without quick degradation and minimum side effects to the receivers (Gregoriadis, 1977). Liposome-mediated efficient uptake of dsRNA molecules and silencing response have been reported in insects (Whyard et al., 2009; Taning et al., 2016b). *D. melanogaster* is known to lack the *sid-1* homologous genes and uptake of the dsRNA happens through receptor-mediated endocytosis (Saleh et al., 2006; Ulvila et al., 2006). This appears to be a slow process, which reduces RNAi efficiency and transfection reagents are required to enhance the dsRNA delivery to gut cells to high enough amounts to induce RNAi effects. For example, it was demonstrated that four different *Drosophila* species (*D. melanogaster, Drosophila sechellia, Drosophila yakuba,* and *Drosophila pseudoobscura*) were selectively killed when larvae were fed on γTub23C-dsRNA encapsulated in cationic liposomes, which target the 3′ UTR of the *γ-tubulin* gene (Whyard et al., 2009). None of the drosophilid species exhibited any RNAi-silencing when fed on non-encapsulated dsRNA.

Feeding of dsRNA expressed in micro-organisms

The delivery of dsRNA using micro-organisms can have many advantages, including protection of dsRNA from degradation in the insect gut. Following reports that RNAi can successfully be elicited in *C. elegans* after feeding with a strain of *Escherichia coli* (HT115(DE3)) engineered for dsRNAs production (Kamath et al., 2003), the same strategy was tested in insects. Colorado potato beetles (*L. decemlineata*) fed on the same strain of *E. coli* (HT115(DE3)) but engineered to express dsRNAs targeting five different mRNAs in the beetles led to target gene silencing and mortality in the beetles (Zhu et al., 2011).

Symbionts can also be engineered to deliver dsRNA to their hosts. In symbiont-mediated RNAi, the relationship between the symbiont and its host is exploited to continuously produce dsRNA and induce RNAi in the host (Whitten et al., 2016). It was recently reported that ingested recombinant bacteria successfully competed with the wild-type microflora in the long-lived hematophagous insect *Rhodnius prolixus* and the short-lived polyphagous insect *Frankliniella occidentalis* (Whitten et al., 2016). In the study, the authors engineered dsRNA expression cassettes suitable for an actinobacterium and a proteobacterium from *R. prolixus* and *F. occidentalis*, respectively. The transformation of the RNase III-deficient bacteria with a plasmid producing insect-specific dsRNA allowed stable synthesis of specific dsRNA molecules, penetration in the insect gut cells and initiation of RNAi.

Virus induced gene silencing (VIGS)

VIGS (Lu et al., 2003) may be used to transiently silence target genes of insects or pathogens of host plants. In VIGS, an RNAi inducer sequence is introduced into an engineered virus, leading to the production of dsRNAs in the virus' host cell. This could be exploited in crop protection, for example by infecting the host plant with a virus specifically expressing a dsRNA targeting an essential target gene of a pest or pathogen of the host plant or the pest/pathogen itself (Nandety et al., 2015). VIGS in an insect or pathogen can be induced when it feeds on a

plant infected with the engineered virus (Kumar et al., 2012; Kolliopoulou et al., 2017). The plant virus Tobacco rattle virus (TRV), expressing the antisense fragments for a dsRNA specific to a chewing insect, *M. sexta*, in *Nicotiana attenuata* plants, was reported to specifically silence three midgut-expressed MsCYP RNAs when the larvae were fed on these plants. In addition to the use of plant viruses as insect-specific dsRNA delivery vectors, another potential VIGS approach is the use of recombinant insect viruses, which can infect and replicate in the host insect. After infection, the engineered virus will spread through the insect cells, triggering RNAi which will then result in the production of insect gene-specific siRNAs and subsequently gene silencing (Kolliopoulou et al., 2017). The application of this technology, however, requires the identification of viruses that can naturally infect and replicate in the target insect, as the replication and/or transcription of the viral genome is essential for triggering RNAi (Kolliopoulou et al., 2017). The classical use of viruses to control insects (e.g. baculoviruses to control caterpillars) depends on finding a virulent and highly pathogenic viral strain and is mostly species-specific. However, non-pathogenic viruses could be engineered to express specific dsRNAs/siRNAs and deliver them to insect populations directly.

Feeding of dsRNA expressed in transgenic plants

The delivery method that has at this moment come the closest to a field application is the use of transgenic crops producing pest-specific dsRNAs. Many studies have demonstrated that transgenic plants can be engineered to express hairpin dsRNAs targeting genes from insects to increase their resistance to herbivorous insects (Baum et al., 2007; Mao et al., 2011). Delivery of dsRNAs through transgenic plants has been reported to effectively silence genes in lepidopterans, coleopterans and hemipterans (Baum et al., 2007; Pitino et al., 2011; Zha et al., 2011). Transgenic cotton plants (*Gossypium hirsutum*), expressing dsCYP6AE14, acquired enhanced resistance to cotton bollworm, which indicated the usefulness of RNAi technology in engineering an insect-proof cotton cultivar (Mao et al., 2011).

The US-EPA recently registered a product containing a new and innovative RNAi-based PIP called ‘SmartStax PRO’ that will help US farmers to control the Western corn rootworm (WCR: *Diabrotica virgifera virgifera*). The RNAi-based trait in SmartStax PRO results in the formation of a dsRNA transcript containing a 240-bp fragment of the WCR *snf7* gene. Upon consumption, the plant-produced dsRNA in MON87411 (transgenic event) is specifically recognized by the RNAi machinery of WCR and other closely related WCR species, resulting in down-regulation of the targeted *Snf7* gene, leading to mortality (Head et al., 2017). However, in many cases, the use of transgenic crops is not always realistic. This can be for political or legislative reasons, or because the crop in question is technically difficult or impossible to transform.

Other delivery methods through feeding

RNA can be translocated through the vascular systems of a plant (Melnyk et al., 2011). Hence, topical application of dsRNA on leaves or soil application for root absorption can be exploited to suppress pest infestation (Hunter et al., 2012; de Andrade and Hunter, 2016). *In planta* dsRNA delivery as a non-transgenic delivery approach has been reported to successfully deliver dsRNAs to target insect pests feeding on the plant (Hunter et al., 2012; de Andrade and Hunter, 2016; Taning et al., 2016a). Full-sized citrus and grapevines trees were treated with dsRNA using a foliar spray, root drenching or trunk injections. Two hemipteran insects as well as a xylem-feeding leafhopper took up the dsRNA after feeding on plants previously treated with dsRNA. This demonstrates that plant roots can take up dsRNA molecules and moreover trunk injections enable the delivery of dsRNA through both vessels (xylem and phloem) (Hunter et al., 2012; de Andrade and Hunter, 2016; Andrade and Hunter, 2017). These findings open up a range of possibilities for several difficult to control pest insects such as root-feeding and sap-feeding insects, especially in perennial crops (e.g. fruits such as grapes and citrus), where plant transformation takes years to develop and is costly.

The topical application of pathogen-specific dsRNA for pest resistance in plants also presents an alternative to transgenic RNAi. However, the instability of naked dsRNA sprayed on plants has been a major challenge to its practical application. Nevertheless, it was recently demonstrated that dsRNA could be protected by loading it on designer, non-toxic, degradable, layered double hydroxide (LDH) clay nanosheets, also known as 'BioClay'. Once loaded on LDH, the dsRNA does not wash off, shows sustained release and can be detected on sprayed leaves even 30 days after application. Furthermore, a single spray of dsRNA loaded on LDH afforded virus protection for at least 20 days when sprayed and newly emerged unsprayed leaves were challenged with virus (Mitter et al., 2017). This confirms that dsRNA can move/translocate to untreated parts of the plant. Topically applied dsRNA, however, only produces a temporary modification of the targeted gene transcript and does not alter the genome. This means that it will require multiple applications as a consequence of degradation by sunlight (Li et al., 2015a), by environmental microbes (Dubelman et al., 2014) and in the cells by the natural dsRNA processing mechanism (Palli, 2014). Nevertheless, this innovative delivery method translates nanotechnology developed for delivery of RNAi for human therapeutics to use in crop protection as an environmentally sustainable and easy to adopt topical spray.

In insects, it is largely accepted that the RNAi machinery is triggered by the presence of dsRNA. Long dsRNAs are required for efficient uptake and biological activity in the insect pest (Bolognesi et al., 2012). However, the dsRNAs expressed *in planta* are diced into siRNAs (Kumar et al., 2009), which are afterward ingested by insects. This might lead to a limited RNAi-effect in many insects, due to the preferential uptake of long dsRNA in some species, which is further elaborated on in section 2.3.3 of this Introduction. To overcome this, potato plants were engineered to express dsRNA in organelles lacking RNAi processing machinery, such as chloroplasts (Zhang et al., 2015a). These chloroplasts (plastids) are derived from free-living cyanobacteria, which have no RNAi pathway, leading to an accumulation of dsRNA in

these organelles. When potato plants, producing ACT-dsRNA in their chloroplasts were fed to Colorado potato beetle (CPB) larvae, a 100% larval mortality was observed, whereas no larval mortality was observed when larvae were fed on ACT-dsRNA expressing nuclear transgenic plants (Zhang et al., 2015a).

2.3 Potential challenges for a successful RNAi in insects

Despite the tremendous utility of RNAi as a promising strategy for studying fundamental biological questions and for the control of insect pests, there are still some common challenges that can prevent a successful RNAi experiment. RNAi efficacy remains variable among different genes, tissues, organisms and life stage of insect. For example, the RNAi effect has been found to be more successful in hemocytes of *D. melanogaster* as compared to other tissues when injected with target gene dsRNAs (Miller et al., 2008). The success of RNAi experiments in different species can also be influenced by many biological variables such as variation in the core RNAi machinery, cellular uptake and propagation of the silencing signal and dsRNA degrading enzymes, as well as other differences in genetic backgrounds (Miller et al., 2008; Kitzmann et al., 2013). Most of these challenges can be pacified by considering different experimental factors during the design of the experiment, some which include the mode of delivery, the dose of the dsRNA molecule and the target gene. The following sections describe some potential challenges which can hamper the achievement of a successful RNAi-based pest control strategy.

2.3.1 Degradation of dsRNA by nucleases in the insect gut and haemolymph

Nucleic acid degrading enzymes found inside the gut of insects form an integral part of the digestive cocktail of insects. This implies that ingested dsRNA molecules are potent substrates for these nucleases inside the gut and can easily be degraded by them. The rapid clearance and degradation of circulating dsRNA (Thompson et al., 2012; Christensen et al., 2013) limits the potential for ingested dsRNA to trigger the RNAi mechanism. In general, dsRNA is much

more stable than single-stranded RNA (ssRNA), but it must be rapidly taken up in the cells and digested into siRNA by Dicer-2 (Katoch and Thakur, 2012). The presence of salivary nucleases in the midgut can quickly degrade the ingested dsRNA molecules, which is considered to be an important barrier for RNAi efficiency (Terenius et al., 2011; Liu et al., 2013; Christiaens et al., 2014; Wynant et al., 2014a). For example, the existence of dsRNases in the saliva of *Lygus lineolaris*, a hemipteran insect pest, which performs an extra-oral digestion of plant material prior to the uptake, was found to quickly digest dsRNA (Allen and Walker III, 2012). Furthermore, it has been proven that dsRNA is degraded by dsRNases in the salivary secretions and also in the haemolymph of the pea aphid, *A. pisum* (Christiaens et al., 2014). The presence of dsRNases in the midgut makes the insect recalcitrant to RNAi by oral feeding. The pest desert locust, *Schistocerca gregaria*, expresses dsRNases in the midgut making it recalcitrant to ingested dsRNA (Wynant et al., 2014a), whereas, an effective systemic RNAi-response to injected dsRNA has been reported for this species (Wynant et al., 2012). Similarly, dsRNAs are rapidly degraded in the haemolymph of RNAi recalcitrant lepidopteran *Manduca sexta* (tobacco hornworm), but not in the RNAi sensitive *Blatella germanica* (German cockroach) (Garbutt, 2011; Garbutt et al., 2013). The study proposed that the rate of persistence of dsRNA in insect haemolymph, which is mediated by the action of one or more nucleases, could be an important factor in determining the susceptibility of insect species to RNAi. A hypothesis which was further supported by two studies conducted on two closely related African sweet potato weevils (*Cylas puncticollis* and *Cylas brunneus*; (Christiaens et al., 2016; Prentice et al., 2017)). These studies showed that the more RNAi-sensitive weevil of the two, *Cylas brunneus*, was characterized by a slower enzymatic degradation of dsRNA in the midgut. Furthermore, the Colorado potato beetle, which is far more sensitive to RNAi than any of these two weevils, exhibited an even much longer stability of dsRNA in the midgut.

2.3.2 Amount of dsRNA molecules delivered to the insect

Various factors such as; the insect species, life stage, delivery method, abundance of the target gene transcript, and the spatial and temporal expression profiles of the target gene, all determine the requisite amount of dsRNA molecules for optimal silencing. The optimal concentration has to be determined for every target gene and insect species, in order to induce silencing. It is not true that surpassing that optimal concentration necessarily leads to higher silencing (Meyering-Vos and Müller, 2007; Shakesby et al., 2009). In addition, the introduction of multiple dsRNAs leads to competition in cellular uptake between the dsRNAs, and also saturation of the RNAi machinery can occur (competitive inhibition), resulting in a poor RNAi response (Parrish et al., 2000; Miller et al., 2012). As the miRNA and siRNAi pathways share components, saturation of these components can interfere with the miRNA pathway leading to phenotypes related with the loss of the miRNA function. This restraint might lead to lethality, since miRNAs are important for growth and development (Grimm et al., 2006; Tomoyasu et al., 2008). In the context of crop protection, mortality will not be a problem, however, in the context of functional genomic studies, this can prevent the observation of a phenotype other than mortality, which is linked to the target gene. Furthermore, in another study, it was reported that dsRNA targeting V-ATPase in the Colorado potato beetle (*L. decemlineata*) also caused silencing in the WCR (*D. virgifera virgifera*) in a concentration-dependent manner (Baum et al., 2007).

2.3.3 Length of dsRNA molecules

An important question which arises when designing RNAi experiments is the length of the dsRNA. The minimal length of dsRNA, required to obtain maximum RNAi silencing, varies among insect species. For example, Bolognesi et al. (2012) found that in the WCR, siRNAs specific to the *snf7* gene do not cause any adverse phenotypical effects, while the use of longer (240 bp) dsRNA led to 100% mortality. Further cell-based experiments demonstrated that, at least in this species, the dsRNA requires a certain minimal length of around 60 bp to be taken

up efficiently into the cells. A study in another beetle, *T. castaneum*, demonstrated that 60 and 30 bp dsRNAs induce 70 and 30% of gene knockdown, respectively (Miller et al., 2012). On the other hand, several successful RNAi studies in other insects, using siRNA instead of dsRNA, have been published as well (Upadhyay et al., 2011; Gong et al., 2013; Chen et al., 2014; Mao et al., 2015). This suggests that the dsRNA length dependency of successful RNAi in insects might vary from species to species. Most RNAi studies in insects have reported dsRNA lengths ranging from 140 to 600 nucleotides as successful for RNAi and some even reported success using a dsRNA of 1842 nucleotides (Huvenne and Smagghe, 2010). Additionally, dsRNA longer than 200 nucleotides after dicer cleavage results in many siRNAs, which contributes to the RNAi response as well as prevents resistance which might arise due to polymorphism variation encoded by the nucleotide sequence (de Andrade and Hunter, 2016). The length of the designed dsRNA influences the uptake and silencing efficiency both in the insect cell lines (Saleh et al., 2006), as well as in whole insects (Mao et al., 2007).

2.3.4 Life stage of insects

Some studies in insects suggest that the RNAi effect might be more prominent in the early stages as compared to the late stages. For example, silencing of nitropin 2 (*NP2*) was 42 % in second instars of *Rhodnius prolixus* as compared to none in the fourth instars even though treated with the same concentration per gram body weight of dsRNA (Araujo et al., 2006). In *S. frugiperda*, a higher gene silencing was observed in the fifth instar larvae as compared to adult moths (Griebler et al., 2008). It has been recently shown that when dsRNA is injected at the last larval stage, the RNAi effect can last for many months and could extend to the entire lifespan of the individual (Miller et al., 2012). However, the role of the life stage in RNAi efficiency has not really been studied extensively, so it is difficult to conclude anything at this moment. In parental RNAi, where the female pupae or adults are injected with dsRNA, the effect is seen in the offspring for several months (Bucher et al., 2002). However, this parental RNAi is less efficient when last instar larvae are injected with dsRNA. One of the possible

reasons for this is that the female reproductive organs do not complete formation until the pupal stage. For the oocytes to efficiently uptake dsRNA, they must be formed at the time of dsRNA introduction to the body (Bucher et al., 2002).

2.3.5 Saturation of the RNAi machinery

The siRNA pathway is an anti-viral mechanism in many plants and animals. This implies that viral infections in the insect can interfere with the efficiency of siRNAs by saturating the RNAi core machinery, as demonstrated for vertebrate studies (Kanasty et al., 2012). Additionally, the co-evolution between these viruses and RNAi defense has also led to the development of RNAi-blocking proteins called viral suppressors of RNA silencing (VSRs) in some viruses (Haasnoot et al., 2007). In lepidopterans, many specific viruses have been reported to be present in the haemolymph (Garbutt, 2011), which may be an additional factor explaining why most are recalcitrant to RNAi, besides the harsh conditions in the gut for dsRNA. Furthermore, it was reported that viruses can interfere with the RNAi mechanism in very specific ways, for example, by producing RNAi suppressor genes and/or RNA decoys, and manipulation of host gene expression (Swevers et al., 2013). These all imply that the presence of viruses may influence RNAi efficiency in insects.

2.3.6 Target gene selection for RNAi in insects

The outcome of a successful RNAi gene silencing in the insect mainly depends upon the selection of an ideal target gene. For pest control purposes, the ideal gene target for RNAi should be vital for insect survival, and must be highly expressed. It should not have functional redundancy, so that the silencing effect has a profound effect on the target insect (Li et al., 2013). Terenius et al. (2011) summarized the response of lepidopteran insects to RNAi and found that out of 130 genes, 50 genes showed robust RNAi. Thus, variation in RNAi response for different genes will depend upon the importance of the target gene to insect survival and redundancy of the gene in its function. Longer half-life of protein/mRNA also contributes to the

weak RNAi responses, e.g. silencing of nicotinic acetylcholine receptor subunit (Da6) gave a very limited phenotypical RNAi response in both *D. melanogaster* and *T. castaneum* due to the long half-life of its protein (Rinkevich and Scott, 2013).

2.3.7 Off-target effects and silencing effects in non-target species

One of the major concerns regarding the use of RNAi in insect pest control includes the specificity of action and off-target effects (Auer and Frederick, 2009). For a gene knockdown in insects, dsRNA is designed to target one particular gene, but off-target effects may occur if siRNAs have sequence homology with genes (especially, 3′ untranslated regions of genes) not intended for RNAi targeting (Birmingham et al., 2006; Kulkarni et al., 2006). Of course, in a pest control context, the presence of off-target effects in the target species might not necessarily be a great concern. However, possible adverse effects on non-target organisms should definitely be considered. These could be either due to sequence-dependent mechanisms (e.g. gene silencing of a homologous gene) or due to non-sequence-dependent effects, such as immunostimulation or saturation of the RNAi machinery (Christiaens et al., 2018).

In terms of sequence-dependent effects on non-target organisms, a number of studies have been published, demonstrating that RNAi can be highly species specific but is not always necessarily so. For example, Bolognesi et al. (2012) reported that feeding WCR-specific dsSnf7 to the closely related CPB (both Chrysomelidae), and vice versa, did not lead to any adverse effects in the non-target species. However, the same setup, but using dsRNA targeting the vATPase A or E subunits in these species did lead to mortality in the non-target species (Baum et al., 2007). Other studies have also shown that silencing effects in non-target organisms could arise, even in more distantly related species such as a dipteran fruitfly (*Bactrocera dorsalis*) and a hymenopteran parasitic wasp (*Diachasmimorpha longicaudata*) (Zhu et al., 2012; Chen et al., 2015). Therefore, dsRNA design will be crucial in avoiding these

kinds of unintended effects. Of course, using more evolutionarily conserved target regions for the dsRNA design could allow targeting a group of species, belonging to a certain taxonomical group, rather than targeting only one species.

2.4 Current status of RNAi applications in insect pest management

A proof-of-concept milestone paper demonstrated that a dsRNA construct in a genetically engineered maize (*Zea mays*) plant could incite larval mortality in WCR (Baum et al., 2007). This research awakened researchers to the potential of dsRNA as a new pest control agent through the use of transgenic plants. The Canadian Food Inspection Agency (CFIA) approved in September 2016 the commercialization and release of a new maize event, MON87411, which expresses a Crystal (Cry) gene and a dsRNA containing a 240-bp fragment of the WCR sucrose non-fermenting 7 (*DvSnf7*) gene (Head et al., 2017). This event will be stacked with two other events, expressing two more Cry proteins and a glyphosate resistance gene, in the commercial SmartStax PRO corn plant. In June 2017, the United States Environmental Protection Agency (US-EPA) followed to also approve this event for commercial planting. The *DvSnf7* gene codes for an essential protein in vacuolar sorting and, until now, no insecticide has been developed that interferes with this process. However, as a consequence of the mechanism of RNAi, the Snf7 dsRNA alone takes a long time to effectively kill WCR. Therefore, the event MON87411 was developed in combination with the faster acting Cry genes from *Bacillus thuringiensis* (Bt) which will target both key lepidopteran pests and WCR as well as the *Diabrotica spp.* complex. The main purpose of the combination of these mechanisms (i.e. Bt and RNAi) is to reduce the occurrence of insects resistant to Bt technology, which has already been reported for the WCR. Furthermore, these combinations will also prevent or postpone possible resistance development to RNAi-based products.

A completely different application route is the approach of using sprayable dsRNAs, which, in comparison to chemical pesticides, would be an environmentally friendly alternative, given their expected short half-life in the environment (Dubelman et al., 2014; Fischer et al., 2016; Fischer et al., 2017). While the dsRNA molecules in foliar application were sufficiently stable for at least 28 days to control CPB, under greenhouse conditions, once dried on the leaves, the dsRNA was not leachable (San Miguel and Scott, 2016). An RNAi strategy to control the root weevil *Diaprepes abbreviates* has been developed which uses foliar spraying of dsRNA onto citrus leaves, and this has shown promise in controlling these biting/chewing insects (de Andrade and Hunter, 2016). These experiments using sprayable dsRNA are encouraging and, although more progress is needed on several fronts, the dsRNA-containing end-use products are expected to reach the market in the coming years.

The applicability of sprayable dsRNA relies on the development of cost-effective methods for the mass production and formulation of the dsRNA. Regular molecular biology kits are not suitable to produce large quantities of dsRNA intended for field application. Luckily, the costs of producing dsRNA are decreasing rapidly. For example, the cost to produce 1 g of dsRNA using NTP synthesis has dropped from $12 500 USD in 2008 to $100 USD in 2016, and to $60 USD today (de Andrade and Hunter, 2016; AgroRNA, 2017). *E. coli* (HT115 (DE3)) deficient in the RNase III enzyme that degrades dsRNAs can be used to produce large quantities of dsRNA. The bacteria-produced dsRNA pesticides can be sprayed on crops at any time, because of the facility of producing large amounts of bacteria expressing dsRNAs. This may be considered the most cost-effective method for production of dsRNA (~ $4 USD per 1 g), as for most countries bacteria-produced dsRNA would provide an affordable production system, which could advance RNAi (de Andrade and Hunter, 2016). More recently, a biotechnology company developed a technology called ‘Apse RNA Containers’ (ARCs) that allows the mass production of encapsulated dsRNA using bacteria with costs near $2 USD per 1 g (APSE, 2017). Plasmids coding for naturally occurring proteins such as capsids are co-

transformed with another plasmid coding for dsRNA sequences plus a ‘packing site’. While bacteria are growing in culture, they produce protein subunits that self-assemble around RNA in the cell, including the packing site sequence. After purification of the bacteria, the resulting RNA is environmentally stable and a ready-to-spray product. The amount of dsRNA required per hectare and the required frequency of application are as yet unknown, as a consequence of the lack of field experiments, but the amount required per hectare is predicted to be 2–10 g ($4 USD–$40 USD/ha). In comparison to spinetoram (spinosyn), zeta-cypermethrin (pyrethroid) and malathion (organophosphate) currently used in the management of *D. suzukii*, and assuming that they are applied at their maximum label rates (by conventional producers) and with generic purchase prices observed in 2015, the per-hectare material costs of these chemical insecticide applications are $179.40 USD, $7.22 USD and $29.78 USD, respectively (Farnsworth et al., 2016). However, the predicted amount of dsRNA required may vary greatly, depending on species sensitivity to RNAi, systemic RNAi and the efficiency of the formulation developed for delivery. Nevertheless, the growing interest in the market for dsRNA is expected to result in better production systems, more efficient formulations and lower costs for dsRNAs.

Certainly, insect resistance is always a concern when new control strategies are introduced. It is known that insects have great phenotypic and genetic plasticity, and some individuals in a corn rootworm population could be more or less sensitive to the DvSnf7 dsRNA trait introduced into the new maize event MON87411. An experiment (Chu et al., 2014) using field-collected populations with and without crop rotation resistance demonstrated a differential response to ingested dsRNA treatments. This demonstrated that phenotypic responses to RNAi-based pesticides vary across corn rootworm populations and confirmed that there is a potential for resistance development. Following the principles of IPM would mitigate this selection in order to delay the onset of resistance. The possible mechanisms of resistance to transgenic RNAi crops discussed at a US-EPA Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) meeting (EPA, 2016) are as follows. Changes in dsRNA target sequence was considered

improbable with a 240 bp target such as in the case of DvSnf7 dsRNA, but could occur with shorter targets, and the presence of single nucleotide polymorphisms could reduce the complementarity of the dsRNA with its target. Longer dsRNAs (>200 nt) may be advantageous, as they result in a larger number of siRNAs matching the target mRNA, and therefore could increase the RNAi response and prevent the selection of individuals as a result of the natural genetic variation (Napoli et al., 1990). More likely routes of resistance development could be changes in the kinetics of dsRNA imposed by natural barriers that are important for events such as uptake, (systemic) transport, among others. Also, changes in the RNAi machinery enzymes or components such as reduction of recognition by the RISC complex of siRNA molecules, malfunction of the RISC complex in degrading the target mRNA, reduction in processing by Dicer ribonucleases, or the blocking of systemic spread of the RNAi signal could be considered (Fishilevich et al., 2016). In addition, insects could develop different mechanisms to compensate for the specific gene silencing by increasing transcription rates or up-regulating other genes that can perform the same function as, or a similar function to, the silenced gene. Also, resistance to dietary dsRNA could arise from reduced uptake during insect feeding on modified plants, which may present different olfactory or gustative cues. However, SmartStax PRO maize showed no difference from non-transgenic lines regarding these parameters. During the US-EPA FIFRA meeting, a report was presented showing that about 134 adults of corn rootworm emerged from crop plots treated with SmartStax PRO, but the attendees at the meeting stated that there was no evidence that those individuals were in fact resistant to dsRNA DvSnf7. Some of the mechanisms mentioned above could be potential resistance mechanisms, but to date none has been identified. Adoption of classical refuges, which are areas within the crop without the trait/pesticidal substance, and the combination of multiple insecticidal substances with different modes of action as well as different control strategies delay the evolution of insect resistance to RNAi genetically engineered crops.

Chapter 2

RNAi evidence and screening of potential target genes in *Drosophila suzukii*

1 Introduction

The development of a new biotechnological approach to manage *D. suzukii*, based on RNAi, could provide environmental benefits compared to the chemical alternatives. RNAi is a gene silencing mechanism at the cellular level triggered by dsRNA and is likely to play a significant role in the next generation of insecticides to be developed. In some studies, successful delivery of dsRNA molecules to insects by ingestion resulted in the target gene being silenced, which led to death or affected the viability of the target insect (Andrade and Hunter, 2017; Prentice et al., 2017; Zotti et al., 2017). However, while RNAi has proven to be very promising in the control of some insect species, RNAi efficiency and specificity varies from species to species and from gene to gene. Therefore, a successful RNAi-mediated pest control strategy requires the validation and evaluation of RNAi for each insect species prior to its development as a pest control tool.

In this chapter, we verify whether the RNAi machinery is functional in *D. suzukii* and whether the sensitivity of *D. suzukii* to dsRNA could induce a significant RNAi response suitable for further developing an RNAi-based management strategy for *D. suzukii*. In the first part, we describe the development of a microinjection protocol for *D. suzukii*. While in the second part, we describe the methods and findings of RNAi bioassays designed to both verify the functionality of the RNAi machinery in *D. suzukii* and to screen for potential target genes to be further exploited in the development of an RNA-based control strategy for *D. suzukii*.

2 Materials and methods

2.1 Microinjection protocol for *D. suzukii* adults

Over the years, entomologists have been developing several bio-manipulation techniques to study various insect species. An important embodiment of bio-manipulation involves the

injection of foreign substances into insects for functional genomic studies. Microinjection is a physical delivery system that offers a unique advantage by incorporating precise amounts of test/foreign substances into the insect with ease. However, microinjection has certain limitations as it is a physical assault to the insect and can directly affect the viability of the target organism. An important feature of all current microinjection protocols is that they vary to a great extent by accommodating the unique physical and developmental characteristics of the target insect. Furthermore, the efficiency of microinjection is greatly dependent on several factors such as injection volume, developmental stage of the target host insect, desiccation, injection pressure, the buffer and its pH, compatibility of the foreign substance with the target insects, post injection care, etc. (Lobo et al., 2006). As such, microinjection can achieve high efficacy only by ensuring higher (>90%) survivability (defined as the number of surviving insects after a span of 24 h with normal developmental characteristics, indicating that they have survived the physical assault) of target insects after injection (Ringrose, 2009). Major constraints encountered during manual microinjection include poor reproducibility and inconsistency. However, this can be solved through optimization. Optimizing a microinjection protocol would include; standardization of injection time, volume, buffer temperature and proper developmental stage, which will then result in higher survivability rates in the injected insects. This chapter presents a modified protocol for the injection of *D. suzukii* adults, based on the protocol for injecting *D. melanogaster* (Obadia and Saleh, 2011).

2.1.1 Microinjection setup

Prior to microinjecting *D. suzukii* adults, the necessary equipment was setup as shown in Figure 9. *D. suzukii* adults are small in size (1-3 mm), implying that only very little amounts ranging in the nanoliters can be injected into their haemocoel. As such, a nanoinjector (Femtojet, Invitrogen) was used as a pressure source during injection. Injected volumes are determined by the combination of a certain injection pressure and injection time. The injection pressure and time are in turn dependent on the aperture size at the tip of the injection needle.

Hence, these parameters have to be calibrated for each capillary needle with different tip aperture size. The injections of *D. suzukii* adults were performed under a Leica stereomicroscope with the help of a Narishige micromanipulator. A micromanipulator is a device in which a capillary needle can be placed, permitting the capillary needle to be carefully moved in three dimensions. This is helpful for the precise movements needed during this procedure. The microinjection setup presented here can equally be used for the injection of other insect species.

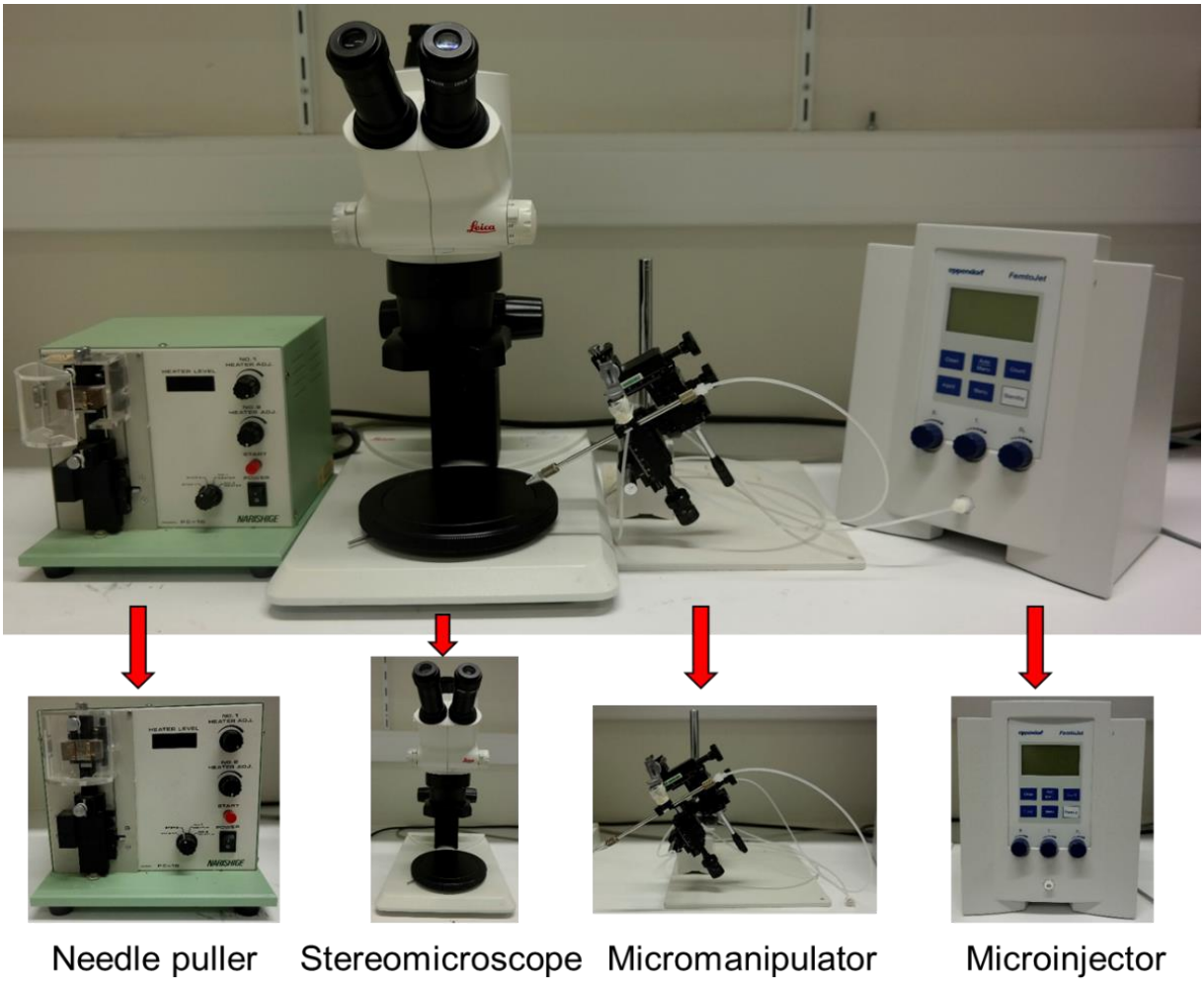


Figure 9: Microinjection setup consisting of a stereomicroscope, a micromanipulator, microinjector and a needle puller (for preparing the glass capillary needles used for injection) (Photo credit: Clauvis NT Taning, Ghent University)

2.1.2 Microcapillaries

An important element in the optimized microinjection protocol for *D. suzukii* adults was the needles used for injection. At first, commercially available glass micro-capillary needles, such as Eppendorf's Femtotip needles, were tested. However, injection of *D. suzukii* adults with these needles proved rather difficult, since the tip of the needle was not really equipped to easily penetrate the tough cuticle of *D. suzukii* adults. The needles either bent or broke while attempting to pierce through the cuticle. This implied that a needle with a much shorter, yet stronger tip was needed for injecting the flies. As such, self-made capillary needles were created, by using glass capillaries (BLAUBRAND IntraMARK, 50 µl) in a two-step pulling procedure, on a Narishige PC-10 needle puller (temperature settings 80 and 83 for step 1 and step 2, respectively). The resulting capillary needle with the shortest tip of the two could be successfully used for injecting *D. suzukii* adults. However, just before use, the tip of the capillary needle was carefully broken with thin tweezers, taking care of making the tip thin enough to avoid major injury to *D. suzukii* adults, but thick enough to ensure easy penetration of the cuticle. One of the main drawbacks of the self-made needles is the lack of uniformity in the aperture size at the tip of the needle compared to commercially available needles, and hence, the need to calibrate the volume for each newly made needle.

2.1.3 Sedation and immobilization

To prevent *D. suzukii* adults from flying away during injection, they had to be immobilized. The use of any type of glue-based method proved difficult given the fragility of these flies, especially the limbs and the wings. This prompted the search for other methods to sedate the flies. A sedation method commonly used to temporally immobilize insects, is the use of cool temperatures, by temporarily placing the insect on ice for a short time. However, in the case of *D. suzukii* adults this proved insufficient. Too long exposure times to cool temperatures led to the death of the fly, while moderate exposure times did not immobilize the flies long enough for injection. Subsequently, the effect of diethyl ether, a common method of anaesthetizing

insects, was tested on *D. suzukii* adults. The sedation effect proved sufficient for the length of the injection procedure and importantly, the procedure was non-toxic for the flies. In order to prevent direct physical contact between the flies and ether, the flies were placed in a 2 ml Eppendorf tube with tiny holes bored in the cap and then introduced into a 50 ml falcon containing cotton soaked with diethyl ether (Fig. 10a). This setup created a sort of 'gas chamber' for the sedation of *D. suzukii* adults for injection. Exposure for 2 min inside the falcon was enough to knock out *D. suzukii* adults for at least 5-9 min, which gave more than enough time to inject the flies. Once sedated, the flies were placed in little gutters on agarose plates that acted as cushion, preventing the flies from being crushed during injection, while also preventing the flies from sliding over the surface while inserting the needle into the body (Fig. 10b).

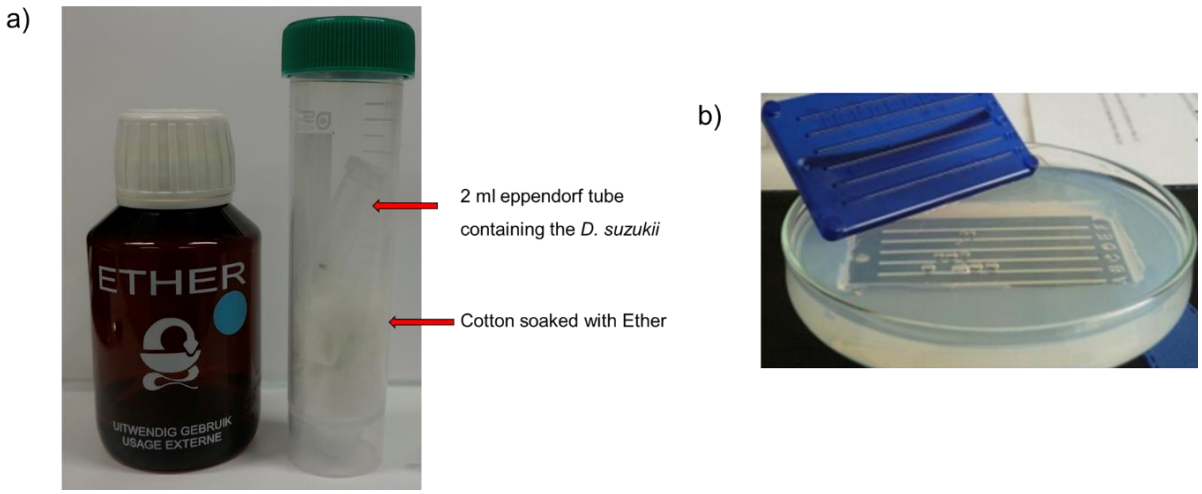


Figure 10: Sedation and injection surface for *D. suzukii* **(a)** Picture of the falcon used for sedation, **(b)** The agarose plate on which the *D. suzukii* adults were placed for injection. Agarose was poured in a glass petri dish and a plastic, grooved dish was floated on the agarose. After the agarose solidified, the plastic floater was taken off. (Photo credit: Clauvis NT Taning, Ghent University)

2.1.4 Needle insertion and volume of injected solution

The shape of the tip of the capillary needle and the angle at which it penetrates the cuticle of *D. suzukii* adults is very important. Not only because it determines the ease by which the

needle penetrates the cuticle, but more importantly, it is vital not to damage anything internally in the *D. suzukii* adults. This is because any form of internal damage, especially to the gut, could have detrimental effects to the fitness of the fly. Theoretically, injecting at a very small angle and as close as possible in a parallel position, along the dorsal or ventral side, would be ideal to avoid internal damage. However, it is practically easier to inject at about 45° through the cuticle. The angle to insert the needle can differ depending on the morphology of each fly individually because some flies are either smaller or have a tougher cuticle than others. However, intrathoracic injection of *D. suzukii* adults between the supra-alar bristles and the presutural bristle, in the intrascutal suture region, should not encounter any resistance (Fig. 11).

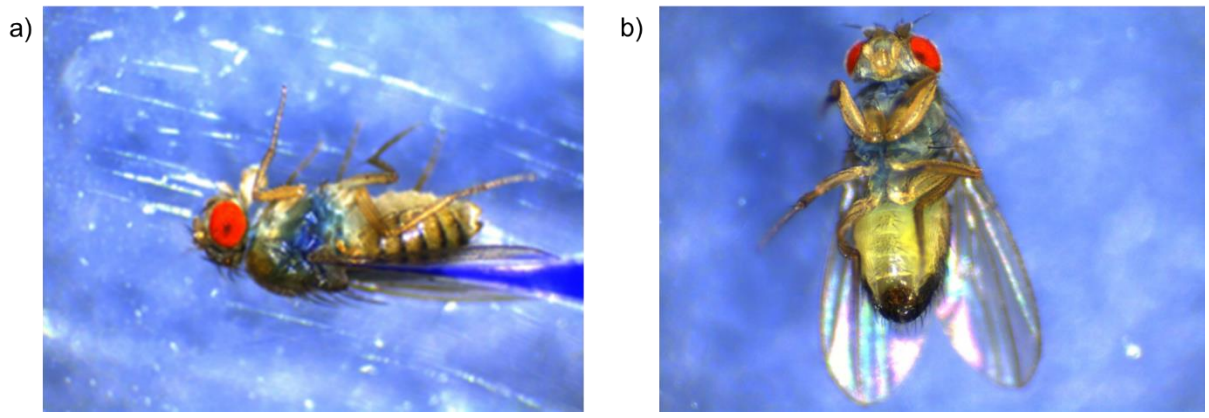


Figure 11: Microinjection of *D. suzukii* adult **(a)** Thoracic injection of blue colorant in the intrascutal suture region, **(b)** Visible blue colorant in the thorax of the fly after injection (Photo credit: Clauvis NT Taning, Ghent University)

Another critical factor which was considered in adapting a microinjection protocol for *D. suzukii* adults was the injected volume. Different volumes of water mixed with a blue colorant dye (bromophenol blue) were injected into *D. suzukii* adults and then survivability was evaluated. Volumes between 50-200 nl did not cause any mortality in *D. suzukii* adults, presenting a wide range of options for injection volumes that could be used in the actual RNAi bioassays. The blue dye used for injection is useful in providing a visual confirmation of a successful injection

(Fig.11). Although the blue dye was not used in the actual RNAi bioassays in this research, it is very useful in optimizing the microinjection of insects and in the training of people who are interested in using this technique.

2.2 RNAi Bioassays

Once the technical aspects of the microinjection procedure were adapted for *D. suzukii* adults, RNAi bioassays were set up to investigate whether RNAi was functional in *D. suzukii*.

2.2.1 Insect culture and life cycle under controlled laboratory conditions

In order to have enough *D. suzukii* adults and larvae for subsequent experiments during the research period, a laboratory culture of *D. suzukii* was initiated. The *D. suzukii* individuals used to initiate the colony were obtained in 2013 from a laboratory culture in France. The flies were originally field collected from infested fruits from the south of France (N: 43.754059 E: 4.4595) and subsequently maintained as a laboratory culture. The flies were reared in 50 ml tubes on an agar-yeast-cornmeal diet slightly modified from Lebreton et al. (2014) (8 g agar–agar, 60 g corn meal, 60 g brewer's yeast, 25 g sucrose, 600 ml distilled water and 2.5 g methyl-4-hydroxybenzoate dissolved in 25 ml of 70% ethanol) at standard laboratory conditions of 25 °C, 65 % relative humidity (RH) and under a 12:12h light: dark photoperiod (incubator model: MIR-154-PE, Panasonic). Prior to the RNAi bioassays, the life cycle of *D. suzukii* was also carefully studied under these controlled laboratory conditions. Three male and female flies were placed in 10 cm diameter Petri dishes for 4 h. An average of 1-3 eggs were laid per Petri dish and the life cycle of the larvae in each Petri dish was followed throughout development (Fig. 12). Adult flies were transferred to 50 ml centrifuge tubes (plugged with cotton) after eclosion and daily observed till death. Understanding the life cycle of *D. suzukii* was vital in the planning of the RNAi bioassays.

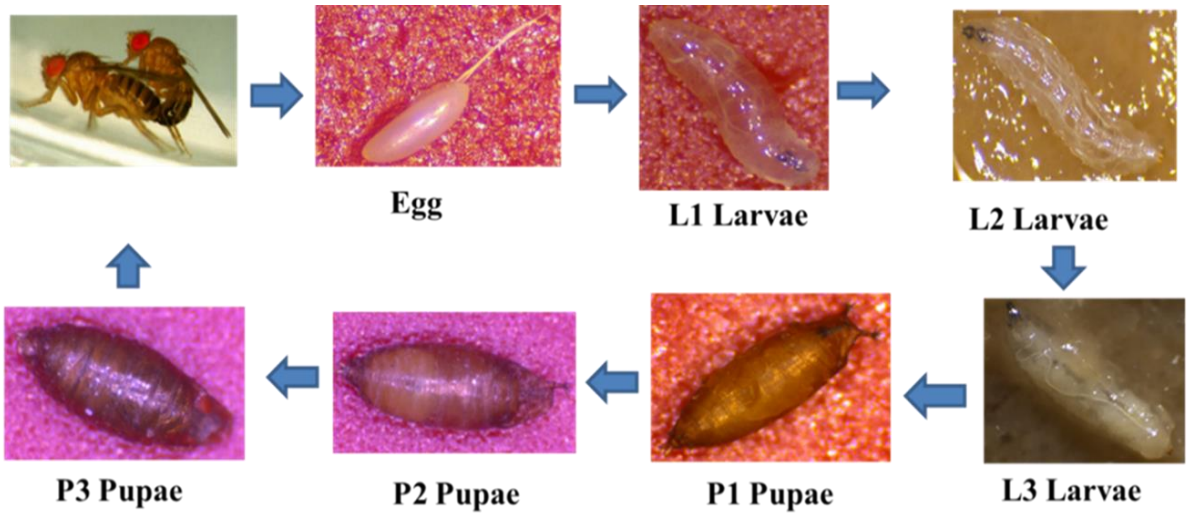


Figure 12: Life cycle of *D. suzukii* under controlled laboratory conditions of 25°C, 65% relative humidity, light intensity of 3LS by a fluorescent lamp and a 12:12h light: dark photoperiod (incubator model: MIR-154-PE, Panasonic). It took an average of 11-14 days from when the eggs were laid till when they emerged as adults. The adults then survived up to 32 days. (Photo credit: Clauvis NT Taning, Ghent University)

2.2.2 *In silico* search and confirmation of RNAi core genes in *D. suzukii*

The siRNA pathway core genes (*R2D2*, *Dicer 2* and *Argonaut*e 2) were searched for in the *D. suzukii* genome database (http://spottedwingflybase.oregonstate.edu/) (Chiu et al., 2013) by BLAST analysis using known query sequences from other insect species. The peptide sequences of the RNAi core genes were aligned with that of other insects and Mega 6.06 was used to generate phylogenetic trees, using the neighbour joining algorithm, examining the evolutionary similarities of the identified genes to that of other known insect species and hence to confirm the correct annotation. Bootstrap analysis with 1,000 replicates for each branch position was used to assess support for nodes in the tree (Felsenstein, 1985).

2.2.3 Target gene selection

To test the functionality of the RNAi machinery in *in vivo* RNAi bioassays, a selection of target genes (Table 2) was made. Some genes were selected on the basis of previous reports on their effectiveness in causing mortality in other insect species (Baum et al., 2007; Zhu et al., 2011) and others on the basis of current running in-house RNAi studies on other insect species. The target gene sequences were retrieved from the *D. suzukii* genome database (http://spottedwingflybase.oregonstate.edu/) (Chiu et al., 2013) by BLAST analysis using known query sequences from other insects. To avoid cross-silencing of other genes, each dsRNA sequence was screened for cross-homologies within the *D. suzukii* genome using BLAST analyses to ensure that there were no shared fragment identities greater than 19 nucleotides in length. The chosen target gene regions were also screened against other genomes by BLAST searches against the Genbank database (NCBI) to check for specificity of the sequences to only the target species, *D. suzukii*.

Table 2: Target genes in *D. suzukii* and their biological and molecular functions

Gene ID	Target gene name	Function	References
DS10_00007684	V/A-type ATP synthase catalytic subunit A (vha68)	ATP binding; proton-transporting ATPase activity, endosomal lumen acidification; imaginal disc growth.	(Baum et al., 2007; Christiaens et al., 2016; Prentice et al., 2017)
DS10_00008303	Adenylate kinase-3 (adk3)	Adenylate kinase activity. ADP biosynthetic process	(Christiaens et al., 2016; Prentice et al., 2017)
DS10_00007384	Gamma-coatomer protein (gamma COP)	Single-organism developmental process; open tracheal system development; cellular component organization or biogenesis; biological regulation; localization; cellular process; system development; gland morphogenesis; cytokinesis; regulation of anatomical structure size; regulation of lipid storage; chitin-based cuticle development; instar larval development; establishment of protein localization.	(Christiaens et al., 2016; Prentice et al., 2017)
DS10_00005153	Delta-coatomer protein (delta COP)	Regulation of tube diameter, open tracheal system; regulation of lipid storage; protein secretion; phagocytosis, engulfment.	(Christiaens et al., 2016; Prentice et al., 2017)

DS10_00004477	Alpha-coatomer protein, isoform A (alpha COP)	Lipid storage regulation. Vesicle-mediated Coat Protein Complex I (COPI) transport complex	(Christiaens et al., 2016; Prentice et al., 2017)
DS10_00012146	lethal (2) NC136 (l(2)NC136)	Nuclear-transcribed mRNA poly (A) tail shortening; neurogenesis.	(Christiaens et al., 2016; Prentice et al., 2017)
DS10_00003341	Mitotics arrest deficient-like 1 (mad1)	Mitotic cell cycle spindle assembly checkpoint; attachment of spindle microtubules to kinetochore involved in mitotic sister chromatid segregation; spindle checkpoint	(Christiaens et al., 2016; Prentice et al., 2017)
DS10_00000374	Ribosomal protein S13 (rps13)	Mitotic spindle elongation; mitotic spindle organization.	(Christiaens et al., 2016; Prentice et al., 2017)
DS10_00009030	ATPase alpha subunit (atpalpha)	Sodium: potassium-exchanging ATPase activity; cation transmembrane transporter activity.	In-house RNAi studies
DS10_00003320	Shrub (shrb) (homolog of snf7)	Neuron projection morphogenesis; dendrite morphogenesis; negative regulation of growth of symbiont in host	(Ramaseshadri et al., 2013; Koči et al., 2014; Christiaens et al., 2016; Prentice et al., 2017)
DS10_00010885	Vacuolar H[+]-ATPase 26kD E subunit (vha26)	Proton-transporting ATPase activity	(Whyard et al., 2009; Zhu et al., 2011; Murphy et al., 2016)

2.2.4 *In vitro* dsRNA synthesis

After the selection of potential target genes, dsRNAs designed to specifically silence these target genes in *D. suzukii* were synthesized using a standard protocol based on the use of commercial kits. Total RNA was extracted from *D. suzukii* adults, using an RNeasy RNA extraction kit (Qiagen), following the manufacturer's instructions. RNA was treated with amplification grade DNase I (Invitrogen) and 1 µg was used to synthesize complementary DNA (cDNA) using a First Strand cDNA Synthesis kit (Invitrogen), following the manufacturer's instructions. DsRNA templates were then produced by PCR using cDNA as template and gene-specific primers with a T7 promoter region (TAATACGACTCACTATAGGGAGA) added to the 5′ end of each primer (Table 3). The primers were designed using the software Primer3, Version 4 (http://bioinfo.ut.ee/primer3-0.4.0/) and the PCR reactions included 0.2 µl of Taq DNA polymerase (Invitrogen), 2 µl of 10x PCR buffer (Invitrogen), 0.6 µl of 10 µM forward primer (Invitrogen), 0.6 µl of 10 µM of reverse primer (Invitrogen), 0.6 µl of 50 mM $MgCl_2$, 0.6 µl of 10 mM dNTPs, 15 µl of nuclease-free water and 0.9 µl of cDNA, in a total volume of 20.5 µl. The amplification conditions were 2 min at 94 °C followed by 33 cycles of 30 s at 94 °C, 30 s at 60 °C and 45 s at 72 °C, and then 10 min at 72 °C and infinity at 10 °C. The resulting PCR products were purified using the CyclePure E.Z.N.A. kit (Omega Bio-Tek) and immediately used *for in vitro* dsRNA synthesis using MEGAscript RNAi kit (Ambion), following the manufacturer's instructions. The synthesized dsRNAs (length 380-500 bp) were eluted with nuclease free water, verified by agarose gel electrophoresis, quantified with a DS-11 spectrophotometer (DeNovix) and stored at –20 °C until use for the RNAi bioassays.

Table 3: Primers used to amplify templates for dsRNA synthesis in injection bioassays

Gene name	dsRNA size	Primer sequence (5'- 3')
V/A-type ATP synthase catalytic subunit A (Vha68)	400bp	F-CGCACCCTGGACAACTACTA R-CCAGCTTGGGATCGATGAAC
Adenylate kinase-3 (adk3)	413bp	F-CCAAGGAGTTTATCGCTGCC R-AACCAGGCCCTTCTCTTTGT
Gamma-coatomer protein (gamma COP)	390bp	F-TCTCTGAAAAGGTGCCCGAG R-TTTCAAAGGATGCATCGCGG
Delta-coatomer protein (delta COP)	381bp	F-GACGTGGCCATTGTCATCC R-ATGGCTAGTGTGACCGAACA
Alpha-coatomer protein, isoform A (alpha COP)	397bp	F-GAATTACAAGACGGCCGCC R-AACTAAACTAAGGGGTCTCGC
lethal (2) NC136 (l(2)NC136)	387bp	F-AATCGAAACCACAGCCACAG R-ATGGGGCTTGGAGATTGACA
Mitotics arrest deficient-like 1 (mad1)	476bp	F-GACTGGAAGGAGGTGACCAA R-AAGTTCTTGTGGCGCTTCAG
Ribosomal protein S13 (rps13)	411bp	F-CAACGTGCCAAAAGTCTCCA R-CTCGACCAGAATCAGACGGA
ATPase alpha subunit (atpalpha)	473bp	F-ACACCCAGACACTCGAGTTT R-CGGAGGGCGAAAGAACAAAA
Shrub (shrb) (homolog of snf7)	394bp	F-CACAAACACTGCCGTCTTGA R-GTTGGACCAGGATAGCAGCT
Vacuolar H[+]-ATPase 26kD E subunit (vha26)	460bp	F-GTGCAGCGATTAGAAGGAGC R-GAGCCTGAAATGATGGTGCA

2.2.5 Microinjection

Using the *in vitro* synthesized dsRNAs, microinjections were performed using 5-day-old *D. suzukii* adult flies. Prior to these bioassays, female and male adult flies were transferred to new diet tubes for 6 h for egg laying. The resulting synchronized mixed population of both male and female flies were then used for the RNAi bioassays. Briefly, *D. suzukii* adults were anesthetized with diethyl ether for 2 min and immobilized in a 1.5 % agarose plate. A volume of 100 nl of the *in vitro* synthesized dsRNA based on the target genes (Table 3) and green fluorescent protein (*gfp*) as control was injected into the haemolymph of the flies. GFP-derived double-stranded RNA was used as control in the RNAi experiments, since its gene does not exists in the genome of *D. suzukii*. This controls for unknown effects which could arise from the simple fact that dsRNA is being introduced into the insect. The injections were performed at a concentration of 3 µg/µl, corresponding to a ratio of 1.36 µg/mg fresh body weight (BW), using a microinjector (FemtoJet, Eppendorf) and needles prepared with glass capillary tubes. A total of 21 adult flies were injected per treatment. After injection, the flies were allowed to recover for 10 min in a horizontally placed 50 ml tube, and then transferred into 50 ml tubes containing 10 ml of diet and incubated at 25 °C and 65% RH. Mortality was evaluated every day for 15 days. The injection assays were repeated twice (total number of injected adults = 42) and for each replication of the experiment, 3 insects per treatment were taken at 48 h post injection, homogenized in RLT buffer (Qiagen) + β-mercaptoethanol for RNA extraction and stored in the buffer at −80 °C until further purification and transcript analysis (total number of injected adults collected for transcript analysis = 6).

2.2.6 Reverse transcription quantitative PCR (RT-qPCR)

Following the RNAi bioassay, transcript analysis was performed to verify if gene silencing had occurred. Total RNA was extracted from the stored homogenized whole insect samples obtained 48 h post treatment, using the RNeasy Mini Kit (Qiagen). From the microinjection RNAi bioassay, two biological replicates of 3 pooled insects were used. After DNase I

treatment (Ambion) to remove residual genomic DNA, the RNA was quantified using a DS-11 spectrophotometer (DeNovix) and verified by 1.5 % agarose gel electrophoresis. Total RNA (1 µg) was reverse transcribed using the SuperScript II kit (Invitrogen) according to manufacturer's instructions. RT-qPCR was performed on a CFX 96™ real-time system and data were analysed using the CFX manager software (Bio-Rad). The reaction included 10 µl of SYBR green Supermix (Bio-Rad), 0.4 µl of 10 µM forward primer (Invitrogen), 0.4 µl of 10 µM of reverse primer (Invitrogen), 8.2 µl of nuclease-free water and 30 ng of cDNA, in a total volume of 20 µl. The amplification conditions were 3 min at 95 °C followed by 39 cycles of 10 s at 95 °C and 30 s at 60 °C. The reactions were set-up in 96-well format Microseal PCR plates (Bio-Rad) in triplicates. Transcript levels of the target genes were normalized to the endogenous reference genes encoding ribosomal protein L32 (*rpl32*) and glyceraldehyde-3-phosphate dehydrogenase 1 (*gapdh1*) by the equation ratio $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001) (See primer sequences in Tables 4 and 5). The target gene transcript levels in the samples from insects treated with the target gene-specific dsRNA were first normalized to the endogenous reference genes and then normalized relative to the level of target gene transcripts in the control samples which were from insects treated with the GFP-specific dsRNA. Appropriate controls, such as no-template controls and no RT control, were also included in the assay.

Table 4: Target genes primer sequences used for quantitative PCR in injection bioassays

Gene name	Product size	Primer sequence (5'- 3')
Shrub (shrb) (homolog of snf7)	100bp	F-AAGACAGCCCACCAGAACAT
		R-GGTTGGAAATGGCATCGGAA
alpha-coatomer protein, isoform A (alpha COP)	104bp	F-GGCAACACACTTCCTGAGGT
		R-CCCTTGGAAACGGACAACAG
lethal (2) NC136 (l(2)NC136)	103bp	F-TGGAGGAGGGGTTGGTGATA
		R-TCGTACGGGTGTTGGCTTAA
ATPase alpha subunit (atpalpha)	118bp	F-TCTGACAAAGCGGCTAGGG
		R-GTGGGTGTGACTGTGGGT
Adenylate kinase-3 (adk3)	104bp	F-GATCGGGTCAAGCATCGTTG
		R-GGTTCTCCCGTGACATCATC
Vacuolar H[+]-ATPase 26kD E subunit (Vha26)	106bp	F-GTGCAGCGATTAGAAGGAGC
		R-CATTTCGGTGTTTCGGCGG
Ribosomal protein S13 (rps13)	99bp	F-TCCAAGATCGGCATCATCCT
		R-CAGACCCACCGACTTCATGA
Mitotics arrest deficient-like 1 (mad1)	99bp	F-GAGGGCTACGATACCGTCAA
		R-GTGCATCATCTCCAGTTCCA
gamma-Coatomer protein (gamma COP)	103bp	F-AGATCGTGTCCTCGATCACC
		R-AAGTGAATACGGGGGTAGGG
delta-Coatomer protein (delta COP)	102bp	F-TTCCGTGTTCGGTCACACTA
		R-ATGGGCGAGGCTTTTAATTC
V/A-type ATP synthase catalytic subunit A (vha68)	101bp	F-GTCCATTGTGGGAGCTGTCT
		R-TCTTGTCCAGTCCCCAGAAC

Table 5: Reference genes primer sequences used in quantitative PCR

Gene ID	Gene name	Product size	Primer sequence (5'- 3')
DS10_00012899	Ribosomal Protein L32 (rpl32)	110bp	F-CCCAAGATCGTGAAGAAGCG R-CGCACTCTGTTGTCGATACC
DS10_00002887	Glyceraldehyde-3-phosphate dehydrogenase 1 (gapdh 1)	101bp	F-GATCACCGTCTTCAGCGAAC R-TGTCGATGGTGGTGAAGACA

2.2.7 Statistical analysis

The data were analysed using the SPSS 21 statistical software. Mortality data of *D. suzukii* larvae and adults from the microinjection assays were analysed using a generalized linear model (GLM) with Log link function and Poisson error distribution. As the data from the RT-qPCR assays could not be fitted to a parametric distribution, a non-parametric analysis was applied, namely bootstrapped median regression. Significant differences between appropriate groups were identified at a significance level of 0.05.

3 Results

3.1 RNAi in *D. suzukii*

Prior to performing RNAi bioassays, the presence of siRNAi core genes such as *r2d2*, *dcr2* and *ago2* were confirmed in the genome of *D. suzukii* through *in silico* sequence analysis. A neighbour joining phylogenetic tree was constructed for the translated amino acid sequences of Dcr2, AGO2 and the dsRNA-binding cofactor R2D2 (Fig. 13). The phylogenetic tree was meant to confirm the identity of the identified genes, by comparing the identified siRNAi core

genes from the *D. suzukii* genome with their homologs in other insect species, hence, confirming the correct annotation of these genes.

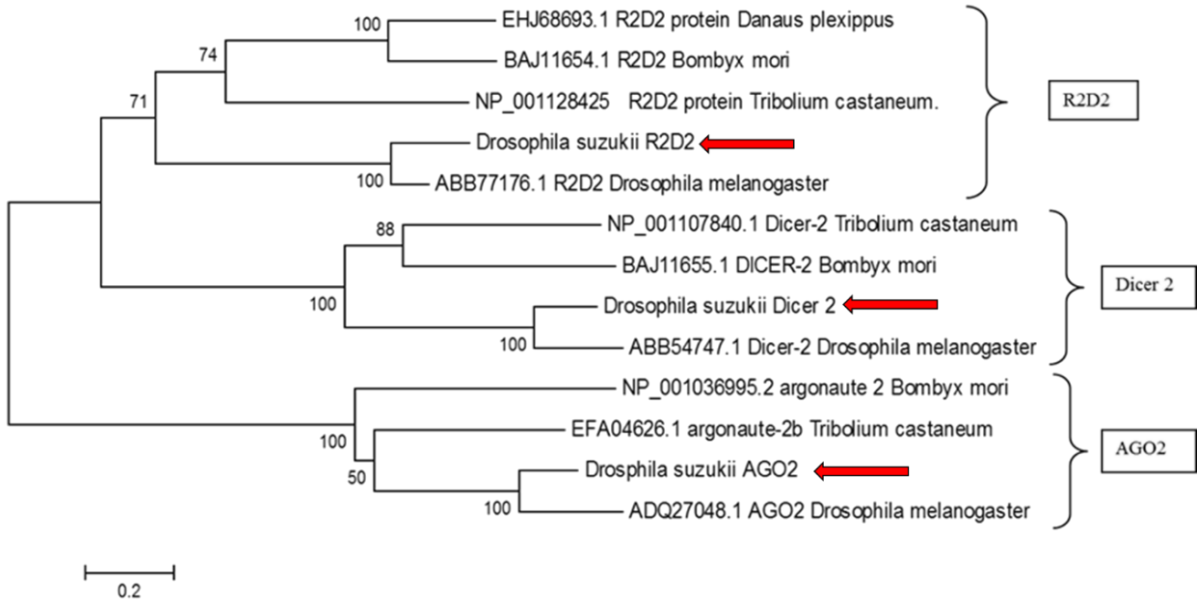


Figure 13: Evolutionary similarity between *D. suzukii* siRNA pathway core genes and that of other insect species. A neighbour joining phylogenetic tree was constructed in MEGA 6, using amino acid sequences of the R2D2, Dcr2 and AGO2 of a selection of insect species: *Tribolium castaneum, Drosophila melanogaster*, *Bombyx mori, Drosophila suzukii, Danaus plexippus.* A bootstrap analysis was performed on 1,000 replicates.

To confirm the functionality of the siRNA pathway, dsRNA targeting *shrb* and *alpha COP* was injected into the haemolymph of the adult flies. These injections resulted in a 94 ± 4% and 51 ± 10% reduction in transcript levels for *shrb* and *alpha COP* in the treated groups, respectively (Fig. 14a). Resulting mortality percentages of 46 ± 9% and 27 ± 8% were observed in both the ds-alpha COP- and ds-Shrb-treated groups, respectively (Fig. 14b).

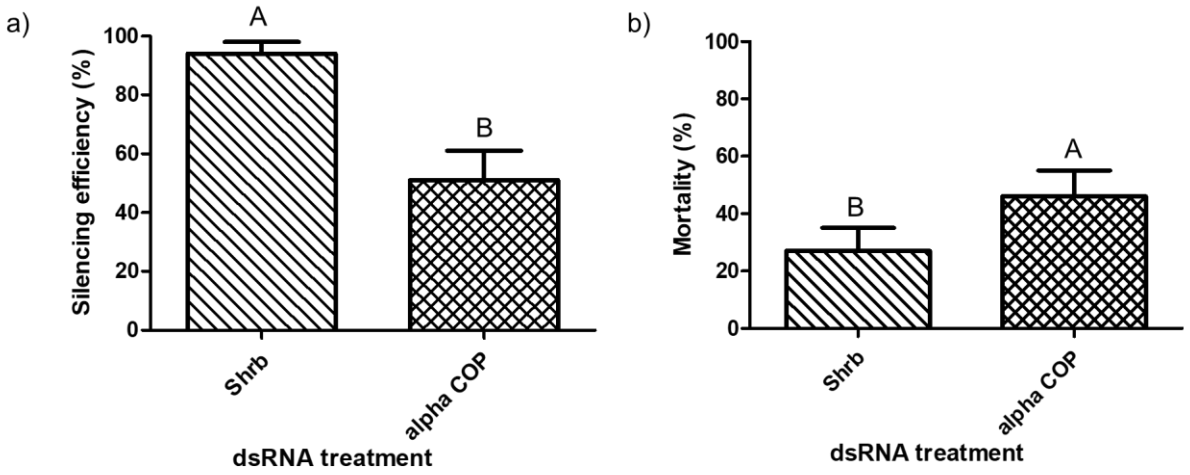


Figure 14: Confirmation of a functional RNAi machinery in *D. suzukii* **(a)** Gene silencing efficiency in adult *D. suzukii* 48 h post injection of gene-specific dsRNA at a concentration of 1.36 µg/mg body weight. The bars in the figure indicate different silencing efficiencies for the different target gene dsRNA treatments relative to the control group (ds-GFP-treated group) with a silencing efficiency of zero. The error bars indicate the standard error of the mean (SEM). All target gene silencing efficiencies differed significantly from the control (Bootstrapped median regression, $p < 0.05$). The silencing efficiency was analysed using two biological replicates of 3 pooled insects (n = 6) per treatment, **(b)** Mortality in *D. suzukii* adult flies following the injection of *in vitro* synthesized gene-specific dsRNA. The bars in the figure indicate the mean ± SEM. Mortality in all the target gene dsRNA-treated groups was significantly different from mortality in the ds-GFP-treated control group (GLM, $p < 0.05$). Mortality was analysed using a total of 36 insects per treatment. Bars labelled with different letters in this figure indicate a significant difference in the mean between the different target gene treatments.

3.2 Selection of potential target genes for RNAi in *D. suzukii*

Once the functionality of the RNAi machinery was confirmed, we sought to screen for additional target genes for subsequent RNAi experiments in *D. suzukii*. DsRNAs targeting nine endogenous *D. suzukii* genes were injected into the haemolymph of the adult flies. These injections resulted in a reduction in transcript level for all target genes (Fig. 15a), however, the

resulting mortality in the treated groups was variable, depending on the gene targeted (Fig. 15b).

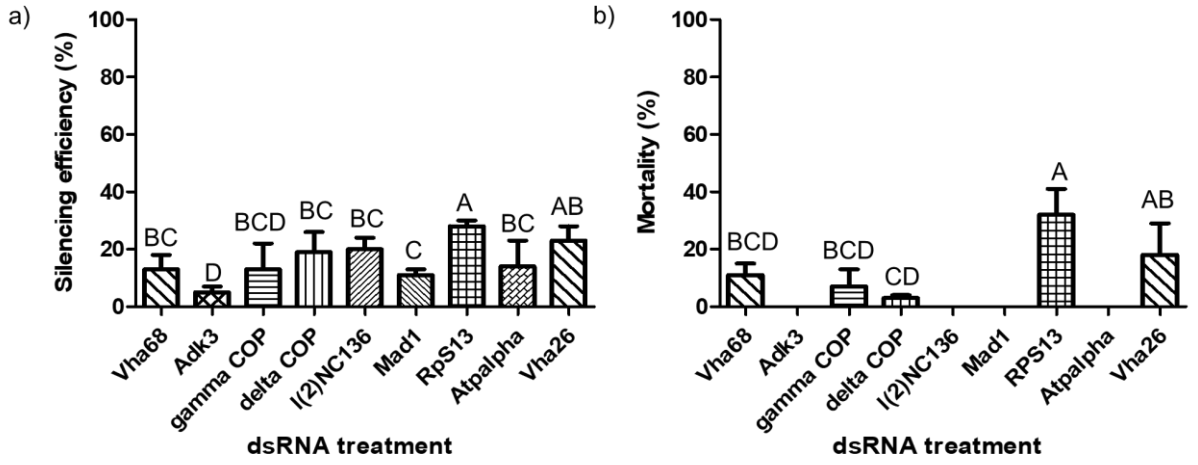


Figure 15: Screening of potential target genes for RNAi in *D. suzukii* **(a)** Gene silencing efficiency in adult *D. suzukii* 48 h post injection of gene-specific dsRNA at a concentration of 1.36 µg/mg body weight. The bars in the figure indicate different silencing efficiencies for the different target gene dsRNA treatments relative to the control group (ds-GFP-treated group) with a silencing efficiency of zero. The error bars indicate the SEM. All target gene silencing efficiencies differed significantly from the control (Bootstrapped median regression, $p < 0.05$). The silencing efficiency was analysed using two biological replicates of 3 pooled insects (n = 6) per treatment, **(b)** Mortality in *D. suzukii* adult flies following the injection of *in vitro* synthesized gene-specific dsRNA. The bars in the figure indicate the mean ± SEM. Mortality in all the target gene dsRNA-treated groups was significantly different from mortality in the ds-GFP-treated control group (GLM, $p < 0.05$). Mortality was analysed using a total of 36 insects per treatment. Bars labelled with different letters in this figure indicate a significant difference in the mean between the different target gene treatments.

No mortality was recorded in the ds-Adk3, ds-I(2)NC136 and ds-Mad1-treated groups following a decrease of 5 ± 2%, 20 ± 4% and 11 ± 2% in their transcript levels, respectively. Furthermore, the ds-Vha68, ds-gamma COP and ds-delta COP- treated groups showed low mortality of less than 16%, following their silencing efficiencies of 13 ± 5%, 13 ± 9% and 19 ±7%, respectively.

Based on these results, these genes were not selected as potential target genes for further development.

4 Discussion

4.1 RNAi is functional in *D. suzukii*

In arthropods, it is well established that the core RNAi pathways consist of Argonaute endonucleases, Dicer enzymes and dsRNA binding proteins (Jinek and Doudna, 2008; Siomi and Siomi, 2009). More specifically, the siRNA pathway is activated by exogenous dsRNA and involves Dcr2/R2D2 and AGO2. The Dcr2/R2D2 complex enables dicing of the long dsRNA into siRNA molecules, while AGO2 is an integral element of the RNA induced silencing complex, eventually leading to the degradation of the target mRNA. The presence of these core genes in the genome of *D. suzukii* stipulated the presence of an siRNAi machinery. However, whether it was functional, still needed to be verified.

Direct injection of dsRNAs has proven to be an effective way to demonstrate RNAi efficacy in numerous insect species (Zhu et al., 2008; Prentice et al., 2017; Wilkins et al., 2017). Our results suggest that RNAi can be induced in *D. suzukii* by microinjection of dsRNA into the haemolymph of the insect. Two essential genes, *shrb* and *alpha COP*, were targeted in the injection assays. The *shrb* gene encodes a class E vacuolar protein sorting (Vps) protein (also known as Vps32), involved in the trafficking of transmembrane proteins to the lysosome via multivesicular bodies (MVBs) (Babst et al., 2002). The *alpha COP* gene encodes the alpha subunit of a non-clathrin-coated vesicular coat protein (COP), which mediates protein transport between the endoplasmic reticulum and Golgi compartments (Gerich et al., 1995).

The first interesting observation is a large difference in silencing efficiency between the two target genes at 48 h after injection. The *shrb* transcript level was reduced by 94% compared

to the control, while the injection of the same amount of dsRNA targeting alpha COP only resulted in a 51% silencing. There are several factors that can explain the difference in silencing between both genes. For example, the gene region and dsRNA fragment itself have been shown to influence silencing efficiency (Wang et al., 2000; Zhou et al., 2002). Other possible factors are differential mRNA transcription rate, leading to a faster recovery of the RNAi silencing effect in the case of *alpha COP* or it is possible that *alpha COP* has many more mRNA copies, so less relative knockdown is observed compared to *shrb*. Although the silencing efficiency in this study was much higher for *shrb* (*snf7* orthologue) than for *alpha COP*, the mortality in the latter was significantly higher. This could indicate that the protein coded for by *shrb* could have a slower turnover than that of *alpha COP*. Alternatively, it could also mean that *shrb* might not be as essential in *D. suzukii*, contrary to its homologue (*snf7*) in a number of beetle species, including *D. virgifera*, where lower levels of silencing by RNAi caused rapid and widespread mortality (Bolognesi et al., 2012; Koči et al., 2014). Based on the results in the injection assays, *shrb* was considered not to be an optimal target gene to induce mortality while *alpha COP* was selected as a target gene for subsequent oral bioassays.

4.2 Potential target genes for RNAi in *D. suzukii*

Two promising target genes were identified, that is, *rps13* and *vha26*. With a silencing efficiency of 28 ± 2%, ds-RPS13 caused 32 ± 9% mortality to the injected insects. This was the most effective target among all nine genes tested. With a silencing efficiency of 23 ± 5%, ds-Vha26 caused the second highest mortality (18 ± 11%) among the nine tested genes. The variation in gene silencing efficiency, for example between ds-shrub and many of the other target genes, is difficult to explain. Several factors have been hypothesized in insects, which could cause differential gene silencing efficiencies between target genes. For example, tissue-specific effects might play a role. Genes which are tissue-specific might result in higher silencing efficiency, for example due to a more efficient uptake of dsRNA in these tissues, a

more active RNAi machinery or even a higher RNA extraction efficiency from these tissues when preparing the qPCR samples. However, since all these target genes are considered essential elements in any cell's functioning, and should therefore be expressed in all cells, it is difficult to say whether this indeed plays a major role. Nonetheless, it is possible that some of these genes do have a higher expression in some tissues than others, which might at least partly explain the differences. Furthermore, the variation observed amongst the targeted genes could also be explained by two hypotheses which are related to interactions between siRNAs and the target mRNA. The first hypothesis is that the local secondary structure of the targeted mRNA may affect the accessibility of the siRNAs generated from the dsRNA (Bohula et al., 2003; Kretschmer-Kazemi Far and Sczakiel, 2003). Bohula et al. (2003) reported that the ability of siRNAs to block the expression of type 1 insulin-like growth factor receptor (*IGF1R*) overexpressed by tumours, correlated with the accessibility of the target sequence within the transcript. Therefore, siRNAs corresponding to weakly hybridizing oligonucleotides caused minor *IGF1R* down-regulation, whereas siRNAs homologous to accessible target regions on the transcripts induced profound sequence-specific *IGF1R* gene silencing. This indicates that secondary structure in the target transcript has a major effect on siRNA efficacy and silencing efficiency. The second hypothesis is that the local protein factor(s) on different regions of the mRNA may cause a positional effect (Holen et al., 2002). That is, the presence of local proteins on the target mRNA may interfere with the binding of siRNAs to the target transcripts, hence, leading to low silencing efficiency. These two hypothesis are not mutually exclusive. However, at present, there is still lack of clear understanding on the mechanisms that determine the gene-silencing efficiency of a given siRNA. Hence, we cannot completely explain the variation and low silencing efficiencies observed in this study at the moment. Nevertheless, our results indicate that by injecting a known amount of dsRNA into adult *D. suzukii*, potential target genes for use in the development of an RNAi-based control method for *D. suzukii* can be screened based on lethality.

5 Conclusion

Based on the results presented in this chapter, we can conclude that RNAi is functional in *D. suzukii* and can be induced through injection of exogenous dsRNA into *D. suzukii*. Furthermore, *alpha COP*, *rps13* and *vha26* are good potential target genes for further optimization in the development of an RNAi-based insecticide for *D. suzukii*, although screening on a larger scale could potentially deliver better target genes. This Chapter also presents a microinjection approach for such screening of potential target genes in *D. suzukii*.

Chapter 3

RNAi induction by oral feeding in *Drosophila suzukii*

Modified from: Taning, C. N. T., Christiaens, O., Berkvens, N., Casteels, H., Maes, M., & Smagghe, G. (2016). Oral RNAi to control *Drosophila suzukii*: laboratory testing against larval and adult stages. Journal of pest science, 89(3), 803-814.

1 Introduction

Having demonstrated that the RNAi machinery is present and functional in *D. suzukii* by microinjection of dsRNA in the haemolymph, the next step in the development of an RNAi-based pest control strategies is examining whether oral delivery of dsRNA is also capable of eliciting a meaningful silencing response. The delivery of dsRNA by oral feeding is comparatively attractive as it is convenient, causes less damage to the insect, is easy to execute, and is a more natural method of introducing dsRNA into the insect body (Chen et al., 2010). However, the efficiency of RNAi by ingestion of dsRNA varies between different species due to many reasons such as; the degradation of dsRNA in the insect body, poor cellular uptake of dsRNA, poor processing of the dsRNA and variation in the expression levels of the RNAi core machinery genes (Terenius et al., 2011; Joga et al., 2016). This implies that RNAi by oral ingestion will have to be evaluated and optimized independently for each insect species. Furthermore, for RNAi to be a useful pest control method in the field, the development of easy and reliable methods for production and delivery of dsRNA will be required. The use of transgenic plants expressing dsRNA has been exploited for the production and delivery of dsRNA (Baum et al., 2007; Mao et al., 2007). However, the major limitation of the transgenic plant approach for RNAi pest management is public hesitancy in accepting transgenic food. Delivering dsRNA by spraying on the crop plants fits well with current insecticide delivery methods and several research groups have explored the possibility of delivering dsRNA to insects using this method (Huvenne and Smagghe, 2010; Joga et al., 2016; Zotti et al., 2017). However, this method will require that large amounts of dsRNA are produced elsewhere and sprayed on the plants. The use of bacteria as biofactories to produce dsRNA could be exploited. In *C. elegans*, an efficient induction of RNAi using bacteria to deliver dsRNA was developed and successfully used for a rapid and effective genome-wide analysis of gene functions (Timmons and Fire, 1998; Kamath et al., 2003). The bacteria-mediated feeding RNAi

is considered as a highly efficacious and cost-effective tool, showing potential in the eventual field control of some insects such as the Colorado potato beetle (Zhu et al., 2011).

In this chapter, we verify whether RNAi can be effectively induced by oral feeding and how we could exploit this to develop an RNAi-based control for *D. suzukii*. In the first part, we describe an oral feeding protocol for laboratory testing of RNAi in *D. suzukii* adults and larvae. In the second part, we discuss the findings of oral RNAi in *D. suzukii* while presenting how bacteria can be engineered to become a micro-factory to produce *D. suzukii* target gene dsRNAs for oral delivery. Furthermore, we report how the use of lipid-based nanoparticles can improve the oral delivery of dsRNAs in *D. suzukii*.

2 Materials and methods

2.1 Development of a feeding assay protocol for *D. suzukii* adults and larvae

Various simulations of oral bioassay setups were performed on *D. suzukii* adults and larvae and the best method for oral feeding was then applied for the actual bioassays with *D. suzukii* adults and larvae. Two main feeding setups were tested; exposure to a known amount of liquid diet and exposure to a known amount of solid diet.

2.1.1 Exposure to liquid diet containing a food dye

This simulation was aimed at verifying whether *D. suzukii* adults and larvae could take up dsRNA from solution within a certain exposure time.

In this simulation, adult *D. suzukii* flies were exposed to 50 µl drops of 5% glucose solution containing bromophenol blue dye for 5 h. The flies were observed every hour to verify if they had fed on the dyed glucose solution within the exposure time. We observed that all flies fed within the second hour after exposure. Dry starving the adult flies in empty 50 ml Falcon tubes

for 1-3 h before the feeding assays caused the flies to feed within the first 30 min after exposure (Fig. 16a and b).

In a similar simulation, larvae were partially submerged in 5% glucose solution containing bromophenol blue dye for 1 h. Larvae were initially wet starved for 1 h by partial submersion in water in a 10 cm Petri dish before the feeding assay simulation. Wet starving prevents desiccation of the larvae, which can lead to mortality. Additionally, partial submersion was important in this simulation to prevent drowning. However, after a 1 h exposure time not all the larvae had fed on the glucose solution (Fig. 16c and d). Based on these results, a different oral bioassay feeding method was setup and tested.

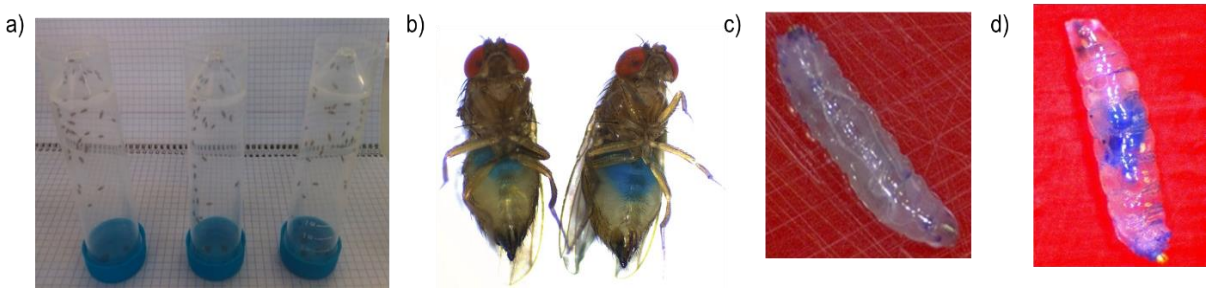


Figure 16: First oral feeding simulation **(a)** *D. suzukii* adult flies exposed to 50 µl drops of 5% glucose solution containing bromophenol blue placed on the cover (blue) of the Falcon tube. Tiny holes bored on the top end of the inverted tubes provide air for respiration, **(b)** Blue dye observed in the abdomen of the flies after feeding on drops of glucose solution containing bromophenol blue, **(c)** Larvae with no visible blue dye in the midgut implying that it did not feed during the exposure time, **(d)** Larvae with blue visible dye in the mid gut indicating that they fed during the exposure time. (Photo credit: Clauvis N T Taning, Ghent University)

2.1.2 Exposure to solid diet containing a food dye

In a second simulation, designed in a way to be applicable to both adults and larvae following the same protocol, *D. suzukii* adults and larvae were exposed to diet containing a bromophenol blue dye for a certain period. The aim of this simulation was to ascertain if, and how fast, the

adults and larvae will feed on their usual corn meal diet within the exposure time. This will let us predict if all the exposed insects will take in dsRNA in the diet during the exposure time when the actual experiment is performed.

D. suzukii adults and larvae were exposed for 5 h to 50 mg of a yeast corn meal diet mixed with 32 µl of bromophenol blue. They were observed every 30 min for food intake. All adults and larvae fed within 30 min after exposure (Fig. 17a-d). This method was then optimized for use in the actual oral bioassays with *D. suzukii*. The only drawback of this setup compared to the previous one is that it requires a lot more dsRNA, making it more costly.

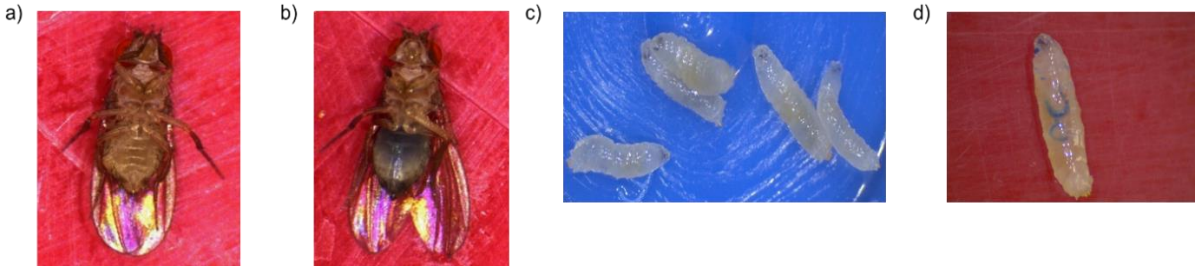


Figure 17: Second oral feeding simulation **(a)** Adult *D. suzukii* prior exposure to diet containing blue dye. No visible dye in the abdomen and flat abdomen from starvation, **(b)** Adult *D. suzukii* post exposure to diet containing blue dye. Visible dye in the swollen abdomen indicating that feeding occurred, **(c)** *D. suzukii* larvae prior exposure to diet containing blue dye. No visible dye in the mid gut, **(d)** *D. suzukii* larvae post exposure to diet containing blue dye. Visible dye in the midgut.

2.2 RNAi bioassays

Once the feeding setup was established, RNAi induction through feeding was evaluated in *D. suzukii* adults and larvae. *Rps13*, *alpha COP* and *vha26* were selected during the screening step in chapter 2 and were used as targets in the oral feeding assays.

2.2.1 DsRNA synthesis

In vitro synthesized dsRNA

In vitro synthesis of dsRNA was performed as described in chapter 2 (section 2.2.4). The primers which were used are shown in Table 6.

Table 6: Primers used to amplify templates for dsRNA synthesis for oral bioassays

Gene name	dsRNA size	Primer sequence (5'- 3')
alpha-coatomer protein, isoform A (alpha COP)	397bp	F-GAATTACAAGACGGCCGCC R-AACTAAACTAAGGGGTCTCGC
Ribosomal protein S13 (rps13)	411bp	F-CAACGTGCCAAAAGTCTCCA R-CTCGACCAGAATCAGACGGA
Vacuolar H[+]-ATPase 26kD E subunit (vha26)	460bp	F-GTGCAGCGATTAGAAGGAGC R-GAGCCTGAAATGATGGTGCA

DsRNA synthesis in engineered bacteria

The dsRNA templates used for cloning were produced by PCR using cDNA and gene-specific primers (Table 6). Restriction enzyme sites for BamH1 (CGGGATCCCG) and EcoR1 (CGGAATTCC) were added to the 5′ end of the forward and the reverse primers, respectively. The PCR products were then cloned into the multiple cloning site (MCS) of the LITMUS 38i plasmid, flanked by two convergent T7 polymerase promoters in opposite orientations (Evans et al., 1995). The 38i plasmid is a small (2814 bp), high copy number *Escherichia coli* plasmid vector designed for efficient transcription of dsRNA. The plasmid vector containing the PCR product was transformed into competent HT115 (DE3) cells, an RNase III-deficient *E. coli* strain with an IPTG-inducible T7 polymerase activity. Single colonies of *E. coli* containing the 38i vector plus insert, cultured on Luria–Bertani (LB) agar plates, were inoculated into 4 ml of LB medium containing 4 µl of ampicillin (100 µg/ml) and 4 µl of tetracycline (12.5 µg/ml), and

cultured overnight at 37 °C while shaking at 200 rpm. The bacterial solution was then diluted 100 times by transferring 250 µl of the overnight culture into 25 ml of fresh LB medium containing ampicillin and tetracycline, and allowed to grow to an OD600 = 0.4. Then, 12 µl of 1 M IPTG was added to the culture medium and incubated for another 3 h at 37 °C on a shaker (200 rpm). The culture medium was centrifuged at 6000 g for 10 min to harvest the bacteria cells, which were then suspended in 0.8 % NaCl solution and stored at -80 °C until the purification of the dsRNA from the bacteria cells. To purify and analyze the dsRNA synthesized in the bacteria, total RNA was extracted from the bacterial cells using TRI reagent (Sigma-Aldrich) with some modifications. After the cell lysis step from the TRI reagent protocol, an extra step where single-stranded RNA was removed by incubating the lysate with 5 µl RNAse A (1000U/µl) and 25 µl of 10X RNase A buffer (4 M NaCl, 0.1 M Tris–HCl) at 37 °C for 25 min was added. After purification, the dsRNA pellets were re-suspended in 25 µl nuclease-free water, and the concentration of the dsRNA was quantified using a DS-11 spectrophotometer (DeNovix). The synthesized dsRNA was also evaluated by loading the suspension onto a 1.5 % agarose/TBE gel, stained with ethidium bromide and photographed to determine integrity and estimate the quantity relative to the standard marker.

2.2.1 Oral feeding bioassays

Second-instar larvae and 4-days-old adult flies were starved for 3 h before the initiation of feeding assay. While the adult flies were dry starved (no water and no food) in 50 ml Falcon tubes, the larvae were partially submerged in water (in 10 cm Petri dish) to prevent desiccation and mortality. The dsRNAs used for the bioassays were encapsulated in liposomes with the intention of facilitating uptake in the gut.

First, RNAi was evaluated by feeding *in vitro* synthesized dsRNA to *D. suzukii* larvae and adults. This was done by placing the insects for 5 h on 50 mg of diet mixed with 32 µg of the *in vitro* synthesized gene-specific dsRNA encapsulated with Lipofectamine 2000 (Invitrogen)

in a mixture of buffered sucrose (20 % sucrose, 10 mM Tris, pH 7.5) and 0.05 mM spermidine. For all assays where Lipofectamine 2000 was used, a 1:1 mixture with dsRNA was prepared as follows: 8 µl of dsRNA (4 µg/µl) was mixed with 7 µl of buffered sucrose (20 % sucrose, 10 mM Tris pH 7.5) containing 0.05 mM spermidine and 1 µl of Lipofectamine 2000. The mixture was incubated at room temperature for 5 min and then mixed with 50 mg of diet.

Secondly, RNAi was evaluated using dsRNA synthesized in and purified from bacteria. Similar to the feeding assay using *in vitro* synthesized dsRNA, the insects were exposed for 5 h to 50 mg of diet mixed with 32 µg of purified dsRNA from bacteria encapsulated with Lipofectamine 2000 (Invitrogen) in a mixture of buffered sucrose (20 % sucrose, 10 mM Tris, pH 7.5) and 0.05 mM spermidine.

Feeding assays with a combination of 2 gene-specific dsRNAs, each targeting a separate gene, were also evaluated in *D. suzukii* larvae and adults. This was done by exposing the insects for 5 h on 50 mg of diet mixed with 32 µg of each of the gene-specific dsRNAs encapsulated with Lipofectamine 2000 (Invitrogen) in a mixture of buffered sucrose (20 % sucrose, 10 mM Tris, pH 7.5) and 0.05 mM spermidine. Both the *in vitro* synthesized and bacterially produced and purified dsRNA were evaluated in these assays.

For all the experimental groups, both the larvae and adults were transferred to fresh diet without dsRNA after the 5 h exposure period. A total of 32 insects were used for each of the treatments with the gene-specific dsRNA and the assays were repeated 3 times (total number of insects per treatment = 96). Mortality was recorded daily for 15 days and for each repetition of the experiment, 2 insects per treatment were collected 48 h post treatment, homogenized in RLT buffer + β-mercaptoethanol for RNA extraction and stored in the buffer at −80 °C until further purification and transcript analysis (total number of insects per treatment collected for transcript analysis = 6). Transcript and statistical analysis were performed in the same way as

described in chapter 2 (section 2.2.6 and 2.2.7, respectively). The primers used for transcript analysis are listed in Table 7 and 8.

Table 7: Target genes primer sequences used for quantitative PCR for oral bioassays

Gene name	Product size	Primer sequence (5'- 3')
alpha-coatomer protein, isoform A (alpha COP)	104bp	F-GGCAACACACTTCCTGAGGT
		R-CCCTTGGAAACGGACAACAG
Ribosomal protein S13 (rps13)	99bp	F-TCCAAGATCGGCATCATCCT
		R-CAGACCCACCGACTTCATGA
Vacuolar H[+]-ATPase 26kD E subunit (vha26)	106bp	F-GTGCAGCGATTAGAAGGAGC
		R-CATTTCGGTGTTTCGGCGG

Table 8: Reference genes primer sequences used in quantitative PCR for oral bioassays

Gene name	Product size	Primer sequence (5'- 3')
Ribosomal Protein L32 (rpl32)	110bp	F-CCCAAGATCGTGAAGAAGCG
		R-CGCACTCTGTTGTCGATACC
Glyceraldehyde-3-phosphate dehydrogenase 1 (gapdh 1)	101bp	F-GATCACCGTCTTCAGCGAAC
		R-TGTCGATGGTGGTGAAGACA

3 Results

3.1 RNAi effects in *D. suzukii* following feeding with naked dsRNA

To verify whether RNAi could be induced in *D. suzukii* adults and larvae through the oral route, dsRNA was administered via feeding by mixing dsRNA with the artificial diet of the insects. Directly feeding *in vitro* synthesized *D. suzukii* target gene dsRNAs to the adults and larvae

did not result in gene silencing and mortality. Similarly, when bacteria expressing the dsRNAs were directly fed to the adults and larvae, no gene silencing and mortality was observed. As such, *in vitro* synthesized dsRNAs encapsulated in a liposome or bacterially synthesized dsRNAs extracted and encapsulated in liposomes were used in all subsequent feeding experiments.

3.2 RNAi effects in *D. suzukii* following feeding with dsRNA encapsulated in liposomes

Upon feeding *in vitro* synthesized gene-specific dsRNA encapsulated in a liposome to the larvae, significant silencing of *rps13* (32 ± 3%), *alpha COP* (34 ± 2%) and *vha26* (42 ± 2%), was observed in the ds-RPS13-, ds-alpha COP- and ds-Vha26-treated groups, respectively (Fig. 18a). Figure 18b shows the resulting mortality from silencing the targeted genes in the larvae. The highest mortality of 42 ± 7% was observed in the ds-Vha26-treated group, while the ds-RPS13- and ds-alpha COP-treated group resulted in 32 ± 7% and 22 ± 6% mortality, respectively. The mortality between the ds-Vha26 and ds-alpha COP group was found to be significantly different *(p* = 0.03). However, no significant difference was found between the ds-RPS13 treatment and the other treatments (Fig. 18b).

The oral treatment of adults with the gene-specific dsRNAs (ds-RPS13, ds-alpha COP and ds-Vha26) also resulted in gene silencing and mortality. However, a much lower transcript silencing (19–24%) and mortality (10–23%) was observed in the adults (Fig. 18c and d).

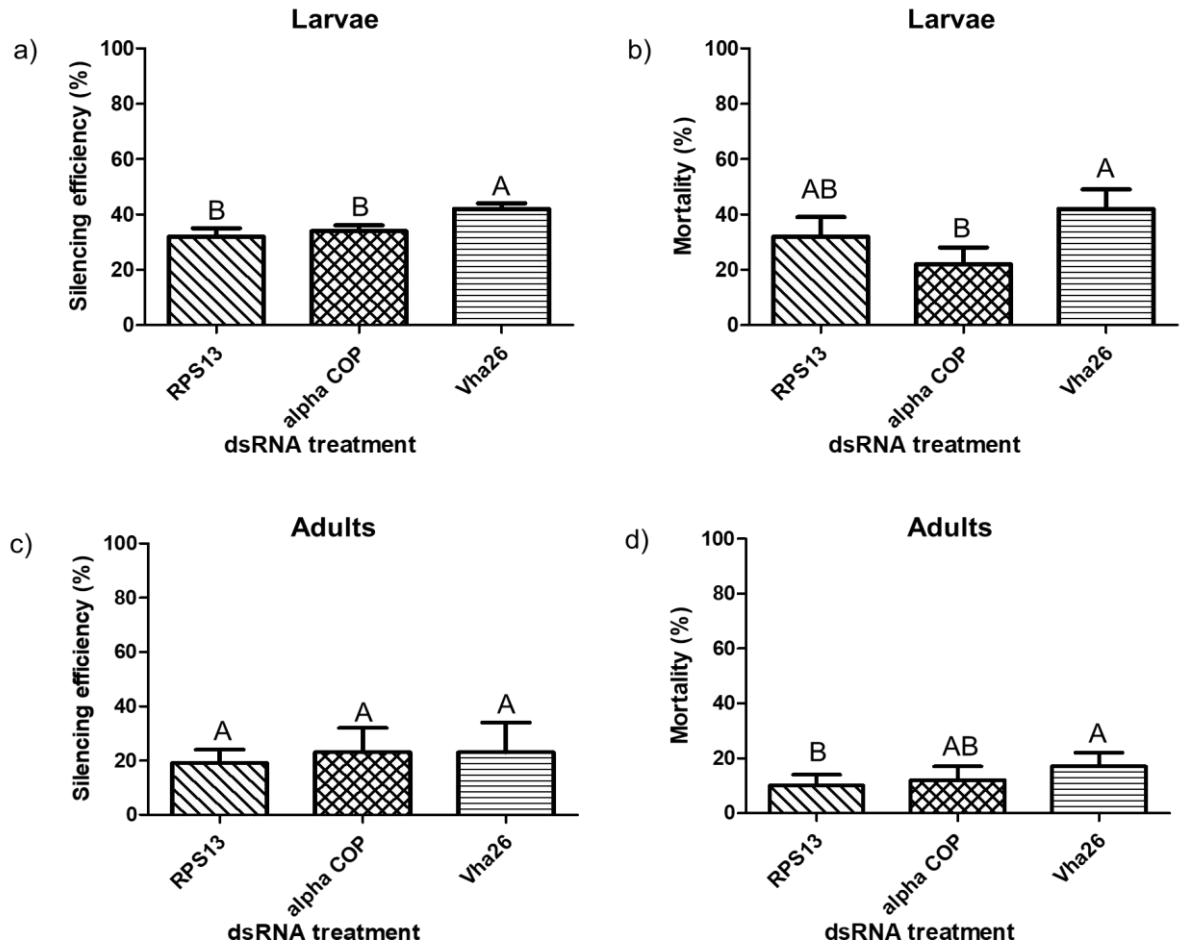
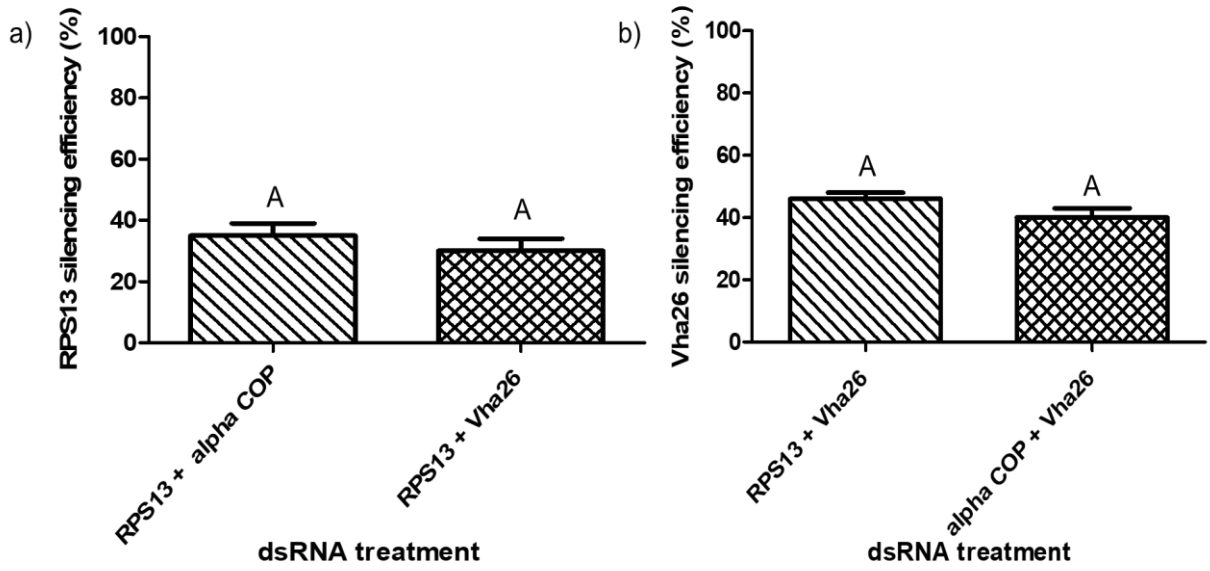


Figure 18: RNAi effects in *D. suzukii* adults and larvae following oral treatment with *in vitro* synthesized dsRNA targeting a single gene; **a)** and **c)** Gene silencing efficiency in *D. suzukii* larvae and adults, respectively, 48 h post feeding with dsRNA. The bars in the figure indicate different silencing efficiencies ± SEM for the different target gene dsRNA treatments relative to the control group (ds-GFP-treated group) with a silencing efficiency of zero. All target gene silencing efficiencies differed significantly from the control (Bootstrapped median regression, $p < 0.05$) and bars labelled with different letters indicate a significant difference in mean between target gene treatments. The silencing efficiency was analysed using three biological replicates of 2 pooled insects (n = 6) per treatment, **b)** and **d)** Mortality in *D. suzukii* larvae and adults respectively. The bars in the figure indicate the mean mortality ± SEM for the different dsRNA treatments targeting single genes (*rps13*, *alpha COP* and *vha26)* in one dsRNA treatment. Mortality was analysed using a total of 90 insects per treatment. Bars labelled with different letters indicate a significant difference in mean mortality rates between treatments (GLM, $p < 0.05$). No mortality was observed in the ds-GFP-treated control group.

3.3 RNAi effects in *D. suzukii* following feeding with combinations of dsRNAs targeting different genes

In a second set of experiments carried out together with the single-target dsRNA treatment assays, combinations of the different dsRNAs were administered orally to *D. suzukii* larvae and adults. The objective was to investigate whether high mortality percentages could be obtained in *D. suzukii* groups treated with two dsRNAs targeting two different target genes in the same insect. Figure 19a–c shows the transcript silencing of *rps13* (30–35%), *alpha COP* (36–38%) and *vha26* (40–46%) when a combination of two dsRNAs targeting two different genes was orally administered to the larvae. The combination of dsRNA specific for *rps13* and *alpha COP* resulted in 40 ± 7% mortality, the combination of ds-RPS13 and ds-Vha26 caused 44 ± 7% mortality and the combination of ds-alpha COP and ds-Vha26 caused 50 ± 7% mortality. No significant difference in mortality was found between the three treatments (Fig. 19d).



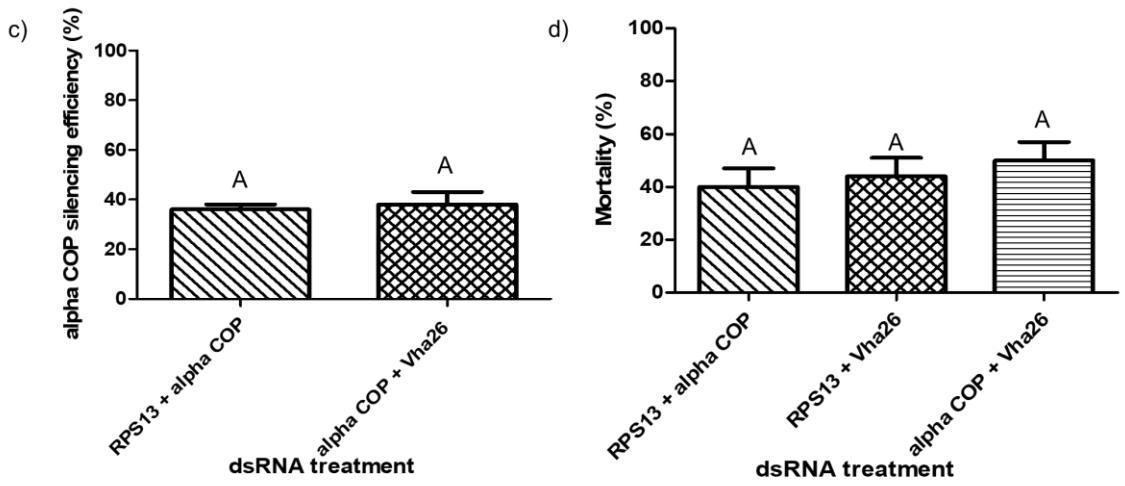


Figure 19: RNAi effects in *D. suzukii* larvae following oral treatment with *in vitro* synthesized gene-specific dsRNA targeting two genes in a single treatment; **a)-c)** Gene silencing efficiency in *D. suzukii* larvae at 48 h post feeding with dsRNAs. The bars in the figure indicate different silencing efficiencies ± SEM for the different target gene dsRNA treatments relative to the control group (ds-GFP-treated group) with a silencing efficiency of zero. All target gene silencing efficiencies differed significantly from the control (Bootstrapped median regression, $p < 0.05$) and bars labelled with different letters indicate a significant difference in mean between target gene treatments. The silencing efficiency was analysed using three biological replicates of 2 pooled insects (n = 6) per treatment, **d)** Mortality in *D. suzukii* larvae. The bars in the figure indicate the mean mortality ± SEM for the different dsRNA treatments targeting two genes (*rps13* + *alpha COP*, *rps13* + *vha26* and *alpha COP* + *vha26*) in one dsRNA treatment. Mortality was analysed using a total of 90 insects per treatment. Bars labelled with different letters indicate a significant difference in mean mortality rates (GLM, $p < 0.05$). No mortality was observed in the ds-GFP-treated control group

The mean silencing efficiencies in adult *D. suzukii* for the different target genes were *rps13* (19–21%), *alpha COP* (20–21%) and *vha26* (21–22%) (Fig. 20a-c), and the resulting mortality for the different combination treatments were ds-RPS13+ds-alpha COP (15–21%), ds-RPS13+ds-Vha26 (17–25%), and ds-alpha COP+ds-Vha26 (17–25%), respectively. No significant difference in mortality was observed between the different treatments (Fig. 20d).

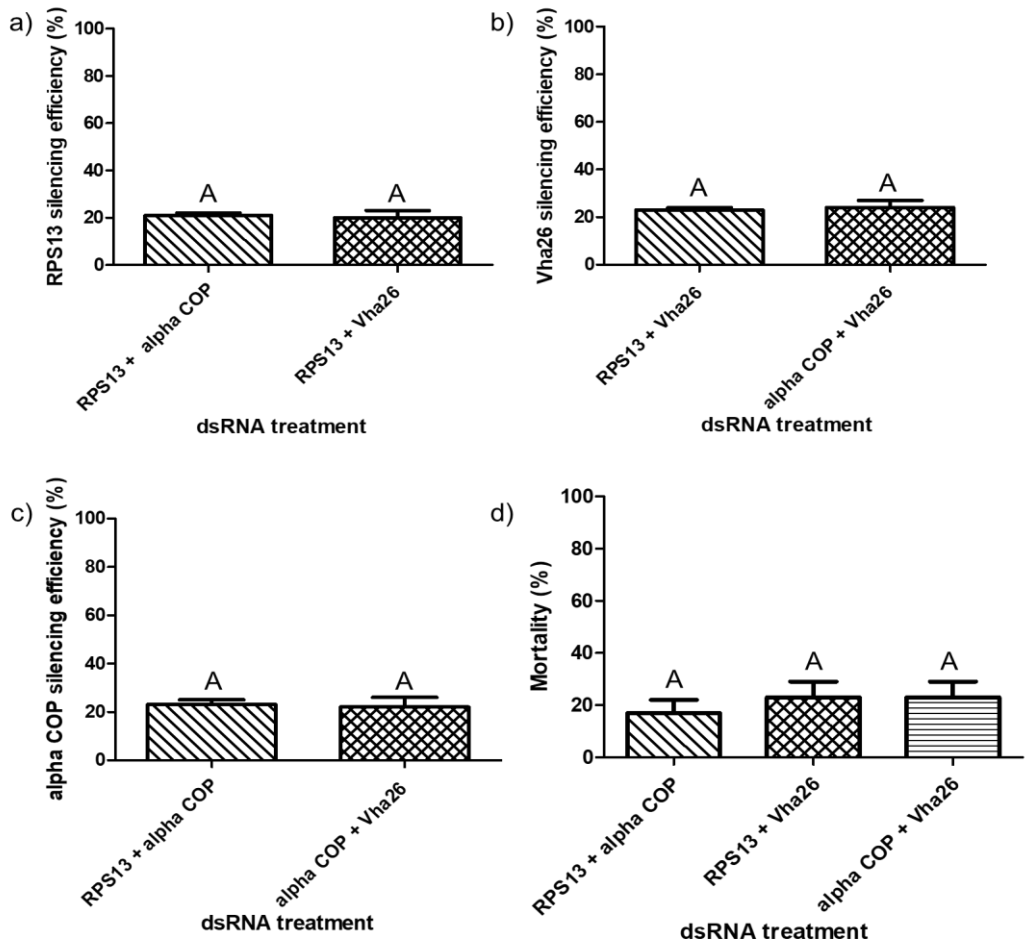
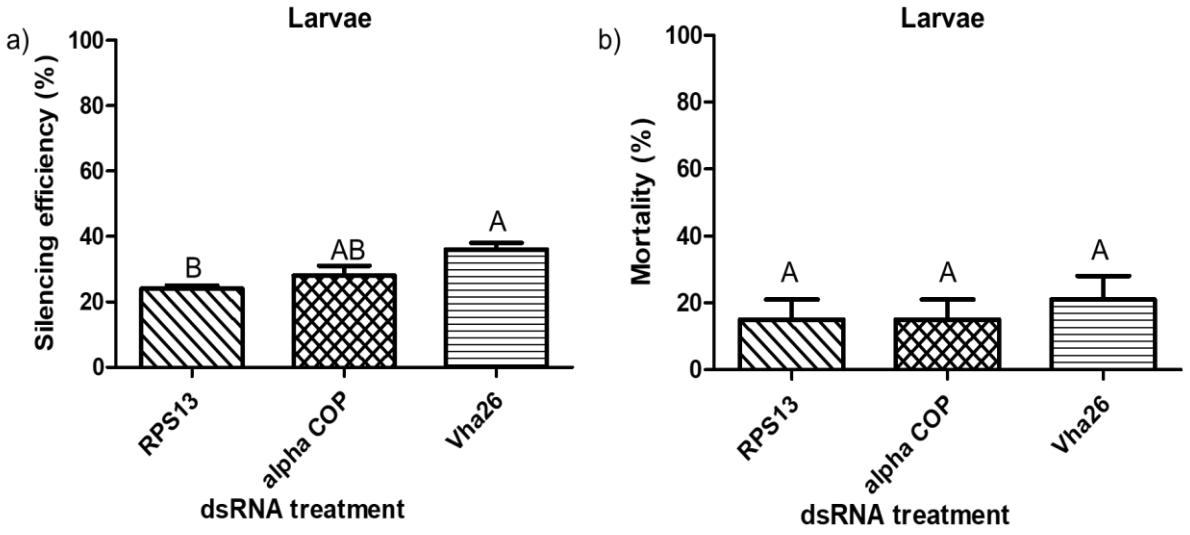


Figure 20: RNAi effects in *D. suzukii* adults following oral treatment with *in vitro* synthesized gene-specific dsRNA targeting two genes in a single treatment; **a)-c)** Gene silencing efficiency in *D. suzukii* adults at 48 h post feeding with dsRNA. The bars in the figure indicate different silencing efficiencies ± SEM for the different target gene dsRNA treatments relative to the control group (ds-GFP-treated group) with a silencing efficiency of zero. All target gene silencing efficiencies differed significantly from the control (Bootstrapped median regression, $p < 0.05$) and bars labelled with different letters indicate a significant difference in mean between target gene treatments. The silencing efficiency was analysed using three biological replicates of 2 pooled insects (n = 6) per treatment, **d)** Mortality in *D. suzukii* adults. The bars in the figure indicate the mean mortality ± SEM for the different dsRNA treatments targeting two genes (*rps13* + *alpha COP*, *rps13* + *vha26* and *alpha COP* + *vha26*) in one dsRNA treatment. Mortality was analysed using a total of 90 insects per treatment. Bars labelled with different letters indicate a significant difference in mean mortality rates (GLM, $p < 0.05$). No mortality was observed in the ds-GFP-treated control group

3.4 RNAi effects in *D. suzukii* following feeding with dsRNA produced in bacteria

In a final experiment, the larvae were fed with lipofectamine-encapsulated dsRNA that was purified from the RNase III-deficient *E. coli* bacteria expressing the dsRNA. Similarly, a significant silencing effect against *rps13* (24 ± 1%), *alpha COP* (28 ± 3%) and *vha26* (36 ± 2%) was observed in the ds-RPS13-, ds-alpha COP- and ds-Vha26-treated groups, respectively (Fig. 21a). The ds-Vha26-treated group showed the highest mortality of 26 ± 7% (p = 0.027), while the ds-RPS13- and ds-alpha COP-treated group resulted in 14 ± 6 and 15 ± 6% mortality, respectively (Fig. 21b).

In adults, a significant silencing effect against *rps13* (15%), *alpha COP* (19%) and *vha26* (19%) was observed in the ds-RPS13, ds-alpha COP and ds-Vha26 treated groups, respectively (Figure 21c). However, very low mortality (<11%) was observed in all treated groups (Figure 21d).



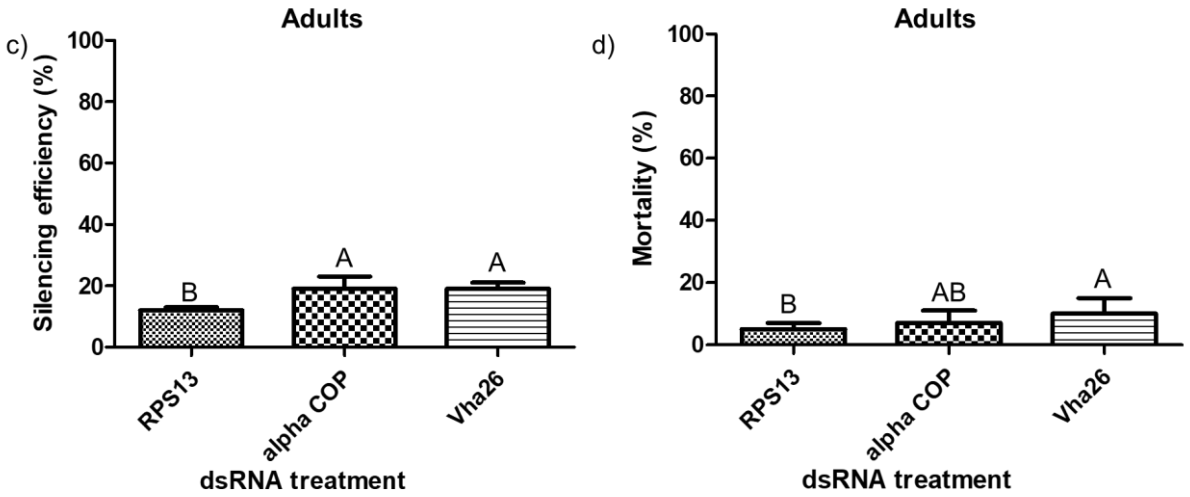


Figure 21: RNAi effects in *D. suzukii* larvae and adults following oral treatment with bacterially synthesized gene specific dsRNA targeting a single gene in a single treatment, **(a)** and **(c)** Gene silencing efficiency in *D. suzukii* larvae and adults, respectively, 48 h post feeding with dsRNA. The bars in the figure indicate different silencing efficiencies ± SEM for the different target gene dsRNA treatments relative to the control group (ds-GFP treated group) with a silencing efficiency of 0. All target gene silencing efficiencies differed significantly from the control (*gfp*) (Bootstrapped median regression, $p < 0.05$) and bars labelled with different letters indicate a significant difference in mean between target gene treatments. The silencing efficiency was analysed using three biological replicates of 2 pooled insects (n = 6) per treatment, **(b)** and **(d)** Mortality in *D. suzukii* larvae and adults. The bars in the figure indicate the mean mortality ± SEM for the different dsRNA treatments targeting single genes (*rps13*, *alpha COP* and *vha26*) in one dsRNA treatment. Mortality was analysed using a total of 90 insects per treatment. Bars labelled with different letters indicate a significant difference in mean mortality rates (GLM, $p < 0.05$).

Administering a combination of two dsRNAs targeting two genes to the larvae resulted in significant transcript silencing of *rps13* (25–26%), *alpha COP* (31–37%) and *vha26* (35–37%) (Fig. 22a-c). The combination of dsRNA specific for *rps13* and *alpha COP* resulted in 21 ± 8% mortality, the combination of ds-RPS13 and ds-Vha26 caused 26 ± 7% mortality and finally, the combination of ds-alpha COP and ds-Vha26 caused 21 ± 8% mortality (Fig. 22d). No mortality was observed in both the ds-GFP- and water-treated control groups.

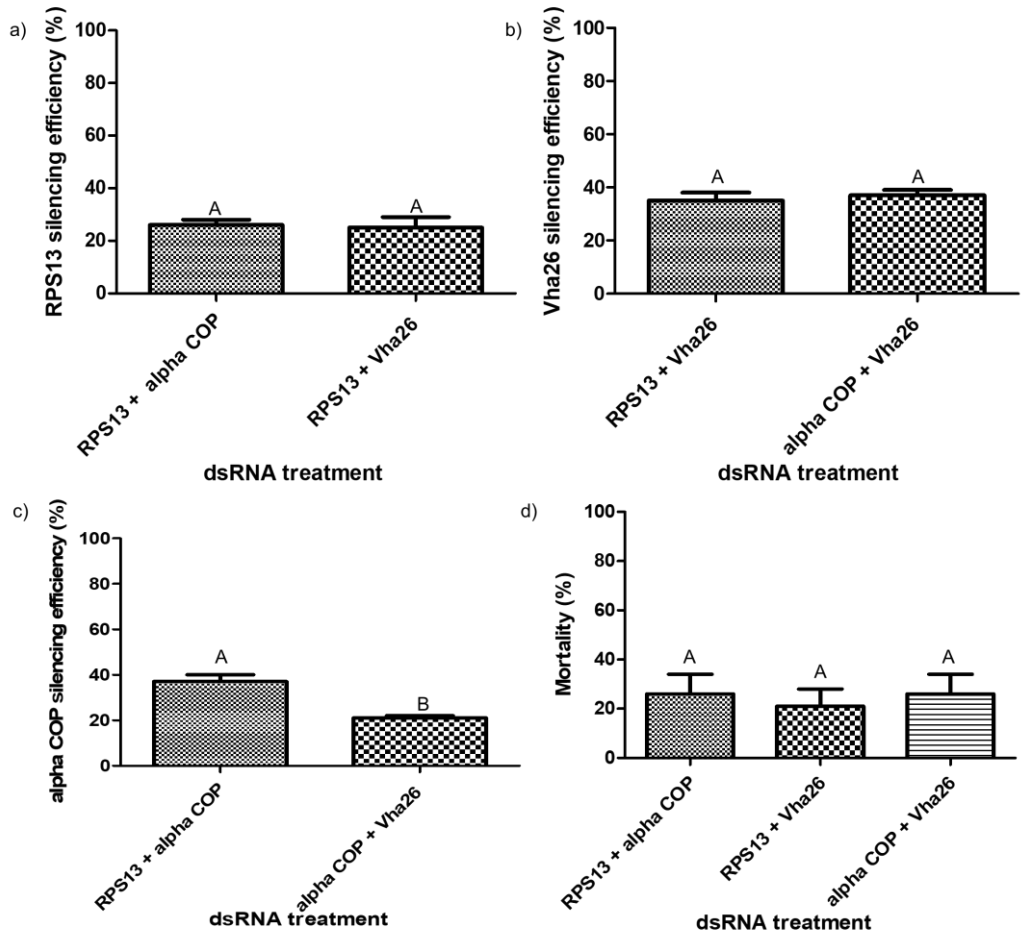


Figure 22: RNAi effects in *D. suzukii* larvae following oral treatment with bacterially synthesized gene-specific dsRNA targeting two genes in a single treatment**; a)-c)** Gene silencing efficiency in *D. suzukii* larvae at 48 h post feeding with dsRNA. The bars in the figure indicate different silencing efficiencies ± SEM for the different target gene dsRNA treatments relative to the control group (ds-GFP treated group) with a silencing efficiency of 0. All target gene silencing efficiencies differed significantly from the control (*gfp*) (Bootstrapped median regression, $p < 0.05$) and bars labelled with different letters indicate a significant difference in mean between target gene treatments. The silencing efficiency was analysed using three biological replicates of 2 pooled insects (n = 6) per treatment, **d)** Mortality in *D. suzukii* larvae following feeding assays with bacterially synthesized gene specific dsRNAs targeting two genes in a single treatment. The bars in the figure indicate the mean mortality ± SEM for the different dsRNA treatments targeting two genes (*rps13* + *alpha COP, rps13* + *vha26* and *alpha COP* + *vha26*) in one dsRNA treatment. Mortality was analysed using a total of 90 insects per treatment. Bars labelled with different letters indicate a significant difference in mean mortality rates (GLM, $p < 0.05$).

In the adults, a significant transcript silencing (<20%, Fig. 23a-c) relative to the control (0% for ds-GFP treatment) and less than 20% mean mortality (Fig. 23d) was observed for all of the treated groups.

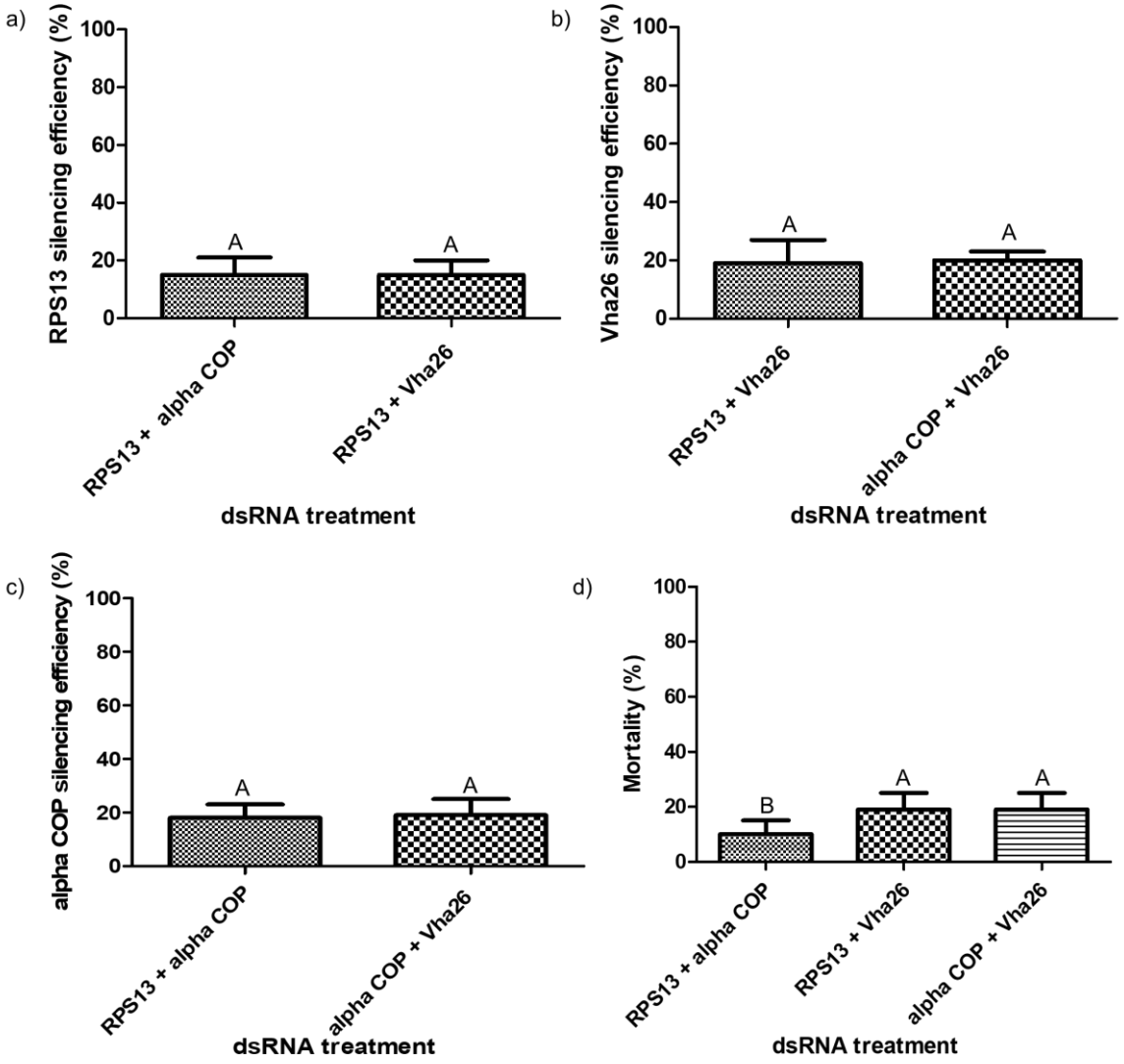


Figure 23: RNAi effects in *D. suzukii* adults following oral treatment with bacterially synthesized gene-specific dsRNA targeting two genes in a single treatment**; a)-c)** Gene silencing efficiency in *D. suzukii* adults at 48 h post feeding with dsRNA. The bars in the figure indicate different silencing efficiencies ± SEM for the different target gene dsRNA treatments relative to the control group (ds-GFP treated group) with a silencing efficiency of 0. All target gene silencing efficiencies differed significantly from the control (*gfp*) (Bootstrapped median regression, $p < 0.05$) and bars labelled with different letters indicate a

significant difference in mean between target gene treatments. The silencing efficiency was analysed using three biological replicates of 2 pooled insects (n = 6) per treatment, **d)** Mortality in *D. suzukii* adults following feeding assays with bacterially synthesized gene specific dsRNAs targeting two genes in a single treatment. The bars in the figure indicate the mean mortality ± SEM for the different dsRNA treatments targeting two genes (*rps13 + alpha COP, rps13 + vha26* and *alpha COP + vha26*) in one dsRNA treatment. Mortality was analysed using a total of 90 insects per treatment. Bars labelled with different letters indicate a significant difference in mean mortality rates (GLM, $p < 0.05$).

4 Discussion

4.1 RNAi can be induced in *D. suzukii* by oral feeding of liposome-encapsulated dsRNA

Feeding dsRNA to insects to induce RNAi has often proven to be less efficient than through microinjection (Rajagopal et al., 2002). Previous studies (Whyard et al., 2009; Zhang et al., 2010; Zhang et al., 2015b) have suggested that the use of RNA carriers or transfection reagents is required to deliver dsRNA orally to several insects including *Drosophila* species, and this was in line with the findings in this study. No significant mortality was observed when naked dsRNAs, targeting *rps13*, *alpha COP* or *vha26*, were fed to *D. suzukii* larvae and adults. However, in the presence of the transfection reagent, Lipofectamine 2000 (Invitrogen), significant silencing and mortality were observed in the treated groups compared to the controls. The mechanisms that facilitate dsRNA uptake in the insect guts are still not yet completely understood and therefore, it is difficult to predict in which species this method of delivery might work. Compared to other insect species where a trans-membrane channel-mediated uptake mechanism, involving *sid1*-like genes, can mediate dsRNA uptake in the gut (Gordon and Waterhouse, 2007; Tomoyasu et al., 2008; Cappelle et al., 2016), drosophilid species apparently lack any *sid* ortholog and endocytosis appears to facilitate cell uptake of dsRNA (Saleh et al., 2006). This process may, however, be too slow to facilitate a strong RNAi

response without the use of transfection reagents to improve delivery to gut cells. Also, naked dsRNA degradation in the gut could contribute to the decrease in the total amount of dsRNA available for uptake in the gut. Studies in other insect species have shown that dsRNA can be degraded in the insect (Allen and Walker III, 2012; Christiaens et al., 2014). More research into *D. suzukii* gut physiology and uptake mechanisms may lead to the development of better helper molecules to facilitate uptake of dietary dsRNA.

The data presented in this chapter show that oral feeding of gene-specific dsRNA, combined with Lipofectamine, to *D. suzukii* larvae and adults can be used to successfully knock down the expression of target genes. Both bacterially expressed and *in vitro* synthesized dsRNA successfully triggered the silencing of target genes and caused significant mortality in the treated larvae. A brief exposure time of 5 h was sufficient to cause significant mortality in the treated individuals. Similar feeding assays in adults also showed successful silencing and mortality, but at much lower levels than in larvae. While the mechanisms by which the dsRNA enters and distributes itself in *D. suzukii* have not been fully elucidated, the fact that ingested dsRNA can induce RNAi in both the adults and the larvae, offers some intriguing possibilities for future applications.

Three target genes, *alpha COP*, *rps13* and *vha26*, were screened in this study for RNAi induction through the oral route. *Alpha COP* codes for the subunit of the cytosolic coatomer protein complex that binds to dilysine motifs and reversibly associates with Golgi non clathrin-coated vesicles, which further mediate biosynthetic protein transport from the endoplasmic reticulum (ER), via the Golgi up to the trans Golgi network (Kitazawa et al., 2012). *Vha26* encodes a subunit of the peripheral V1 complex of vacuolar ATPase essential for assembly or catalytic function. V-ATPase is responsible for acidifying a variety of intracellular compartments in eukaryotic cells (Dow, 1999). *RPS13* codes for a constituent of the small ribosomal subunit. Ribosomes translate all mRNAs produced from nuclear genes and perform the majority of

cellular protein synthesis (Alonso and Santarén, 2006). Among the three tested target genes, silencing *vha26* resulted in the highest mortality in these feeding assays (42%), compared to 32% for *rps13* and 22% for *alpha COP*. Transcript silencing efficiencies did not differ greatly between the three target genes indicating that, from the three genes that were evaluated in this analysis, *vha26* is the best possible target gene for further RNAi optimization. In a second set of experiments, combinations of the different dsRNAs were also tested. The dsRNA doses used in these experiments were the same as used for each dsRNA in the single-target gene treatments, leading to a double amount of total dsRNA added to the diet. While the combination of dsRNA specific for *rps13* and *alpha COP* resulted in 40% mortality, the mortality observed for the separate single dsRNA experiments for *rps13* and *alpha COP* were 32% and 22%, respectively. Given the fact that the total amount of dsRNA that was administered in the combination assays was double the concentration given in the single target gene assays, we could not prove that using combinations is a better strategy than targeting single genes. Nevertheless, further research testing different genes, combinations and doses to *D. suzukii* will allow us to assess better whether combinations of gene targets could have potential for future applications.

4.2 Bacterially produced dsRNA can induce RNAi in *D. suzukii*

In view of the possibility of applying RNAi to control *D. suzukii* using an economically viable and a non-transgenic approach, we evaluated dsRNAs produced in a microbial system and also evaluated the silencing efficiency and mortality resulting from oral feeding bioassays using these dsRNAs. The dsRNAs were produced by transforming an RNase III-deficient *E. coli* strain (HT115) having an IPTG-inducible T7 polymerase activity, with a 38i plasmid (2814 bp) containing the gene-specific sequence to be transcribed. However, directly feeding the bacteria expressing the dsRNAs to adults and larvae did not lead to gene silencing and mortality. A possible explanation to this observation could be that when the dsRNA is released

from the bacteria in the insect gut, it faces a similar drawback of slow cellular uptake by the gut cells. Hence, the dsRNAs were purified from the bacteria and encapsulated into a liposome, to facilitate uptake into the insect gut cells. Like the feeding assays with *in vitro* synthesized dsRNA encapsulated in a liposome, RNAi effects were observed, with the ds-Vha26-treated group showing the highest mortality (26%). A possible explanation for the low mortality in the groups treated with bacterially synthesized dsRNA could be the overestimation of the amount of dsRNA administered to the treated groups. The overestimation could have been caused by the presence of residual bacterial nucleic acids in the sample, which when measured spectrophotometrically gave a false positive result. Furthermore, the mortality values obtained in these experiments are the result of a single 5 h long exposure of the insects to the dsRNA. The situation in the field, where a continuous supply of dsRNA could be provided, could lead to a higher mortality, provided that the dose to which the insects are exposed to is high enough. Since dsRNA has been shown to be vulnerable to fast degradation in several insects (Allen and Walker III, 2012; Christiaens et al., 2014), protection of the dsRNA by nanoparticles could provide a more efficient silencing and cause more toxicity. Further research towards new formulation methods, which could improve the dsRNA persistency and stability both inside and outside the insect, might therefore lead to better results.

4.3 Can RNAi be exploited as a crop protection strategy against *D. suzukii*?

Concerning delivery of RNAi products against *D. suzukii* in the field, one of the biggest challenges is the fact that the larvae feed and grow inside the fruit, making it difficult for most control methods to get in contact with the larvae. This results in a limited range of current control methods to target the adult flies and prevent them from laying eggs. The transgenic delivery option presents the possibility of targeting the larvae in the crop since it constantly expresses the dsRNA against the pest. However, in many cases, the use of transgenic crops is not realistic. This can be due to political or legislative reasons, or because the crop in

question is technically difficult or unable to be transformed. Another major challenge is that the dsRNA expressed in the transgenic plant against *D. suzukii* will probably encounter the same difficulties in the gut of *D. suzukii* as the *in vitro* synthesized naked dsRNA. However, considering that *D. suzukii* (especially the larvae feeding inside the fruits) will constantly be exposed to the target gene dsRNA expressed in the transgenic plant, this might result to optimal levels of dsRNA being taken up by the insect cells to induce RNAi effects. Nevertheless, the transgenic delivery option will still face an additional challenge which is the wide food range for *D. suzukii,* offering shelter for the flies in or around the field. Besides the transgenic option, new alternative routes for dsRNA delivery such as the use of recombinant virus vectors, engineered to express the desired insect gene-specific dsRNA could be exploited in the future to control the larvae in the fruits. Kumar et al. (2012) demonstrated that the plant virus, tobacco rattle virus (TRV), expressing the antisense fragments for a dsRNA specific to a chewing insect, *Manduca sexta*, in *Nicotiana attenuata* plants, could specifically silence three midgut-expressed MsCYPs RNAs when the larvae were fed on these plants. Similarly, Wuriyanghan and Falk (2013) also used recombinant tobacco mosaic virus (TMV) to induce RNAi effects in *Bactericera cockerelli* on tomatillo plants. Plant root drenching and direct injection of dsRNA into the plant also present alternative delivery methods to target the in-fruit *D. suzukii* larvae. Hunter et al. (2012) demonstrated that dsRNA introduced into citrus trees through root drenching or stem injection could be detected in the leaves of the trees 53 days post treatment. In these experiments, the persistence of dsRNA in psyllids and leafhoppers was also detectable for 5–8 days post ingestion from treated citrus plants. Similarly, Li et al. (2015a) also demonstrated that exposing the brown plant hopper (*Nilaparvata lugens*) and Asian corn borer (*Ostrinia furnacalis*) to rice or maize that had been irrigated with a solution containing the dsRNA of an insect target gene, resulted in a significant increase in the insect's mortality rate. Nevertheless, targeting in-fruit pest will require sufficient amounts of dsRNA to reach the fruits and be taken up by the larvae, and this remains to be verified.

In the framework of an integrated pest management (IPM) system, a developed dsRNA-based insecticide against *D. suzukii* can be used in several ways and in combination with other control methods. Besides direct spraying on the plant, the dsRNA-based insecticide in combination with attractants could be used to lure the flies towards traps placed around the field. Landolt et al. (2012a) showed that using a combination of food materials in place of the recommended baits of apple cider vinegar or grape wine (Beers et al., 2010) would provide a significant increase in the power of a trap-bait system for *D. suzukii*. One possibility of applying a dsRNA-based insecticide will be through the use of bait sprays. Bait sprays are mainly based on phagostimulants (mainly proteins and sugars) combined with small amounts of insecticides (Vayssieres et al., 2009; Böckmann et al., 2014). They can substantially reduce insecticide use in agriculture because the mode of application is by only partial treatment of the canopy, as spots or bands. Bait sprays containing the target gene dsRNAs against *D. suzukii* could be applied in small amounts (an optimal concentrations) on the trunk of the trees to attract and kill the flies just before fruit maturation. Although this method will mostly target the adults, the fly population could be significantly reduced depending on the efficacy of the dsRNA-based insecticide and the attractiveness of the lure. Moreover, alternating control methods will also reduce the probability of resistance to the insecticide arising.

Field usage of dsRNA for pest control will only be feasible if it is species specific and cost effective. Given that species are defined by the uniqueness of their gene sequences, it is possible to target portions of genes unique to one species but not to other closely related species (Baum et al., 2007; Whyard et al., 2009). Singh et al. (2013) also reported that even for a highly conserved gene such as β-tubulin, it was possible to design a dsRNA that can kill one species (*A. aegypti*) but not adversely affect another (*D. melanogaster*).

Production of dsRNA in large scale will have to be cost effective. Microbial biofactories producing gene-specific dsRNAs present a cheaper option. There are many advantages in

using bacteria to produce and deliver dsRNA in insect pest control when compared with using *in vitro* synthesized dsRNA or plant-mediated dsRNA delivery. The most significant advantage is the lower cost per application of bacteria-expressed dsRNA when compared with that of *in vitro* synthesized dsRNA. Mass production of target gene dsRNAs may be achievable using engineered microorganisms as biofactories to produce the dsRNA *in vivo*. Since RNAi is not a knockout, but a knockdown method which is generally transient, to maximize the potential use of RNAi in crop protection, continuous and large-scale delivery of dsRNA for target gene silencing might be necessary to kill the insect pests (Huvenne and Smagghe, 2010). With the ease in manufacturing large quantities, the bacteria-expressed dsRNA could be used whenever necessary. Furthermore, the possibility of using either heat-killed bacteria containing dsRNA or dsRNA purified from the bacteria in the field may facilitate its public acceptance compared to the transgenic option. Although RNAi technology provides vast opportunities, species specificity is a critical issue that needs to be addressed before the use of RNAi-based pest control measures in the field. However, RNAi technology has the potential to address this problem by using sequence-specific and species-specific dsRNAs (Whyard et al., 2009).

6 Conclusion

Based on the results presented in this chapter, we can make several conclusions. Feeding naked dsRNA (either synthesized using an *in vitro* system or in bacteria) to *D. suzukii* adults and larvae does not induce RNAi effects in the insects. However, encapsulating either the *in vitro* or bacterially synthesized dsRNA in a liposome and feeding to *D. suzukii* adults and larvae leads to clear knockdown of the target genes. This implies that the protection of dsRNA in the insect gut and/or the facilitated uptake of dsRNA into the insect gut cells are vital for a successful RNAi-based approach for *D. suzukii*. Nevertheless, RNAi can be induced through the oral route in *D. suzukii* using both *in vitro* and bacterially synthesized dsRNA. This can be exploited to develop better delivery systems for RNAi-based control strategies for *D. suzukii.*

Chapter 4

Engineered flock house virus for targeted gene suppression through RNA interference in fruit flies: A proof of concept in *Drosophila melanogaster*

Modified from: Taning, C. N. T et al. (2018). Engineered Flock House Virus for targeted gene suppression through RNAi in fruit flies (*Drosophila melanogaster) in vitro* and *in vivo*. Frontiers in physiology (submitted).

1 Introduction

The oral feeding bioassay experiments, presented in Chapter 3, indicated that in *D. suzukii*, oral RNAi using naked dsRNA is not highly efficient. The successful use of liposomic nanoparticles, which encapsulate the dsRNA and facilitate cellular uptake, to improve oral RNAi efficiency suggested that this insect does not possess an efficient cellular uptake mechanism for naked dsRNA in the gut. Looking at possible application in the field, spraying of liposome-encapsulated dsRNAs is not very practical. Firstly, because these nanoparticles still carry a high cost but, equally important, also because the larvae, which are the most RNAi-sensitive life stages (see chapter 3), are living inside the fruits and will therefore be difficult to expose to a sprayable dsRNA. This last problem could be remediated using transgenic plants, but as was discussed in the previous chapter, this is not always an option. Furthermore, transgenic plants would express naked dsRNA which might not be efficacious anyway, even when the larvae are constantly exposed to a high dose of dsRNAs.

One possible alternative delivery method, which has proven successful in insects is to employ engineered micro-organisms that produce dsRNA molecules. While several studies have shown the successful use of engineered *E. coli* to produce and deliver dsRNA in insects (Zhu et al., 2011; Kumar et al., 2013; Taning et al., 2016b; Whitten et al., 2016), the same constraints as with sprayable dsRNA apply here. The dsRNA either needs to reach the larvae in the fruit, or it has to elicit a strong enough RNAi response in the adults, which has proven to be difficult. However, among the use of engineered micro-organisms for improved delivery, the possible application of engineered insect viruses for this purpose has received much less attention. Nevertheless, viruses have several attractive properties that make them excellent delivery vehicles for nucleic acids such as, efficiency and specificity of infection and the evolved avoidance of the immune response. We hypothesized that virus-induced gene silencing (VIGS)

could be a practical delivery method for *D. suzukii*, since it could cause a high expression of insecticidal dsRNAs inside virus-infected individuals (larvae and adults).

VIGS is an RNA silencing-based technology that can be exploited to silence genes of interest in insects (Kolliopoulou et al., 2017). Briefly, infection by a virus triggers RNA silencing, an insect innate defense pathway that specifically degrades the viral genome. If the virus is engineered to carry a fragment of an insect gene transcript, RNA silencing would be directed to target this particular endogenous gene. In the past decade, a number of viral vectors have been developed as a powerful reverse genetic tool for the functional characterization of genes in plants (Kumagai et al., 1995; Ruiz et al., 1998; Purkayastha and Dasgupta, 2009; Lange et al., 2013). However, the majority of the published VIGS vectors have a host range limited to some plant species, and very few have been developed for application in insects (Kolliopoulou et al., 2017). Currently, only two insect virus-based RNAi delivery systems have been developed. The baculovirus system based on *Autographa californica* nuclear polyhedrosis virus (AcMNPV) (Huang et al., 2007; Kontogiannatos et al., 2013) and the densovirus system based on *Aedes aegypti* Densovirus (AeDNV) (Gu et al., 2011). Briefly, AcMNPV and AeDNV are DNA viruses which have a limited host range (lepidopterans and mosquitoes, respectively) and can be easily manipulated and produced in cell lines. These properties have driven research on these viruses for potential applications in environmentally safe pest control, and as gene transduction and RNAi delivery vectors. However, their specificity to a limited number of insect species is also a drawback in research, since it limits the exploitation of these virus-based RNAi delivery systems for use in many other insects.

In this chapter, we aimed to design a proof-of-concept for VIGS in fruit flies by engineering Flock House virus (FHV) as a virus-based RNAi delivery system in *Drosophila*. Since FHV is known to infect and replicate in many other insect species (Selling and Rueckert, 1984; Gallagher and Rueckert, 1988; Swevers et al., 2016), this will additionally provide an ideal

delivery system for functional studies in different RNAi recalcitrant insects. FHV belongs to the Nodaviridae family and the Alphanodavirus genus, and was first isolated from the grass grub *Costelytra zealandica* (Coleoptera) in New Zealand (Dearing et al., 1980). Although no information is available about its ecological epidemiology, it is a well-studied insect model virus in the laboratory. FHV has a simple genome organization composed of two positive-sense, single-stranded RNAs packaged by a single capsid into a non-enveloped icosahedral virion (Scotti et al., 1983; Schneemann et al., 1998). RNA1 is 3.1 kb in length and encodes the autonomous viral RNA-dependent RNA polymerase (RdRp, protein A; 112 kDa) (Friesen and Rueckert, 1981; Poch et al., 1989; Price et al., 2000). During FHV replication, a subgenomic RNA3 (0.4 kb) is also synthesized which encodes two proteins, B1 and B2 (Guarino et al., 1984). The function of translated B1 protein is poorly defined, but may be important for maintenance of RNA replication (Ball, 1995), whereas protein B2 is responsible for suppressing Dicer-mediated RNA silencing (Li et al., 2002). Genomic RNA2 (1.4 kb) encodes the viral capsid protein precursor, CP-α (43 kDa), that is later cleaved into 40 kDa (β) and 4 kDa (γ) fragments after provirion assembly (Friesen and Rueckert, 1981; Schneemann et al., 1998). The autonomous ability of the FHV RNA1 to replicate and the robust intracellular genome synthesis and protein expression directed by subgenomic promoters make FHV an ideal candidate for amplifying heterologous sequences.

A FHV plasmid-based system was designed, whereby an expression cassette that transcribes RNA1 with precise 5′- and 3′-ends can initiate high levels of FHV replication. The precise 5’- and 3’-ends of RNA1 were realized by positioning a promotor sequence and a self-cleaving ribozyme at the 5’ and 3’ end of RNA1, respectively. In the presence of RNA2, the replication system will generate functional virions. Since FHV infection results in production of viral siRNAs (Gammon and Mello, 2015), an insertion of foreign sequences in the FHV genome could therefore be employed to deliver specific RNAi effects in infected cells (Fig. 24).

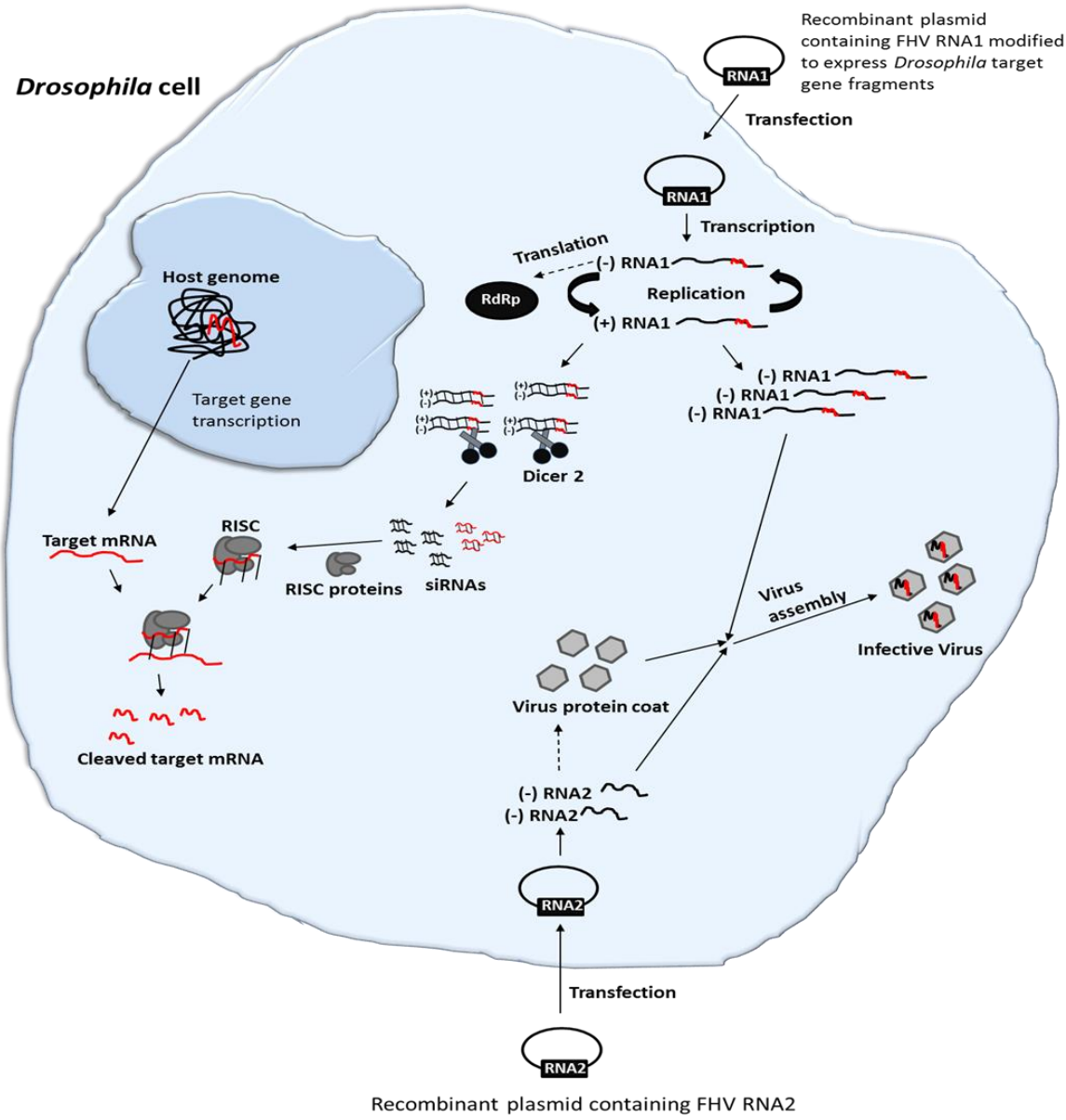


Figure 24: Schematic illustration of FHV-based RNAi delivery in *Drosophila*. Cell transfection and activation of FHV RNA1 and RNA2-based plasmids lead to the expression of the FHV genome. During the replication of FHV RNA1, dsRNAs are formed. Through the production of viral siRNAs, an insertion of target gene sequences in the FHV genome could lead to a specific RNAi effect in infected cells. Furthermore, the system will generate functional recombinant virions that can infect neighbouring cells, spreading the RNAi signal.

Herein, using *D. melanogaster* for the proof of concept, since no cell lines are currently available for *D. suzukii*, we first developed a recombinant FHV expressing selected *D.*

melanogaster target gene sequences and then assessed whether it could replicate and induce targeted gene suppression in *D. melanogaster*. To this end, we first examined whether the engineered FHV plasmids could express the recombinant FHV clones in S2 cells. We then investigated whether the resulting infective recombinant FHV can induce gene silencing *in vitro* in S2 cells and *in vivo* in adult *D. melanogaster*.

2 Materials and Methods

2.1 Cell culture

Schneider 2 (S2) cells, derived from a primary culture of *D. melanogaster* embryos (Schneider, 1972), were maintained at 27 °C in InsectXpress culture medium (Lonza) supplemented with 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich) at the Laboratory of Agrozoology, Ghent University, Belgium.

2.2 Insect culture

D. melanogaster adults and larvae used in this research came from the same culture and were reared on a similar agar-yeast-cornmeal diet as described in chapter 2, section 2.2.1.

2.3 Target gene selection

The target genes used in this study, *alpha COP*, *rps13* and *vha26*, were selected based on our findings in chapter 2 and 3, which reported on their effectiveness in causing mortality to the closely related species, *D. suzukii*. Homologous target gene sequences for *D. melanogaster* were retrieved from the database of *Drosophila* genes and genome (http://flybase.org/) by BLAST analysis using known query sequences from *D. suzukii*. The chosen target region from each gene selected was amplified using designed synthetic primers

containing restriction sites for AsiSI (GCGATCGC) and BsrGI (TGTACA) flanking both the 5' and 3' ends, respectively (Table 9). The Enhanced green fluorescent protein gene (*eGFP*) was used as a reporter gene in this study. The entire *eGFP* sequence was amplified with synthetic primers including restriction sites for NsiI (ATGCAT) and AsiSI flanking its 5' and 3' ends, respectively (Table 9)

Table 9: Primers for genes used in designing the FHV-based RNAi delivery system

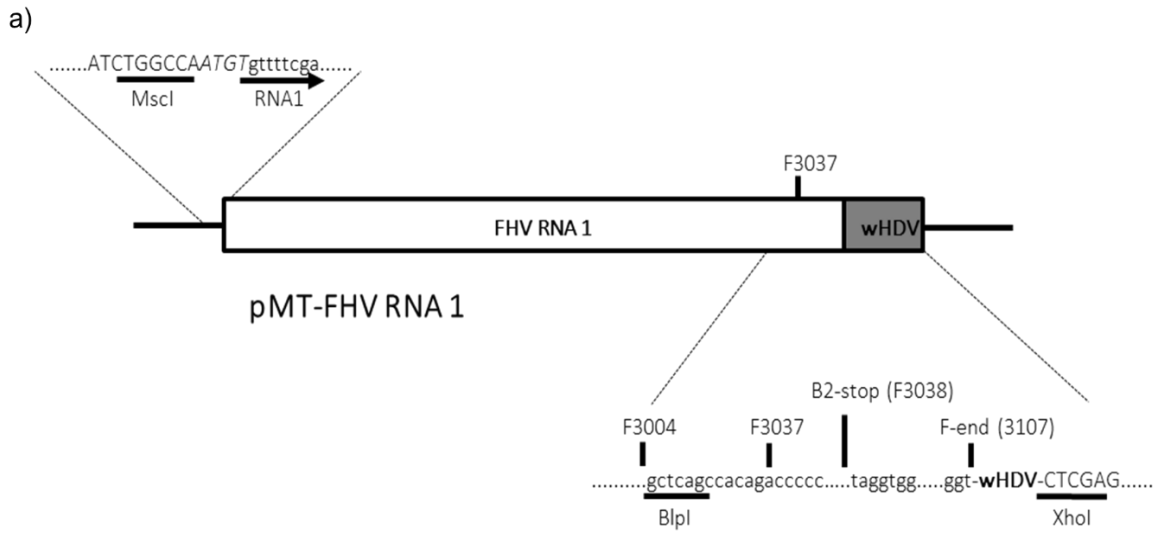
Target genes	Accession	Primer sequence (5′-3′)*	Product size
alpha-coatomer protein, isoform A (alpha COP)	NM_058047.5	F-TGATCGCCTTGTGAAGT R- GATCGTAGGTGCTGTTCTCCA	499bp
Ribosomal protein S13 (rps13)	X91854.1	F-GCAGATGATGTCAAGGA R- ATGTAGGACCCCGCAAGAC	421bp
Vacuolar H[+]-ATPase 26kD E subunit (vha26)	U38198.1	F-AGCACCGAAATGGACCT R-ATTGGCGAACATGCGAATA	449bp
Enhanced Green Fluorescent protein (Reporter) (egfp)	/	F-ATGGTGAGCAAGGGCGAGGA R-TTACTTGTAGAGCTCGTCCA	720bp

*Restriction enzyme sites are not included in the primer sequence

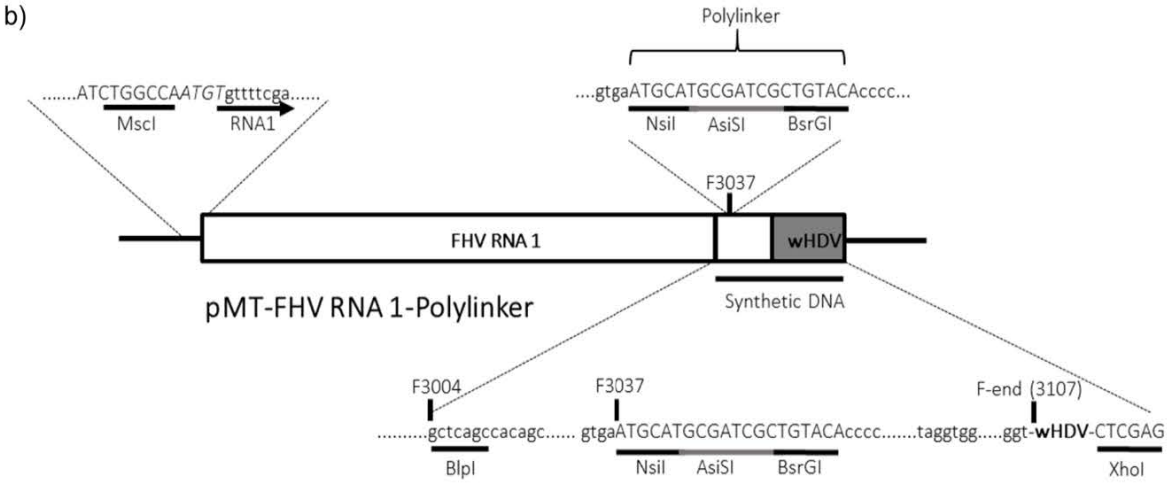
2.4 Plasmid constructs for the expression of both eGFP and target gene sequences

Standard molecular cloning techniques were used unless otherwise stated. A plasmid (pMT/V5-His A) containing the full FHV RNA1 genome and a ribozyme sequence derived from hepatitis delta virus (HDV) attached to its 3' end was kindly provided by Professor Ronald Van Rij (Radboud Institute for Molecular Life Sciences, Nijmegen, the Netherlands) (Fig. 25a). Self-cleaving ribozymes such as the HDV ribozyme catalyse sequence-specific intramolecular cleavage of RNA. Hence, by placing the HDV ribozyme sequence directly after the 3' end of the FHV RNA1 sequence in the recombinant plasmid, the exact 3' end of FHV RNA1 will be

maintained in the expressed FHV RNA1 transcript (required for the generation of a functional virus), after the HDV ribozyme has catalysed its own scission from the transcript. Based on Maharaj et al. (2014), an insertion site was created at position 3037 bp of the pMT-FHV RNA1 genome for the introduction of the reporter gene (*egfp*) and subsequently a *D. melanogaster* target gene sequence for dsRNA production during viral replication. First, a polylinker, ATGCATGCGATCGCTGTACA, composed of three restriction sites, NsiI, AsiSI and BsrGI was inserted into position 3037 bp of the pMT-FHV RNA1 genome (Fig. 25b). After confirmation by sequencing and restriction digest analysis, *egfp* was introduced in between the NsiI and AsiSI restriction sites to create pMT-FHV RNA1-GFP replicons (Fig. 25c). Additional expression constructs were generated where *D. melanogaster* target genes (*vha26*, *rps13* and *alpha COP*) were inserted after *egfp* in between AsiSI and BsrGI restriction sites (Fig. 25d). The FHV RNA2 sequence tailed at its 3' end by the HDV ribozyme sequence (Fig. 25e) was synthesized (by gene synthesis: ThermoFisher Scientific) and cloned into the vector backbone of the pMT-FHV RNA1 plasmid (plasmid without FHV RNA1). A non-virus positive control construct for eGFP expression was made by replacing the FHV RNA1 genome in the plasmid with *egfp* (Fig. 25f). All constructs were sequenced to ensure maintenance of sequence identity and to assess for spurious mutations in the constructs.



b)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NsiI
AsiSI
BsrGI
F3037
FHV RNA 1
wHDV
pMT-FHV RNA 1-Polylinker
Synthetic DNA
F3004
F3037
F-end (3107)
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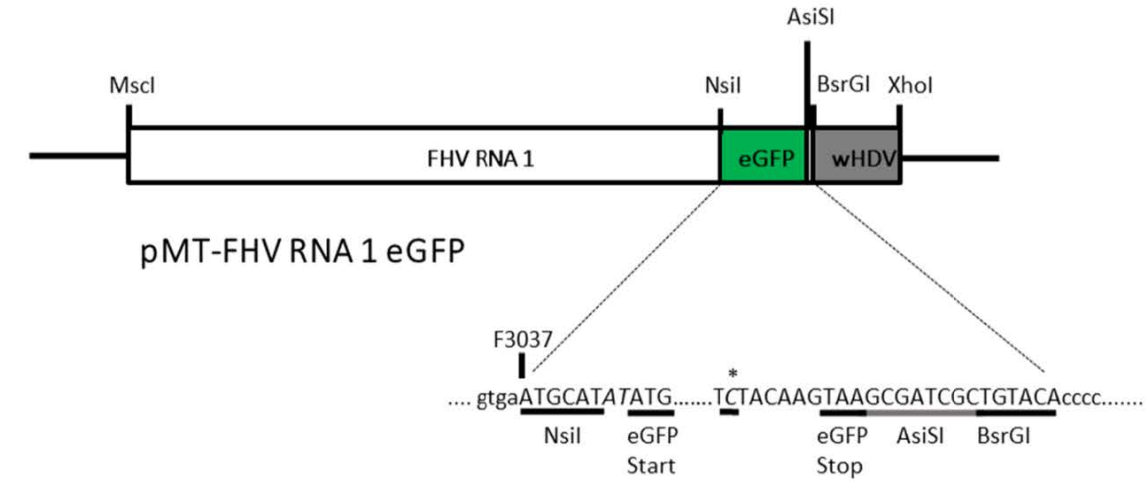
BlpI
NsiI
AsiSI
BsrGI
XhoI

c)


AsiSI
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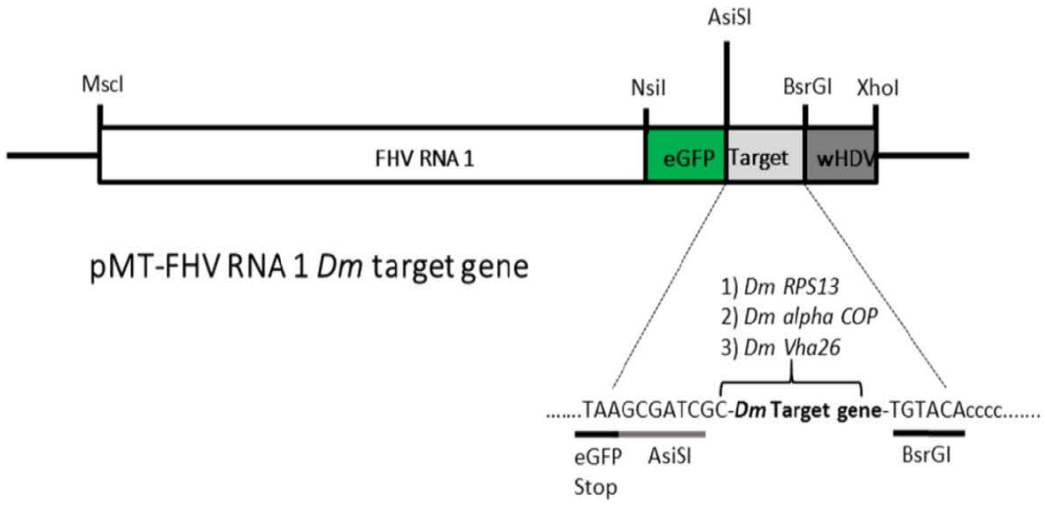
d)


AsiSI
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1) Dm RPS13
2) Dm alpha COP
3) Dm Vha26
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eGFP
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AsiSI
BsrGI

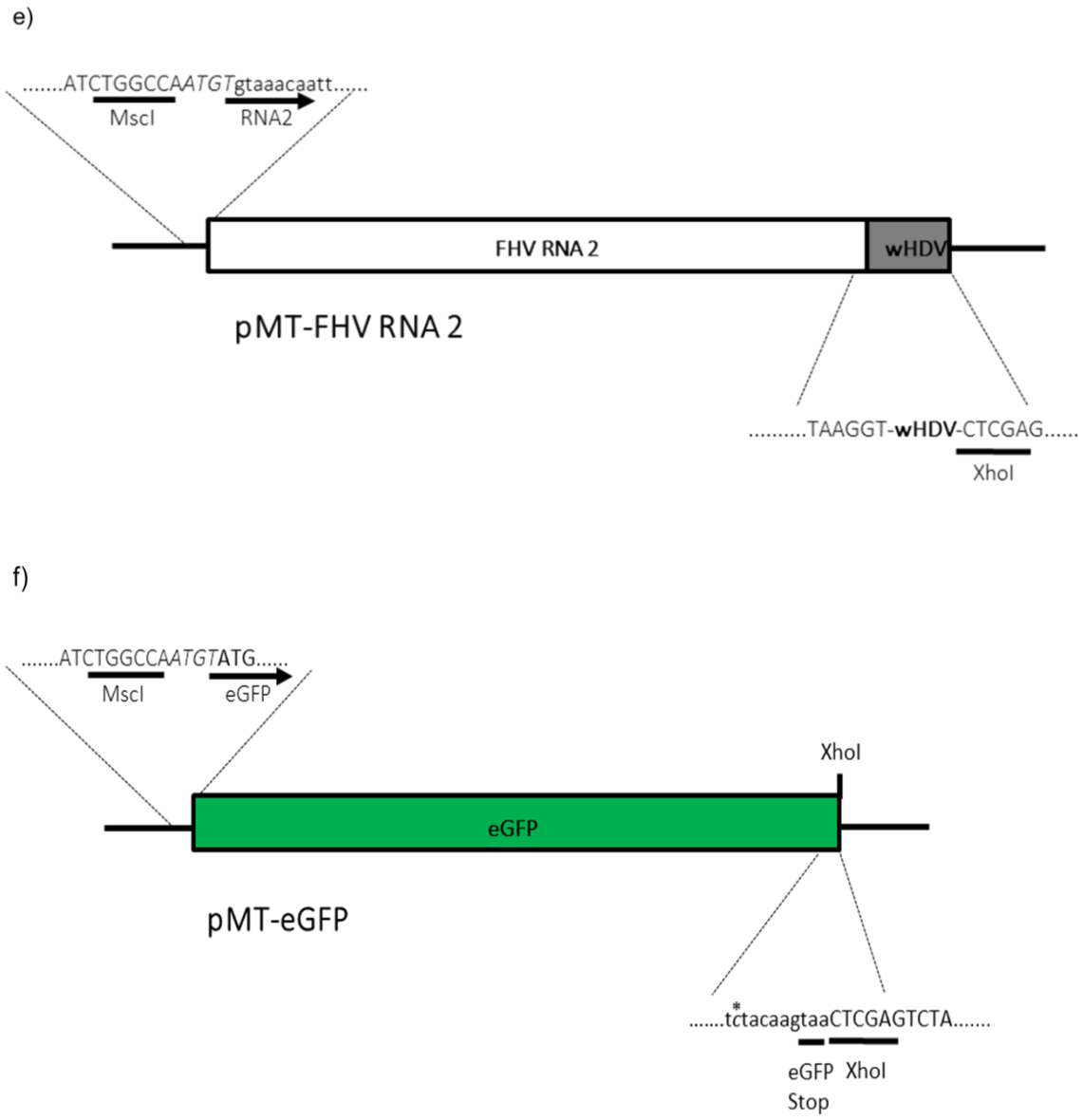

Figure 25: Schematic illustration of FHV RNA1-based plasmid systems for foreign gene expression in *Drosophila* S2 cells.

2.5 Transfection of S2 cells and virus detection by fluorescence microscopy

S2 cells were transiently transfected with the engineered plasmids using Escort IV (Sigma-Aldrich), according to the manufacturer's instructions. Briefly, each well of a 6-well plate was filled with 3 x 10^6 S2 cells and washed twice with serum-free culture medium. 14 µL of the Escort IV was first pre-incubated alone for 30-45 min in serum-free culture medium and then

30 min together with 1.5 µg of each of the pMT-FHV RNA1 plasmids plus 1.5 µg of the pMT-FHV RNA2 plasmid (1:1 ratio). The transfection medium was added to the cells and incubated for 16 h at 27°C. After incubation, the transfection medium was replaced with fresh medium containing serum (10% FBS) and further incubated for 24 h for cell recovery. Following cell recovery, copper sulfate ($CuSO_4$) was added to the medium to a final concentration of 700 µM to activate the plasmid promoter to express FHV RNA1 and 2. S2 cells were then observed for eGFP expression at 72 h post-activation using a Nikon Eclipse TS-100 microscope (Melville, NY, USA) and NIS Elements BR 4.11.00 imaging software (Nikon, Melville, NY). The plasmid containing only *egfp* (pMT-eGFP) was used as positive control for transfection and plasmid activation for eGFP expression, while the plasmid containing the wild type (WT) genome, pMT-FHV RNA1, was used as a negative control (no eGFP expression).

2.6 Detection of FHV expression by PCR

After confirmation of eGFP expression by imaging, cells obtained from each treatment were lysed and total RNA was extracted using the RNeasy Mini Kit (Qiagen). After DNase I treatment (Ambion) to remove residual genomic and plasmid DNA, the RNA was quantified using a NanoDrop ND-1000 (Thermo Scientific) and verified by 1.5% agarose gel electrophoresis. Total RNA (1 µg) was reverse transcribed using the SuperScript IV kit (Invitrogen) according to manufacturer's instructions. The resulting complementary DNA (cDNA) was used as a template in a PCR reaction for the detection of FHV using designed primers (Table 10). The primers used for FHV detection were designed to detect the negative strand of the virus, to further confirm for virus replication. The PCR reactions included 0.2 µl of Taq DNA polymerase (Invitrogen), 2 µl of 10x PCR buffer (Invitrogen), 0.6 µl of 10 µM forward primer (Invitrogen), 0.6 µl of 10 µM of reverse primer (Invitrogen), 0.6 µl of 50 mM $MgCl_2$, 0.6 µl of 10 mM dNTPs, 15 µl of nuclease-free water and 0.9 µl of cDNA, in a total volume of 20.5 µl. The amplification

conditions were 2 min at 94 °C followed by 33 cycles of 30 s at 94 °C, 30 s at 60 °C and 45 s at 72 °C, and then 10 min at 72 °C and infinity at 10 °C.

Table 10: Primers used for PCR detection of FHV RNA1 and 2

Target genes	Primer sequence (5′-3′)	Product size
FHV RNA1	F-GTTGGGACGGTTTATTCAGC	400bp
	R-ATCGGTATGGGACACAAGGA	
FHV RNA2	F-ATCAAGAGGTGGCGAGTCAT	500bp
	R-GCATTTACCCAACGTCGAAC	

2.7 Virus amplification

While some cells were collected for FHV detection by PCR, the infectious viral particles were harvested from the remaining cells and supernatant (72 h post-transfection). First, the cells from each treatment were centrifuged at 1000 rpm for 5 min and then 90% of the medium was taken out. The cells and residual medium were subjected to two cycles of freeze-thawing and later centrifugation at 8000 rpm for 10 min to remove cell debris. The unpurified infectious virus supernatant was used to infect virus-free S2 cells and the cells were then incubated for 72 h at 27 °C. After incubation, the infectious virus was extracted from the cells as described above and the process was repeated three times with the aim of increasing the viral load. Based on preliminary experiments, concentrating the viral load three times was just enough to avoid more than 90% S2 cell mortality after 72 h. The supernatant containing the infective virus, obtained from the third repeat, was used to infect S2 cells and adult *D. melanogaster* in the *in vivo* and *in vitro* bioassays, respectively. This step was repeated four times for each separate biological repeat per treatment. Prior to the bioassays, qRT-PCR was used to confirm that the viral titer was the same between the gene targeting and non-gene targeting FHV inoculums (Table 11).

Table 11: Primers used for detecting the virus in the inoculums

Target	Primer sequence (5′-3′)	Product size
FHV RNA1	F-GCCTGGTGTAGGCGTTAAAA	101bp
	R-CAGGATGCTCAAAGGTCAGAG	

2.8 Cell viability assays

For these assays, 100 µl of the unpurified viral supernatant (for each treatment) was added to each well of a 6-well plate filled with 3 x 10^6 S2 cells and then incubated at 27 °C. The infected cells were observed daily under a light microscope. After 72 h, a time point where a clear difference in cell growth could be visually observed between the different treatments, live and dead (stained with 0.4% Trypan blue) cells were enumerated manually under a light microscopy (10 X magnification), using a Neubauer haemocytometer according to Decombel et al. (2004) and Chan et al. (2015). Cell viability was calculated as the ratio of live cells to death cells in the total cell population. This experiment was repeated four times for each separate biological repeat per treatment.

2.9 Survival bioassay

Survival bioassays were performed by infecting *D. melanogaster* adults with the engineered virus and then monitoring their survival over time. Three to four-day-old *D. melanogaster* adult flies were anesthetized with diethyl ether for 2 min, immobilized in a 1.5% agarose plate and injected with the unpurified virus supernatant. A volume of 100 nl of the treatments (RNA1 RS13, RNA1 Vha26, RNA1 alpha COP) and controls (RNA1 eGFP, RNA1, no virus), obtained as described above, was injected into the haemolymph using a microinjector (FemtoJet, Eppendorf) and needles prepared with glass capillary tubes. Since the virus was produced in an *in vitro* cell based system where the cells were incubated in a growth medium, the medium

was included as an extra control in the experiment. This controls that the medium has no effect on the survival of the insects after injection. Sixteen adult flies were injected per treatment and this was repeated four times to give a total number of 64 adults injected per treatment. After injection, the flies were allowed to recover for 10 min in a horizontally placed 50 ml tube, and then transferred into 50 ml tubes containing 10 ml of diet and incubated at 25 °C and 65% RH. The flies were evaluated for mortality every day for 12 days. Four surviving insects per treatment were taken on the fourth day (the day with the highest observed mortality), pooled and homogenized in RLT buffer (Qiagen) + β-mercapto ethanol for RNA extraction, and stored in the buffer at -80 °C until further purification and transcript analysis. This was repeated for each replication of the bioassay (total number of infected adults collected per treatment for transcript analysis = 16).

2.10 Reverse transcription quantitative PCR (RT-qPCR) of insect mRNA

Transcript analyses were performed as described in chapter 2 and 3 using the primers in Table 12.

Table 12: Primers used in quantitative RT PCR

Target genes	Accession	Primer sequence (5′-3′)	Product size
alpha-coatomer protein, isoform A (alpha COP)	NM_058047.5	F-GGGTCAGAGCATCATTGCTT R-CTCCAGAGCGAGTCCAAATC	100bp
Ribosomal protein S13 (rps13)	X91854.1	F-CCGTCTGATTCTGGTCGAGT R-GCAGTGCTCGACTCGTATTTC	99bp
Vacuolar H[+]-ATPase 26kD E subunit (vha26)	U38198.1	F-GCACGCGACACTTAATACCC R-GTGAAAGCTGCACTTGATGG	99bp
Alpha-tubulin at 84B (αTub84B)	NM_057424.4	F- TGTCGCGTGTGAAACACTTC R-AGCAGGCGTTTCCAATCTG	96bp

The endogenous control gene, alpha-tubulin at 84B (*αTub84B*) was used for normalization of the data. The relative amounts of the target gene transcripts in the S2 cell samples with the engineered FHV containing the *D. melanogaster* target gene specific sequence were normalized to the endogenous reference gene by the equation ratio $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001). Appropriate controls, no-template control and no reverse transcriptase control, were also included in the assay.

2.11 Statistical analysis

Cell viability data between the treated groups was tested using ANOVA followed by Bonferroni's multi comparison tests. Survival data from treated *D. melanogaster* adults was analyzed according to the Kaplan–Meier method (Kaplan and Meier, 1958). The Gehan–Breslow–Wilcoxon and log-rank (Mantel–Cox) tests were used to compare the statistical significance ($p < 0.05$) between the datasets (controls and treatments). The Gehan–Breslow–Wilcoxon test measures more at early time points, while the log-rank (Mantel–Cox) test measures equally at all time points. The analyses were performed using GraphPad Prism v5.0 software (GraphPad, La Jolla, CA). For the qRT-PCR analysis, the differences between groups were calculated by an unpaired t-test ($p < 0.05$) and performed in qBase+ software.

3 Results

3.1 Organization and generation of recombinant FHV expressing *eGFP* as a reporter gene

The modified FHV vectors for targeted gene suppression in *D. melanogaster* were designed by inserting a reporter gene (*egfp*) and *D. melanogaster* target gene sequences under the control of the B2 subgenomic promoter as shown schematically in Fig. 25d. More specifically,

the insertion occurs after the critical residues necessary for the functioning of the B2 protein (Chao et al., 2005) (Fig. 26).

```
Wild type FHV B2      MPSKLALIQELPDRIQTAVEAAMGMSYQDAPNNVRRDLDNLHACLNKAKLTVSRMVTSLL
Recombinant FHV B2    mpsklaliqelpdriqtaveaamgmsyqdapnnvrrdldnlhaclnkakltvsrmvtsll
                      ************************************************************

Wild type FHV B2      EKPSVVAYLEGKAPEEAKPTLEERLRKLELSHSLPTTGSDPPPAKL
Recombinant FHV B2    ekpsvvaylegkapeeakptleerlrklelshslpttgseciw---
                      ************************************:
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Figure 26: Comparison between wild type FHV B2 and Recombinant FHV B2 protein. Alignment of recombinant B2 Protein amino acid sequence from recombinant FHV with wild type FHV B2 Protein amino acid sequence (P68831-B2_FHV) using CLUSTAL OMEGA (1.2.4)

This design results in the expression of *egfp*, which provides a robust marker for confirming the expression of the recombinant vectors in the transfected cells. Direct visualization of green fluorescence in S2 cells at 72 h post-transfection confirmed the expression of the recombinant FHV (Fig. 27a). As expected, eGFP fluorescence was not observed in the cells expressing the wild type FHV genome (pMT-FHV RNA1). However, we could confirm the presence of FHV by PCR in wild type FHV transfected cells which showed no fluorescence (Fig 27b).

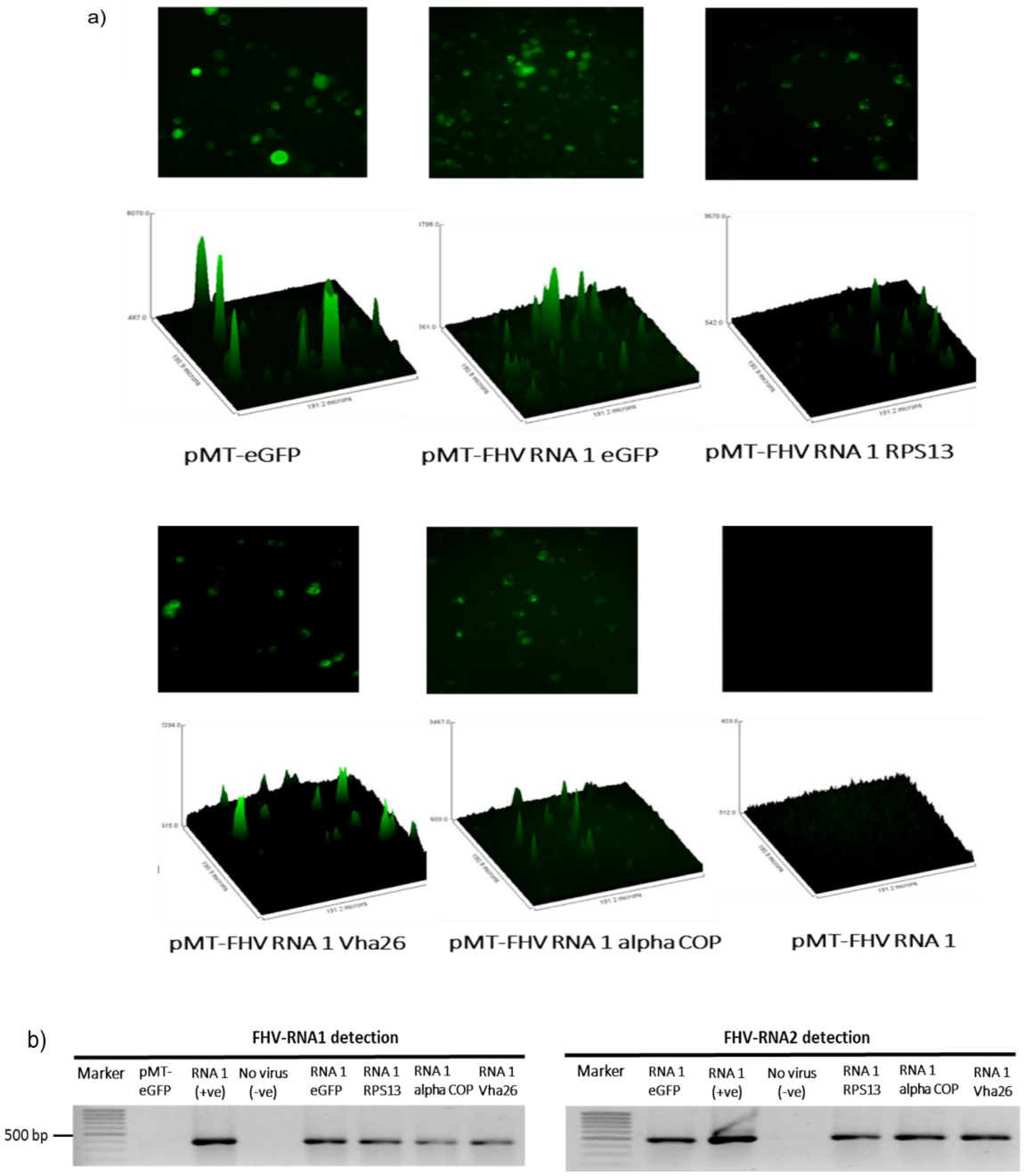

Figure 27: Detection of recombinant FHV RNA1 by fluorescence and PCR **(a)** Fluorescence microscopy analysis of S2 cells expressing the engineered FHV plasmids, containing an eGFP construct, indicating successful expression of the inserted gene. The FHV RNA1 plasmid, not containing an eGFP fragment, was used as negative control, while the plasmid expressing only eGFP without FHV was used as a positive control for eGFP expression. Nikon Eclipse TS-100 microscope and NIS Elements BR 4.11.00 imaging software (Nikon, Melville, NY) were used for the microscopy analysis, **(b)** PCR detection of FHV RNA1 and RNA2 expression.

3.2 Recombinant FHV can induce targeted gene suppression and mortality in *Drosophila* S2 cells

With the aim of evaluating the potential bioactivity of the infectious recombinant FHV, the infective virions were harvested from the cells and used to inoculate virus-free S2 cells. qRT-PCR analysis confirmed that the viral titers in the different treatments were comparable (Fig. 28).

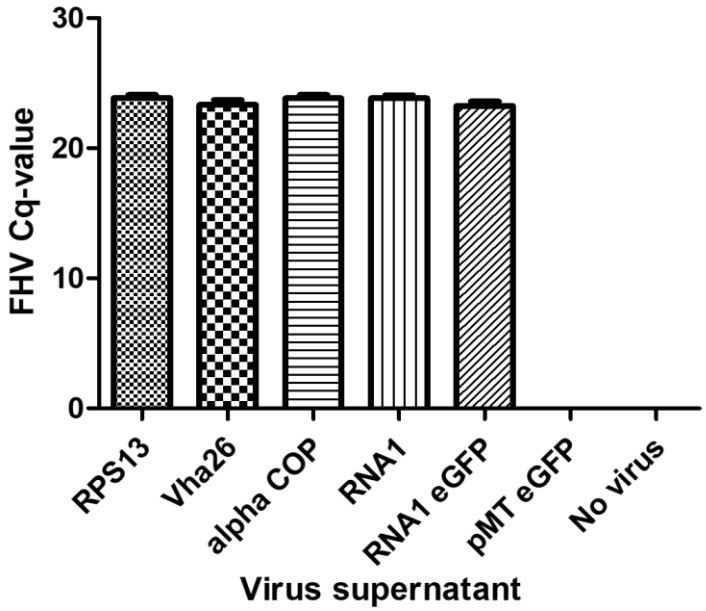

Figure 28: qRT-PCR confirmation that the viral titer was the same between the gene- targeting and non-gene targeting FHV inoculums for the *in vitro* and *in vivo* bioassays. Bars represent the mean ± SEM of the quantification cycle value (Cq-value). Different letters indicate statistically significant differences ($p < 0.05$).

At 72 h post-inoculation, a significant decrease ($p < 0.05$) in cell viability was observed for all the samples infected with the gene targeting recombinant virus, RPS13 (27 ± 9%), Vha26 (48 ± 13%), alpha COP (40 ± 10%) compared to the non-gene targeting controls, RNA1 eGFP (83 ± 10%), RNA1 (76 ± 11%), pMT eGFP (92 ± 6%) and No virus (94 ± 5%) (Fig. 29a and b).

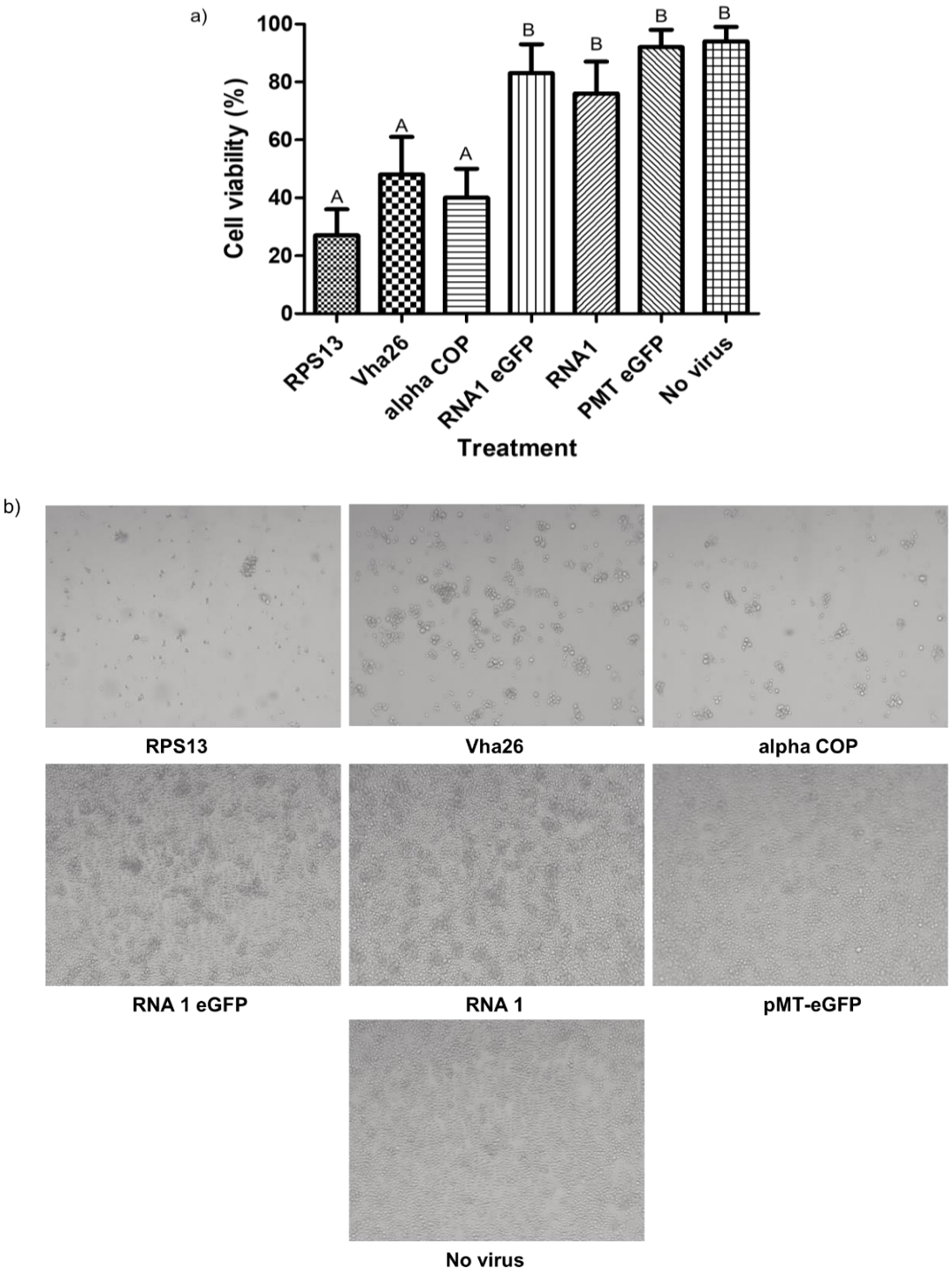

Figure 29: Cell viability at 72 h post-infection with a virus supernatant containing either the wild type FHV (RNA1), either of the four recombinant FHV (RPS13, Vha26, alpha COP and RNA1eGFP), or No virus controls (pMT eGFP and No virus) **(a)** Cell viability post-infection. Bars represent the mean ± SEM. Different letters indicate statistically significant differences ($p < 0.05$), **(b)** Images showing cell viability at 72 h post-infection.

Next, we verified whether the observed significant decrease in cell viability for the FHV target gene treatments was linked to the silencing of the targeted genes. To this end, qRT-PCR was performed on samples collected from the infected cells. The transcript level for RPS13 in the FHV RPS13 infected cells was reduced to 13 ± 3% compared to its transcript level in the FHV eGFP infected cells (Fig. 30). Similarly, for the FHV Vha26- and FHV alpha COP-infected cells, the transcript levels for Vha26 and alpha COP were reduced to 33 ± 12% and 35±16%, respectively, compared to their transcript levels in the FHV eGFP control (Fig. 30). No significant difference ($p > 0.05$) in expression of the targeted genes was observed between the controls, FHV eGFP, FHV WT (wild type) and No virus.

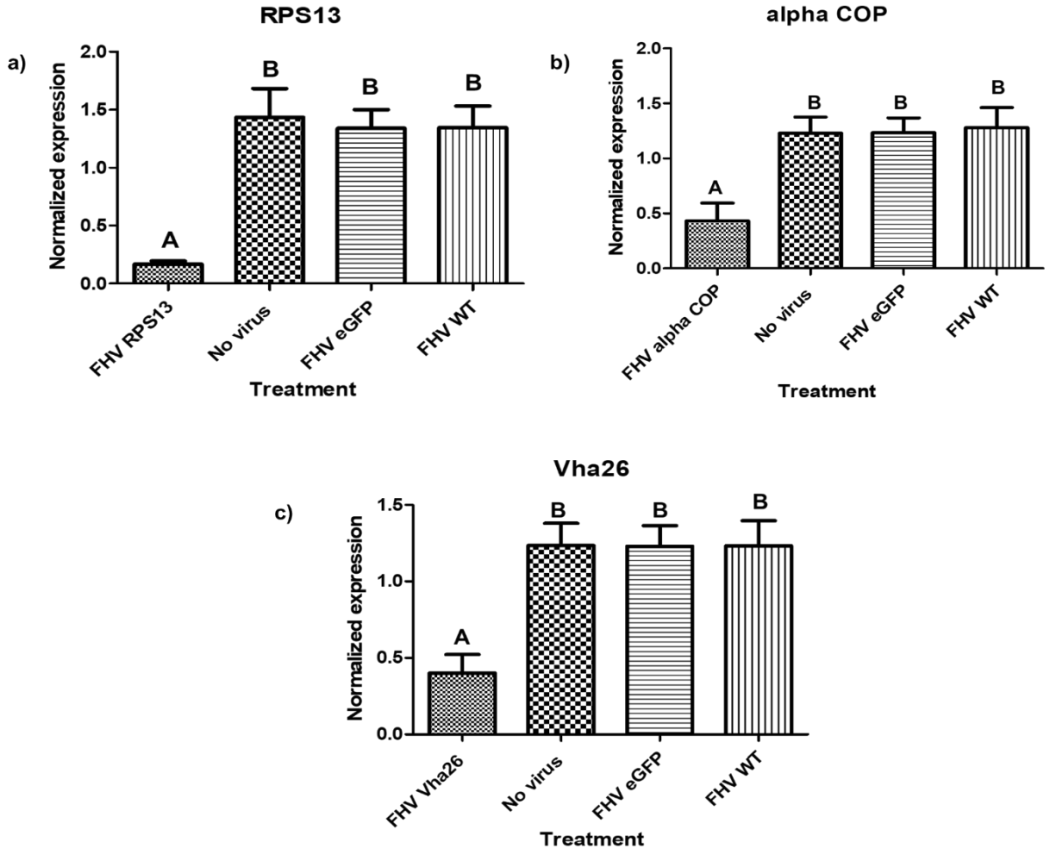

Figure 30: Target gene knockdown **(a)** *alpha COP*, **(b)** *vha26* and **(c)** *rps13* in *D. melanogaster* S2 cells at 72 h post-infection with the respective recombinant FHV (FHV alpha COP, FHV Vha26 and FHV RPS13) compared to the controls (No virus, wild type FHV and FHV eGFP). Bars represent the mean ± SEM. Different letters indicate statistically significant differences ($p < 0.05$).

3.3 Recombinant FHV can induce targeted gene suppression and mortality in adult *D. melanogaster*

Once the bioactivity of the recombinant FHV was confirmed *in vitro* in S2 cells, we next aimed to verify whether similar results could be obtained *in vivo* in live insects. To this end, *D. melanogaster* adult flies were infected by microinjection of FHV into the haemocoel and observed daily. Interestingly, between days four to six post-infection, a big difference could be observed in the survival rates between the groups treated with the recombinant virus expressing the target gene sequences (*rps13*, *vha26* and *alpha COP*) compared to the control groups (FHV eGFP, FHV WT, Medium and Water) (Fig. 31).

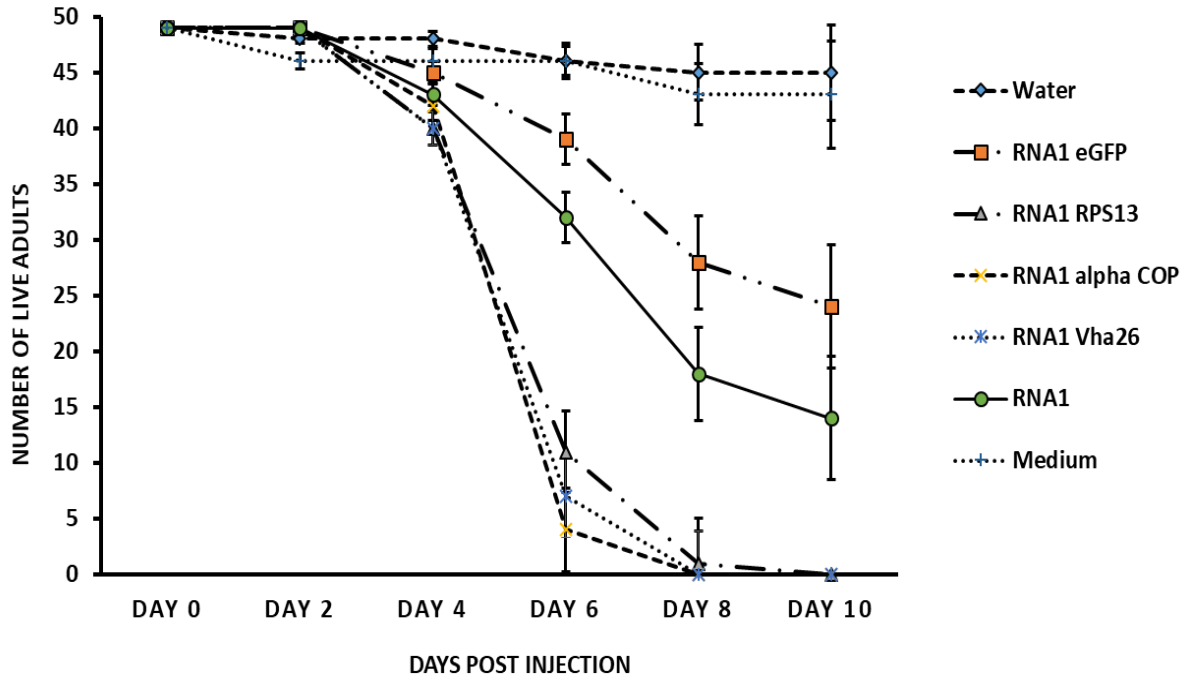

Figure 31: Effect of virus induced gene silencing (VIGS) in *Drosophila melanogaster* flies, using a designed FHV-based RNAi delivery system. Survival of adult flies over 10 days post-injection with a virus supernatant containing either the wild type FHV (RNA1), either of the four recombinant FHV (RNA1 RPS13, RNA1 Vha26, RNA1 alpha COP and RNA1eGFP), water or S2 cell culture medium. Mortality was analysed using a total of 48 insects per treatment.

By 10 days post-infection, none of the flies infected with the recombinant virus expressing the target genes survived (0%) in contrary to the control groups, where a significant proportion of the flies survived; FHV eGFP (49%), FHV WT (29%), Medium (88%), Water (92%) (Fig. 31).

To determine whether this observed difference in mortality between the recombinant FHV target genes treated groups and the control groups was linked to target gene silencing, samples were collected on the fourth day (day with first high mortality in test groups) for gene expression analysis. For the FHV Vha26-treated insects, a significant decrease ($p < 0.05$) in the transcript level for Vha26 (18 ± 5%) compared to the FHV eGFP-treated control was observed (Fig. 32).

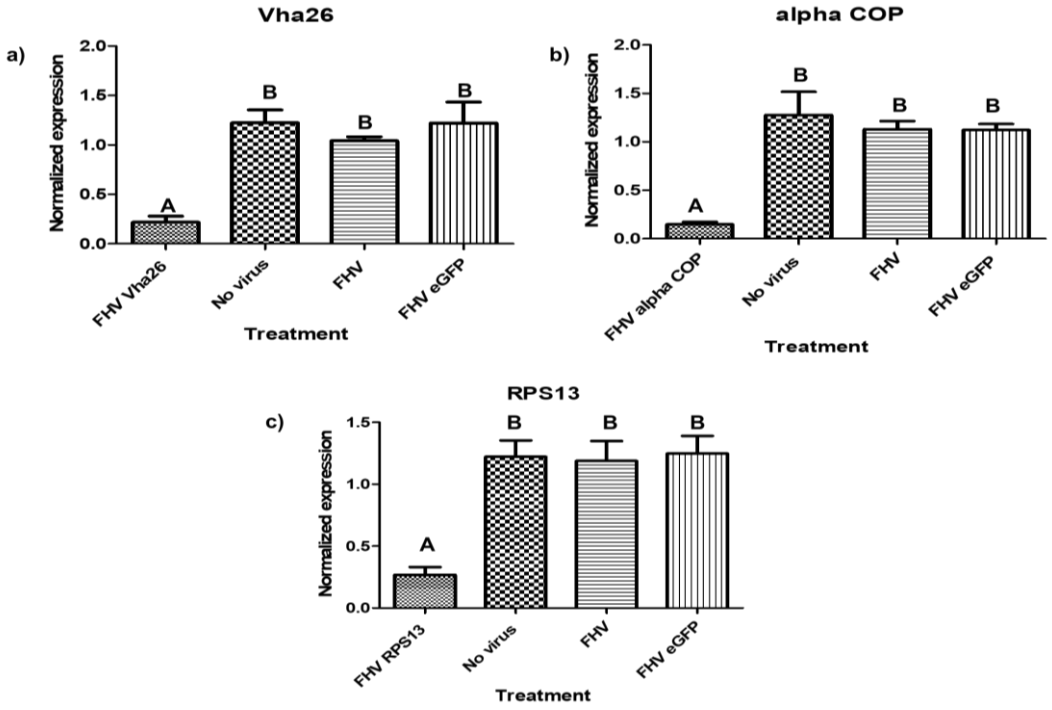

Figure 32: VIGS in *D. melanogaster* adults infected with the recombinant virus. Inhibition of the expression of the target genes **(a)** *vha26,* **(b)** *alpha COP* and **(c)** *rpS13* in *D. melanogaster* adults at 4 days post-infection with the respective recombinant FHV (FHV Vha26, FHV alpha COP and FHV RPS13) compared to the controls (No virus, wild type FHV and FHV eGFP). Transcript levels were analysed using 4 biological replicates of 4 pooled insects (n = 16) per treatment. Bars represent the mean ± standard error. Different letters indicate statistically significant differences ($p < 0.05$).

Similarly, for FHV alpha COP and FHV RPS13-infected insects, the transcript levels for alpha COP (11 ± 1%) and RPS13 (22 ± 2%) were significantly ($p < 0.05$) lower when compared to the control FHV eGFP-infected insects (Fig. 32). No significant difference ($p > 0.05$) in expression of the targeted genes was observed between the controls (FHV eGFP, FHV WT and Medium-injected insects (No virus)) (Fig. 32).

4 Discussion

In this study, we hypothesized that engineering FHV to express *D. melanogaster* target gene sequences could lead to VIGS when *D. melanogaster* cells are infected by recombinant clones of the virus. The findings of this study have three important implications. First, we show that a single stranded RNA insect virus can be engineered as a virus-based delivery system, for both *in vitro* and *in vivo* RNA silencing in *Drosophila*. Second, FHV is known to infect a wide range of insect species, hence this system could be easily adapted for RNAi- related studies in RNAi-recalcitrant insect species. Finally, by using RNAi mediated by the recombinant FHV, we have shown that targeting essential genes such as *rps13*, *alpha COP* and *vha26* causes cell mortality, which in turn leads to the death of the insect.

4.1 Generation of infective recombinant virions

In an efficient process, FHV RNA1 combines the properties of a message for an RNA replicase subunit with those of a template for replication by the same enzyme, to specifically direct its own replication in the cytoplasm of appropriate cells. To reconstruct this autonomous RNA replication system from cDNA clones, we based our strategy on Maharaj et al. (2014), where we inserted either *egfp* alone or *egfp* and one *D. melanogaster* target gene sequence (*rps13*, *alpha COP* or *vha26*) at position 3032 bp of FHV RNA1. Through a $CuSO_4$ inducible pMT vector system, primary transcripts of FHV RNA1 were expressed in the cytoplasm of the *Drosophila* S2 cells. These transcripts were designed to undergo ribozyme-mediated autolysis

to generate competent templates for self-directed RNA replication. Like FHV RNA1, FHV RNA2 transcripts were also designed to undergo ribozyme-mediated autolysis to generate competent templates for capsid protein expression. This was done in accordance with previous studies which have shown that minimizing terminal extensions at the 3'end of the FHV primary transcript is critical in generating RNA molecules which can replicate (Ball, 1995). Using eGFP as a convenient reporter, we demonstrated that all recombinant FHV RNA1 transcripts with an eGFP open reading frame (ORF) were expressed, as evidenced by green fluorescence emitted by the cells. Additionally, the replication of the virus was confirmed by the detection of the reverse genome of the virus. These findings are in line with previous studies, which have also demonstrated successful FHV replication and eGFP expression, by using a similar strategy as described in this study (Cho and Dreher, 2006; Zhou et al., 2015; Zhou and Kearney, 2017). Nevertheless, eGFP fluorescence was only used in this study for detecting the expression of the virus. We were more interested in obtaining infective virions which could infect and replicate in *Drosophila* cells. To this regard, we prepared an unpurified virus supernatant through cycles of freeze-thawing and finally centrifugation. This virus supernatant was used to infect virus-free cells, which were then verified after three days for the presence of the virus through eGFP fluorescence and transcript detection. The observation of fluorescence and detection of the reverse genome of the virus, confirmed its replication in the newly infected cells.

4.2 FHV-based RNAi delivery system is efficient *in vitro*

Besides the infectivity of the virus, we were also interested to know if the recombinant virus could induce targeted gene suppression in S2 cells. To this regard, we infected virus-free S2 cells with the unpurified virus supernatant and then evaluated the transcript level of the targeted genes when a visible decrease in cell viability was observed. Preliminary experiments had indicated that after concentrating and infecting new virus-free cells successively three

times, the resulting virus supernatant would cause less than 100% mortality after three days in newly infected cells. This data was vital for the planning on when to evaluate both cell viability and the transcript levels of the targeted genes in the surviving cells. Our results indicated a significant decrease in cell viability for all recombinant virus-infected cells, expressing *D. melanogaster* target gene sequences in comparison to the controls, which consisted of the recombinant virus expressing only eGFP and the wild type virus. Furthermore, transcript analysis of the treated samples indicated that mRNA levels for the targeted genes significantly decreased in the recombinant virus-treated samples in comparison to the controls. The correlation of a decrease in cell viability to the decrease in target gene transcripts could be explained by the essential role played by the expression products of these genes in the cell. Three target genes, *alpha COP*, *rps13* and *vha26*, were chosen to evaluate the designed FHV-based RNAi delivery system based on their essential functions, making them good RNAi targets (see chapters 2 and 3). By using a mosquito recombinant densovirus RNAi-based system, Gu et al. (2011) also reported that a 90% decrease in the expression of V-ATPase in C6/C36 cells led to increased cell mortality. However, in this case RNAi was triggered after expression of a short RNA hairpin by an RNA polymerase III promoter, which is predicted to be processed to a single siRNA by Dicer. In the FHV system, on the other hand, many different siRNAs are expected to be produced from the targeted gene region during RNA1 replication. Whether this results in more efficient gene silencing may require a direct comparison of both VIGS systems.

4.3 FHV-based delivery RNAi system is efficient *in vivo*

Once the ability of the virus-based RNAi delivery system was confirmed *in vitro* in cells, we proceeded to evaluate its efficiency *in vivo* in adult *D. melanogaster* flies. We used a simple bioassay set up, where we injected the flies with the same batch of unpurified virus supernatant used in the cell bioassays, and then evaluated for target gene silencing at the first signs of high

mortality. On day four after infecting the flies with the virus, we observed a slight increase in insect mortality. This was however not significantly different between the test groups (insects treated with the recombinant virus expressing the target genes) and the control groups (treated with either the recombinant virus expressing only eGFP or the wild type virus). Interestingly, from day six till day ten, we observed a significant difference in insect mortality between the test groups and the control groups. Samples collected on day four for transcript analysis, exhibited over 70% reduction in the transcript level for the target genes, explaining the significant increase in mortality observed in these test groups compared to the controls.

These data demonstrate that our engineered VIGS system successfully induces a highly efficient gene silencing, which leads to an increase in mortality compared to the controls, including the wild type virus infection. A possible limitation for the use of the designed FHV-based RNAi delivery system for functional genomics in live insects could arise from the fact that the FHV will eventually cause mortality in the insect, as it multiplies. This will make it difficult to study a phenotype other than mortality, which arises at a further point in the development of the insect, particularly for insects with long life cycles. Our results in this study indicated that up to 30% of the adult *Drosophila* flies survived for more than thirteen days following infection with the wild type FHV. Therefore, the FHV-based delivery system will only be practical if the expected phenotype arises before the virus causes mortality in the insect in question. A possible solution to decrease insect mortality and improve the FHV-based delivery system will be the use of a mild form of FHV to construct the delivery system. This could for instance be achieved through altered expression or mutations of the B2 protein (Han et al., 2011; van Cleef et al., 2014), which will lead to less pathogenic effects in its host. Picorna-like viruses, such as iflaviruses and dicistroviruses (to which FHV belongs), have been reported to often occur as quasi-species in which multiple viral forms complement each other to support infection (Ojosnegros et al., 2011). In this quasi-species population, mild variants/mutants are present and can be selected for further modification and production in cells lines, given

appropriate genetic methods. Once this mild recombinant virus infects the cell, the difference in pathogenic effect with the mild wild type-infected cell will be determined by the expected phenotypic effect from target gene silencing. In such case, recombinant FHV could trigger specific gene silencing and concomitant phenotypes in the relative absence of non-specific effects due to viral replication. In another study, where recombinant BmNPV was used to deliver dsRNA targeting a juvenile hormone esterase-related (JHER) gene in the Mediterranean corn borer, *Sesamia nonagrioides*, many non-specific pathogenic effects associated with disrupted metamorphosis were observed in both the tests and controls (Kontogiannatos et al., 2013), despite the restricted host specificity of BmNPV (Maeda et al., 1993). A solution for the wild type virus pathogenic effects was either the isolation or generation of incapacitated baculoviruses (that is baculoviruses that are deficient for an essential gene in the infection cycle, such as *ie-1* or *lef-8* (Efrose et al., 2010; Ioannidis et al., 2016)). In this way, the incapacitated baculoviruses can enter target cells and initiate early gene expression without progressing to the late phase of the infection cycle and cell lysis.

In this study, the unpurified virus was used for both the *in vitro* (cell) and *in vivo* (live flies) bioassays. This is not an uncommon source of inoculum for infection. In fact, Gu et al. (2011) reported that when *Aedes albopictus* larvae were infected with unpurified recombinant *Aedes aegypti* densovirus (AeDNV), the expression of V-ATPase was downregulated by nearly 70% compared to controls. Virus-based expression systems are particularly useful for their easy manipulation, higher transfection efficiency, longer-term expression, and more persistent silencing effects *in vivo* (Sliva and Schnierle, 2010). Further studies to improve the use of this FHV-based RNAi delivery system will involve evaluating the infectivity of the virus through the oral route. Although injection, as used in this study, is one of the most commonly used delivery methods for *in vivo* delivery, it is technically demanding. Hence, optimizing an oral delivery method will facilitate the usage of this novel virus-based RNAi delivery system. Another aspect to be evaluated is the mutational rate of the recombinant virus. This is important to verify the

changes that may occur in the inserted sequence in the virus genome after long term maintenance of the recombinant virus in cell cultures or in live *Drosophila* populations. This is because RNA viruses are known to exhibit high mutation rates (Elena and Sanjuán, 2005). Nevertheless, as reported in this study, this novel FHV-based RNAi delivery system can be exploited, for functional genomic studies in *Drosophila*, including the agriculturally important spotted wing drosophila (*D. suzukii*). Furthermore, the ability of FHV to infect many insect species, gives this developed virus-based delivery system a unique ability to be broadly used for functional genomic studies in different RNAi-recalcitrant insect species.

Chapter 5

General discussions and perspectives for future research

1 RNAi induction in *D. suzukii* through feeding in the context of field application

For RNAi to be used in pest control in the field, the target pest must ideally ingest dsRNA through the oral route. In many insects, it has been observed that RNAi through oral uptake of dsRNA works less efficiently than by injecting into the haemolymph (Upadhyay et al., 2011; Luo et al., 2013; Prentice et al., 2017). This is also the case in many dipteran species to which *D. suzukii* belongs. Several factors have been implicated to explain why oral RNAi often works less efficiently (Christiaens et al., 2014; Wang et al., 2016b; Spit et al., 2017). One of the most important ones is that many insects are capable of rapidly degrading dsRNA in their digestive system, by nucleases which are either present in the saliva, or expressed in the gut itself (Christiaens et al., 2014; Wynant et al., 2014b; Garcia et al., 2017; Spit et al., 2017). In Diptera, the stability of dsRNA in the gut has never been investigated, to the best of our knowledge. However, another important factor has been shown to affect RNAi efficiency in these species. A recent review has highlighted that all successful oral RNAi studies in Diptera were obtained using certain formulations, similar to the ones we used in our study (Christiaens et al., 2018). Whyard et al. (2009) reported successful silencing of the ubiquitously-expressed γ-tubulin gene (*γTub23C*) in four *Drosophila* species (*D. melanogaster*, *D. sechellia*, *D. yakuba*, and *D. pseudoobscura*) following feeding with conspecific dsRNA encapsulated in liposomes. Similarly, in the African malaria mosquito (*Anopheles gambiae*), successful silencing of two chitin synthase genes (*CHSI* and *CHSII*) was achieved by feeding the larvae with dsRNA/chitosan self-assembled nanoparticles. These studies all affirm the need to optimize cellular uptake of dsRNAs in Diptera for an improved RNAi efficiency.

In our oral RNAi experiments in *D. suzukii*, we also observed that feeding naked dsRNA did not elicit a strong enough silencing response for RNAi to be useful for future pest control applications. However, mixing the dsRNA with a liposome, often used to facilitate or enhance cellular uptake of nucleic acids, resulted in a clear silencing response and associated

phenotypical effects, implying that also in *D. suzukii*, cellular uptake might be a limiting factor for efficient oral RNAi using naked dsRNA. While the use of liposomes might not be ideal for field application, given the high cost of these products, alternatives could be further investigated as well, such as polymers, which are usually much cheaper to produce. In vertebrates, these polymers have shown to not only protect nucleic acids against nucleolytic degradation, but in some cases, can also improve cellular uptake (Patil and Panyam, 2009; Ardana et al., 2015; Sarett et al., 2016; Wang et al., 2016a; Zhou et al., 2018). The perceived advantages in using polymers (e.g. chitosan) such as being low-cost, enabling high-throughput evaluation of phenotypes (Mysore et al., 2014), and being nontoxic besides their biodegradable nature (Dass and Choong, 2008) make them a novel tool for dsRNA delivery in the context of pest control (Zhang et al., 2010). Chitosan polymers have been used to demonstrate gene knockdown effects in *A. gambiae* (Zhang et al. 2015) and diet-based delivery of chitosan nanoparticles suppressed gene expression in the Asian corn borer (He et al., 2013). Recently, topical application of pathogen-specific dsRNA for virus resistance in plants was reported (Mitter et al., 2017). DsRNA was loaded on designer, nontoxic degradable, layered double hydroxide (LDH) clay nanosheets, and the complex is referred to as “BioClay.” BioClay offered protection against cucumber mosaic virus (CMV) and pepper mild mottle virus (PMMov) in both local lesion and systemic infection assays. In a similar way, by spraying fruits with BioClay containing dsRNA targeting genes in *D. suzukii*, LDH-based nanoparticle technology could also be exploited to offer protection to fruits against *D. suzukii*.

The use of virus-like particles (VLPS) presents another interesting way of potentially delivering dsRNA into *D. suzukii* cells. VLPs are molecular vehicles assembled from key structural components of viral origin that have been repurposed to deliver a cargo different from the initial viral genome. The VLPs' components are proteins which participate in the formation of the viral capsid, and sometimes of the envelope as well. Although they do not retain their infective properties, VLPs are empty shells that are able to enter the respective target cells (Ludwig and

Wagner, 2007; Lund et al., 2010). Agricultural biotechnology could use VLPs and exploit certain properties that these particles possess, such as their capacity for packaging of foreign RNA to improve dsRNA delivery in *D. suzukii*. An example of a popular VLP system is derived from the tobacco mosaic virus (TMV) particle, which consists of its coat protein (CP) and has been the first macromolecular structure identified as capable of self-assembling *in vitro*. TMV carries a 300 nt stem-loop signal sequence in its RNA (origin of assembly, Oa), that interacts with CP to initiate packaging and capsid formation. Importantly, propagation of packaging in TMV is independent of RNA sequence, allowing for packaging of heterologous RNAs since only the initial nucleation requires the Oa sequence, while the process of elongation of packaging is independent of the sequence (Butler, 1999). This implies that foreign sequences engineered to contain the Oa sequence of TMV can be trans-packaged *in vitro*, inside the capsid protein of TMV, forming pseudo-virions (Smith et al., 2007, Maharaj et al., 2014). However, despite the efficiency in encapsidation, TMV does not infect or transverse the gut in insects and further engineering is required to achieve this goal, for instance by incorporation of peptide sequences in the CP protein to allow interaction with receptors in the insect gut epithelial cells. An alternative approach will be the use of viral CPs from viruses that can replicate in the plant host but are transmitted by the insect vector. Such viral CPs could be used as delivery vehicles of insecticidal molecules in the absence of formation of VLPs. For example, the RNA viruses of the family Luteoviridae replicate in plant hosts but are transmitted via a hemipteran vector in a persistent circulative non-replicative manner (Whitfield et al., 2014). After fusion of the CPs with an insect-specific peptide toxin and expression in *Arabidopsis* plants, the fusion proteins were found to cross the gut barrier into the haemocoel of the insect vector to deliver their aphicidal cargo (Bonning et al., 2014). Instead of fusion with a toxin, this property could be exploited by engineering CPs to transport dsRNA targeting *D. suzukii*, for instance by fusion with a dsRNA binding domain. However, for this to work several challenges will first need to be addressed such as, finding a virus that can infect both the fruit plant and *D. suzukii*, and engineering the viral CP to successfully carry the target dsRNA.

The exploitation of cell-penetrating peptides (CPPs) could also present an alternative way to improve dsRNA uptake in *D. suzukii*. The hydrophobic nature of the cell membrane makes it impenetrable to most peptides, proteins, and oligonucleotides. However, certain peptides known as CPPs have been observed to have the ability to pass through cell membranes (Cermenati et al., 2011; Chen et al., 2012; Hughes et al., 2012; Parsons et al., 2018). In general, CPPs are short (less than 35 amino acid residues), water-soluble and partly hydrophobic, and/or polybasic peptides with a net positive charge at physiological pH. Their ability to translocate across cell membranes is attributed to their highly cationic and partly hydrophobic structure and this could be exploited to transfer dsRNAs into insect cells. Chen et al. (2012) demonstrated that three arginine-rich CPPs (SR9, HR9 and PR9) were able to form stable complexes with plasmid DNA and deliver DNA into *in vitro* cultured *Spodoptera frugiperda* cells (Sf9 cells) in a non-covalent and non-cytotoxic manner. Similarly, in a recent study with live insects, Gillet et al. (2017) reported that a chimeric CPP, PTD-DRBD (peptide transduction domain-dsRNA binding domain), combined with dsRNA targeting *ChSII* in the cotton boll weevil (*Anthonomus grandis*), forms a ribonucleoprotein particle (RNP) that improves RNAi efficiency through feeding in the insect. They further reported that the RNP slowed down nuclease activity (probably by masking the dsRNA) and internalization in insect gut cells was achieved within minutes after plasma membrane contact, limiting the exposure time of the RNPs to gut nucleases. The RNP therefore provided an approximately 2-fold increase in the efficiency of insect gene silencing upon oral delivery when compared to naked dsRNA. Taken together, these studies demonstrate the potential of CPPs in improving dsRNA stability and cellular entry, representing a path towards the design of enhanced RNAi strategies against insect pests, including *D. suzukii*.

Nevertheless, moving towards an in-field application for *D. suzukii*, there are other aspects to consider as well. These fruit flies spend their larval life stages feeding inside the fruit in which they hatched from their egg. Therefore, conventional spraying applications of dsRNA will not

allow farmers to target these larvae, no matter which formulations are used to improve cellular uptake. And while there are several ways to get naked dsRNA into the plant, either via transgenic plants, via plant viruses expressing dsRNA or via root absorption/stem injection, these will only allow to expose the larvae to naked dsRNA. An alternative approach could be to target only adults, since they do not live inside the fruit, and could be targeted directly. In such an approach, bait sprays could be used. Bait spraying is a common method used in the control of fruit flies in the field (Vayssieres et al., 2009; Böckmann et al., 2014). The bait contains a protein attractant that is laced with an insecticide and is applied to the trunk of trees to attract and kill both male and female fruit flies. In the bait spray approach against *D. suzukii*, the insecticidal compound will be dsRNA-based and will be applied on the trunk of the trees to attract and kill the flies just before fruit maturation. However, the effectiveness of the bait approach will depend on both the ability of the bait to effectively lure the flies and on the efficacy of the dsRNA-based product as an insecticide. Current trapping strategies for *D. suzukii* are based on the use of traditional “fruit fly” traps baited with apple cider vinegar, vinegar and wine, or yeast/sugar water mixtures (Kleiber et al., 2014). Lure research has focused mainly on attractants based on fermentation products (Landolt et al., 2012b), and a synthetic lure based on these fermentation products, which is attractive to *D. suzukii*, has been formulated (Cha et al., 2014). The four-component (acetic acid, ethanol, acetoin and methionol) synthetic lure has been optimized to reduce the trapping of non-target insects (Cha et al., 2015) and has been developed into a commercially available product (TRÉCÉ Inc., Adair, OK, USA). Moreover, host plant volatiles from both fruit and leaves have also been identified as *D. suzukii*-specific feeding and oviposition attractants (Keesey et al., 2015). Another important aspect to consider when using baits is that insects vary in their response to baits depending on their physiological status and behavioural priorities (Browne, 1993). In *D. suzukii*, three studies have addressed its reproductive status and behavioural response to baited traps or odours. Burrack et al. (2015) reported that reproductively mature females with mature eggs, were generally caught in higher numbers early in the season before fruits were ripe and suitable for oviposition. Later

in the season, reproductively mature flies were caught in lower numbers when fruits were presumably more attractive than most of the baits tested. A recent follow-up study reported that *D. suzukii* aspirated off caneberries had higher egg loads than females caught in nearby traps (Swoboda-Bhattarai et al., 2017). Additional results from a Y-tube olfactometer bioassay further confirmed that mated *D. suzukii* females with mature eggs are highly attracted to fresh fruit odours (Revadi et al., 2015), although these odours were not tested against fermentation odours. The findings from these studies could be exploited to plan when in the crop growing season, baits used in combination with the developed RNAi-based product, could be placed in the field to achieve a maximum lethal effect in the *D. suzukii* population.

2 Virus induced gene silencing can be exploited to control *D. suzukii*

In this project, we opted to investigate a novel way of dsRNA delivery, namely virus-induced gene silencing (VIGS). Hoping to achieve an efficient and direct delivery of dsRNA into *D. suzukii* cells in this way, we developed and evaluated a FHV-based RNAi delivery system for *Drosophila*. We decided to use the model insect *D. melanogaster* for our proof of concept, since cell lines are available for this species. Our results in chapter 4 clearly indicated that the virus delivery system was functional and was efficient in inducing both target gene silencing and mortality in *Drosophila* cultured cells and live adults. However, we did not evaluate the effects of delivering the recombinant virus through the oral route yet. Future experiments where a known concentration of the virus is mixed with diet (using a similar experimental setup as described in chapter 3) and directly fed to *D. suzukii* will provide extra information to properly evaluate the potential of the FHV-based RNAi delivery system. Nevertheless, these results present the possibility of exploiting *Drosophila* specific viruses in developing virus-based RNAi control strategies for *D. suzukii*. In such a scenario, a naturally occurring *Drosophila* specific virus will be modified to express *D. suzukii* dsRNAs. Then, when this recombinant virus infects *Drosophila* spp, it will specifically cause faster mortality in *D. suzukii* due to target gene

silencing. Furthermore, such a system could be cost effective for farmers as they will just have to collect the dead infected *D. suzukii* (from infested fruits), crush them, dilute in water and spray on the field again to further control the residual *D. suzukii* populations on the field. Another route of applying the virus-based RNAi product could be through baited traps as discussed above. Rather than using a “lure and kill” strategy, a “lure and infect” strategy could be employed, where the baits could be designed to provide maximum exposure of the flies to the virus-based RNAi product without immediately killing the flies. This will allow the infected flies to spread the virus-based RNAi product to uninfected flies in the field, ultimately leading to a decrease in the fly population. The use of viruses in insect pest control is not an uncommon practice. Baculoviruses have been used for years and in many regions in the world to combat the infestation of corn fields by several Lepidopteran pests (Moscardi and Sosa-Gomez, 1992; Moscardi, 1999; Lacey et al., 2001; Szewczyk et al., 2006; Sosa-Gómez, 2017; Arthurs and Dara, 2018).

Although no natural viruses have been isolated from *D. suzukii*, potential target viruses can be inferred from numerous studies in *D. melanogaster*. *D. melanogaster* is susceptible to numerous viruses and is a powerful model to study host-pathogen interactions (Lemaitre and Hoffmann, 2007). The study of viral infection and antiviral defence in *Drosophila* spp. (Huszar and Imler, 2008; Webster et al., 2015) indicates that the majority of Drosophila viruses are RNA viruses, including the Sigmaviruses (Rhabdoviridae) (Berkaloff et al., 1965), drosophila C virus (DCV) (Jousset et al., 1972), the picorna-like nora virus (Habayeb et al., 2006), drosophila X virus (DXV) (Teninges et al., 1979), drosophila F virus (DFV) (Plus et al., 1975) and drosophila P virus (DPV) (Jousset et al., 1972; Plus et al., 1975). Two recent studies have reported the susceptibility of *D. suzukii* to two RNA viruses, DCV (which naturally infects drosophilids) and FHV (which only infects drosophilids under controlled conditions) (Cattel et al., 2016; Lee and Vilcinskas, 2017). The specificity of DCV to naturally infect only drosophilids and its moderate genome size of 9 kb makes it ideal for developing a virus-based RNAi delivery

system for *D. suzukii*. In such a system, the DCV (also a single stranded RNA virus as the FHV) will be modified to express *D. suzukii rps13*, *vha26* and *alpha COP* dsRNAs in a similar way to FHV, when it infects *Drosophila* cells. However, like with most genetically engineered organisms, several biosafety aspects will have to be evaluated prior to the use of such a virus-based RNAi delivery system for the field control of *D. suzukii*.

3 Safety aspects associated to the use of recombinant viruses for pest control

The evaluation of potential risks associated with the use of recombinant viruses should include at least two levels of enquiry: the virus which is genetically modified and the fate of the recombinant material (summarized in Table 13).

Table 13: Safety aspects associated with the use of recombinant viruses for pest control.

	Biosafety issues	Recommendation
Virus specificity	Infection of non-target species	Selection of viruses with restricted host range. Host range of used virus strain (infectious clone) should be evaluated during risk assessment, by performing when necessary in vitro or in vivo infection studies (such as infection of the cells of non-target organisms) in addition to traditional PCR and sequencing methods
Transgene	**1)** Transgene may present hazardous properties or change the vector properties **2)** Non-target effects of dsRNA sequence	**1)** Risk assessment should take into account the characteristics of the transgene (nature, stability, condition of expression), the construction/production process and the characteristics of the final recombinant vector (absence/presence of new properties compared to the virus backbone) and possible or known side effects related to the expression of the transgene. **2)** Bioinformatic analyses of sequence complementarity between the pool of siRNAs and target genes in non-target species
Recombination	Establishment of a new vector with novel biological and genetic properties: **a)** Genes that are interrupted or deleted in virus could be rescued during recombination, **b)** Transgene could be transferred to replication competent closely related viruses	Epidemiological data concerning the occurrence of natural closely related viruses in the area of administration should be analysed to consider the necessity for in vitro or in vivo co-infection studies (between the recombinant vector and the potential natural closely related virus)

At the virus level, the specificity aspect has to be seriously considered if the recombinant virus is to be exploited for pest control in the field. For example, several reports have shown that most baculoviruses are not infectious toward predatory or beneficial insects outside of the order Lepidoptera, or toward other non-targeted organisms. Hence, baculoviruses have unanimously been concluded in many studies as safe for use as pest-controlling agents (Kroemer et al., 2015). In contrast to baculoviruses, FHV is known to infect and replicate in insect cells of different insect orders (Dasgupta et al., 2003; Dasgupta et al., 2007). Therefore, while FHV might make a good model for genetic modifications as viral delivery vehicles of dsRNA under confined laboratory experiments, it could pose potential risks if applied as a pest control agent. The use of DCV in this case presents a better biosafety option.

Besides the specificity of the recombinant virus to its target insect pest, special attention should also be put into the selection of the target gene sequence which will be exploited through the virus to control the insect pest. Non-target effects could arise if the RNAi targeted gene in the pest insect shares high sequence similarity to that of other insects, especially beneficial insects. The risk in this scenario could arise when beneficial insects, such as generalist predators, feed on the primary pest insects containing the virus-derived dsRNAs targeting a gene in the pest. As such, if a high degree of similarity exists between the target gene sequence in the pest and the beneficial insect, this could lead to gene silencing in the beneficial insect as well.

Furthermore, the ecological consequences of the release of recombinant viruses must be experimentally addressed in terms of the competitive characteristics of recombinant vs. the wild-type viruses, both in the greenhouse microcosm and in the field. Insertions into a virus genome could reduce the replication efficiency of the resulting recombinant virus, could affect the ability of the viral nucleic acid to be encapsidated properly, or could limit the ability of the recombinant virus to move from cell-to-cell or long distance within the host by affecting the

folding of a native viral protein. However, considering that the virus-expressed insect-dsRNA is meant to accelerate the speed of kill of the target insect, and hence the recombinant virus itself in the process, this will imply that the modification does not confer any selective ecological advantage to the recombinant virus in comparison to the wild-type virus. In other words, compared with the wild-type virus, the recombinant virus is expected to show reduced fitness, resulting in lower concentration or even complete removal from the system if left alone.

Another possible concern associated with recombinant viruses is the potential of genetic recombination resulting in the foreign gene “jumping” from the recombinant virus to another organism. This could pose a risk if genes that are interrupted or deleted in a recombinant virus are either rescued during recombination or the transgene is transferred to replication competent closely related viruses. Recombination is a widespread phenomenon in viruses and can have a major impact on their evolution. Indeed, recombination has been associated with the expansion of viral host ranges, the emergence of new viruses, the alteration of transmission vector specificities, increases in virulence and pathogenesis, the modification of tissue tropisms, the evasion of host immunity, and the evolution of resistance to antivirals (Martin et al., 2011; Simon-Loriere and Holmes, 2011). Recombination seems to be highly frequent in some dsDNA viruses, where recombination is intimately linked to replication and DNA repair and can prevent the progressive accumulation of harmful mutations in their genomes. In contrast, recombination occurs at variable frequencies in (+)ssRNA viruses, with some families showing high rates (e.g. Picornaviridae), while others show only occasional (e.g. Flaviviridae) or nonexistent (e.g. Leviviridae) occurrence. The evolutionary reasons for the occurrence of recombination in RNA viruses are not clear. Since RNA viruses exhibit high mutation rates and large population sizes, it is more likely that these factors, rather than recombination, drive their evolutionary fate, as they regularly produce advantageous mutations and protect themselves from the accumulation of deleterious ones. Nevertheless, this does not exclude the possibility that natural selection can favour specific genotypes generated by recombination.

Several key factors limit or exclude the occurrence of genetic recombination between donor and recipient DNAs, including physical proximity (that is, localization in the same compartment within a single cell), degree of homology and similar modes of replication. However, if a recombinant virus pesticide is used long enough and at high concentrations in the field, it is expected that genetic recombination can eventually occur. In the field, as in laboratory conditions, such an occurrence is expected to be highest between highly homologous viruses (that is, the recombinant virus donor and wild-type virus recipient) that are infectious within the same host. The key question in terms of safety, however, is whether such recombination will result in an environmentally detrimental trait that will become fixed in the population. This will likely not be the case for recombinant viruses carrying a transgene for insect dsRNA expression, since a strong negative selection pressure arising from the rapid death of the target insect will lead to the recombinant virus being quickly outcompeted by the wild-type. Nevertheless, factors such as the homogeneity of the virus to be modified, the transgene and the possibility of recombination will have to be properly evaluated for each recombinant virus, prior to field application.

4 Conclusion

In conclusion, this PhD accomplished three major goals. First, we confirmed the functionality of the RNAi machinery in *D. suzukii*. Secondly, in view of potential field application, we demonstrated that oral RNAi was feasible in *D. suzukii*. Finally, we developed an RNAi delivery system to improve RNAi efficiency in *Drosophila* spp. These findings all confirmed the potential of RNAi technology as a possible tool in the development of a management strategy for *D. suzukii*. However, as discussed above, several more factors will have to be evaluated before an RNAi-based product targeting *D. suzukii* is available for field application.

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Summary

Drosophila suzukii, commonly known as spotted wing drosophila, was first described in Asia, but has recently become an invasive pest in many regions around the world, including Europe. Its movement into these new regions poses substantial risk to the fresh-fruit industry because, unlike most drosophila species, *D. suzukii* larvae feed on ripening and ripe fruit rather than overripe or rotting fruit. Moreover, *D. suzukii* is highly polyphagous and has been recorded feeding on many economically-important crops. Therefore, the dramatic expansion of *D. suzukii's* range has been accompanied by significant crop losses and large increases in pesticide usage. Because heavy pesticide use is unsustainable from economic and environmental standpoints, non-chemical strategies to manage *D. suzukii* are needed. Hence, this PhD evaluates the potential of RNAi technology as a tool in the development of a management strategy for *D. suzukii*.

We began the study by verifying whether the RNAi machinery was functional in *D. suzukii*. Both *in silico* and experimental data shown in **Chapter 2** indicated that the siRNAi core genes were present and that dsRNA introduced into the haemocoel of *D. suzukii* by injection leads to target gene silencing. The results clearly confirmed that the RNAi machinery is functional in *D. suzukii*, suggesting that it could be further developed and lead to an RNAi-based management strategy for *D. suzukii*. Moreover, the optimized microinjection protocol for *D. suzukii* that we developed in this study was also used to screen for potential target genes in *D. suzukii*. Based on gene silencing efficiencies and the resulting mortality caused in *D. suzukii, rps13*, *vha26* and *alpha COP*, were selected as promising target genes for use in further RNAi experiments designed to deliver dsRNAs through the oral route.

In view of potential field application, in **Chapter 3**, feeding assays were developed and used to deliver dsRNA targeting, *rps13*, *vha26* and *alpha COP* in *D. suzukii* adults and larvae. Surprisingly, feeding naked dsRNA did not cause any silencing or mortality to both *D. suzukii* adults and larvae. However, by encapsulating the dsRNAs in liposomes, cellular uptake was

significantly improved as evidenced in target gene silencing and the resulting mortality. Furthermore, when bacteria engineered to produce dsRNAs for targeting *D. suzukii rps13*, *vha26* and *alpha COP* were directly fed to adults and larvae, no gene silencing and mortality was observed. However, when the dsRNA was purified from the bacteria, encapsulated in liposomes and fed to adults and larvae, significant gene silencing and mortality was observed. These results showed that dsRNA can induce RNAi effects following delivery through the oral route in both adults and larvae. However, efficient uptake of the dsRNA from the gut into the cells was key to obtaining a successful RNAi effect in both adults and larvae. This implied that to further develop an RNAi-based control method for *D. suzukii*, a better RNAi delivery system was required.

Chapter 4 describes the engineering of FHV for improved delivery of RNAi to fruit flies, using *D. melanogaster* as a proxy for *D. suzukii*. Virus induced gene silencing presents an opportunity to exploit the natural ability of viruses to get into cells and replicate, to both deliver and mass produce dsRNAs targeting endogenous insect genes. As an initial proof of concept, we engineered FHV because of its small genome (hence easier to manipulate) and then tested it in the model system for fruit flies, *D. melanogaster*. Our results indicated that the FHV-based RNAi delivery system, designed to target *rps13*, *vha26* and *alpha COP* in *D. melanogaster*, could successfully lead to gene silencing and mortality, both *in vitro* in *Drosophila* cultured S2 cells and *in vivo* in adult flies. These results confirmed the possibility of exploiting virus induced gene silencing in developing an RNAi-based control strategy for *D. suzukii.* However, the further development of a VIGS system to control *D. suzukii* in the field will require a virus which specifically infects only *Drosophila*. Additionally, several biosafety aspects will have to be evaluated prior to its use in the field. Nevertheless, the data presented in this study all together confirms that RNAi could be exploited to develop a non-chemical RNAi-based control method for *D. suzukii.*

Samenvatting

Drosophila suzukii, algemeen bekend als de Aziatische fruitvlieg, werd voor het eerst beschreven in Azië, maar is recent een invasieve plaag geworden in heel wat regio's over de hele wereld, waaronder Europa. De introductie in deze nieuwe regio's vormt een aanzienlijk risico voor de fruitindustrie omdat, in tegenstelling tot de meeste *Drosophila*-soorten, *D. suzukii* larven zich voeden met rijpend en rijp fruit in plaats van overrijp of rottend fruit. Bovendien is *D. suzukii* zeer polyfaag en kan het zich voeden op heel wat economisch belangrijke gewassen. De introductie van *D. suzukii* in Europa en elders gaat gepaard met aanzienlijke verliezen van gewassen en ook met een aanzienlijke toename in het gebruik van pesticiden. Omdat het gebruik van chemische pesticiden vanuit economisch en ecologisch oogpunt niet houdbaar is, zijn niet-chemische strategieën voor het beheer van *D. suzukii* nodig. Dit doctoraat onderzoekt om die reden het potentieel van de RNAi-technologie als een hulpmiddel bij de ontwikkeling van een plaagbestrijdingsstrategie voor *D. suzukii*.

Onze studie begon met het nagaan of de RNAi-machine functioneel was in *D. suzukii*. Zowel *in silico* als experimentele data, weergegeven in **Hoofdstuk 2**, toonden aan dat de genen die centraal staan in de siRNAi-pathway aanwezig zijn en dat dsRNA, via microinjectie geïntroduceerd in het hemoceel van *D. suzukii*, leidt tot succesvolle silencing van het doelwitgen. De resultaten bevestigden dat de RNAi-machine functioneel is in *D. suzukii*, wat suggereert dat het verder zou kunnen worden ontwikkeld om een op RNAi gebaseerde bestrijdingsstrategie *voor D. suzukii* te bekomen. Bovendien werd het geoptimaliseerde protocol voor micro-injectie van *D. suzukii* in deze studie ook gebruikt om te potentiële doelwitgenen in *D. suzukii* te selecteren. Op basis van gensilencing-efficiëntie en de resulterende sterfte veroorzaakt in *D. suzukii*, werden *rps13*, *vha26* en *alfa-COP* geselecteerd als veelbelovende doelwit-genen voor gebruik in verdere RNAi-experimenten waarin dsRNA via de orale route wordt toegediend.

Met het oog op mogelijke veldtoepassing, werden in **Hoofdstuk 3** voedingsassays ontwikkeld en gebruikt om dsRNA, specifiek voor *rps13*, *vha26* en *alfa-COP*, toe te dienen in volwassen en larvale stadia van *D. suzukii*. Echter leidde het voeden van naakt dsRNA niet tot een verminderde genexpressie, noch tot fenotypische gevolgen bij volwassen of larvale stadia van *D. suzukii*. Gebruik van liposomen bij de toediening van dsRNA, om cellulaire opname te bevorderen, zorgde wel voor een duidelijke gen-silencing en de daaraan gekoppelde sterfte. Wanneer bacteriën, die genetisch gemanipuleerd werden om rps13-, vha26- en alfaCOP-specifieke dsRNA's te produceren, rechtstreeks gevoed werden aan *D. suzukii* adulten, werd geen gen-silencing of mortaliteit waargenomen. Toen het dsRNA echter werd opgezuiverd uit de bacteriën, en vervolgens opnieuw samen met liposomen werd gevoed aan adulten en larven, werd wel een significante gene silencing en mortaliteit waargenomen. De resultaten bekomen in dit hoofdstuk toonden aan dat orale toediening van dsRNA RNAi-effecten kan induceren bij zowel adulten als larven. Efficiënte cellulaire opname van het dsRNA uit de darm bleek echter de sleutel tot het verkrijgen van een succesvol RNAi-effect bij de Aziatische fruitvlieg. Dit had als gevolg dat om een RNAi-gebaseerde controlemethode voor *D. suzukii* verder te ontwikkelen, een geoptimaliseerd RNAi-toedieningssysteem vereist was.

Hoofdstuk 4 beschrijft het modificeren van Flock House Virus (FHV) voor een efficiënte toediening van dsRNA aan fruitvliegen. Hierbij werd het modelorganisme *D. melanogaster* gebruikt als een proxy voor *D. suzukii*. Virus-geïnduceerde gensilencing (VIGS) biedt een mogelijkheid om het natuurlijke vermogen van virussen om cellen binnen te komen en te repliceren, te gebruiken voor intracellulaire productie van dsRNA's die zich richten op endogene insectengenen. Voor een eerste proof-of-concept gebruikten we FHV vanwege zijn kleine genoom (dus gemakkelijker te manipuleren) en hebben we het vervolgens getest in het modelsysteem voor fruitvliegen, *D. melanogaster*. Onze resultaten toonden aan dat het op FHV gebaseerde RNAi-toedieningssysteem, ontworpen om *rps13*, *vha26* en *alfa-COP* in *D. melanogaster* te targeten, met succes kon leiden tot gen-silencing en mortaliteit, zowel *in vitro*

in *D. melanogaster* S2-celcultuur als *in vivo* bij volwassen fruitvliegen. Deze resultaten bevestigden de mogelijkheid om VIGS te gebruiken bij het ontwikkelen van een op RNAi gebaseerde controlestrategie voor *D. suzukii*. De verdere ontwikkeling van een VIGS-systeem voor de bestrijding van *D. suzukii* in het veld vereist echter een virus dat specifiek alleen *Drosophila* infecteert. Bovendien moeten verschillende bioveiligheidsaspecten worden geëvalueerd voorafgaand aan het gebruik ervan in het veld. Desalniettemin bevestigen de in deze studie gepresenteerde resultaten dat RNAi kan worden gebruikt voor de ontwikkeling van een niet-chemische RNAi-gebaseerde controlemethode van *D. suzukii*.

Curriculum vitae

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EDUCATION

2014 - 2018 **Faculty of Bioscience Engineering, Ghent University, Belgium**

PhD in Applied Biological Sciences
Thesis: RNA interference technology as a potential control method for Spotted Wing Drosophila (*Drosophila suzukii*)

2011 - 2012 **Faculty of Sciences (Plant Biotechnology and Bioinformatics), Institute of Plant Biotechnology for developing countries – Ghent University, Belgium**

Postgraduate Diploma - Biosafety in Plant Biotechnology

2009 - 2011 **Faculty of Bioscience Engineering, Ghent University, Belgium**

MSc. in Nutrition and Rural Development – Tropical Agriculture – Plant production
Thesis: Molecular analyses of *Agrobacterium* spp. T-DNA insertions in *Ipomoea batatas* (Sweet potato) lines.

2007 - 2009 **Faculty of Science, University of Dschang, Cameroon**

MSc. in Biochemistry – Clinical Biochemistry and Pharmacology
Thesis: *In vitro* Antisalmonellal properties of *Euphorbia prostrata* and the evaluation of its acute and sub chronic toxicity.

2003 - 2006 **Faculty of science, University of Buea, Cameroon**

Bsc. honours in Biochemistry (Major) and Medical Laboratory Technology (Minor)

LIST OF A1 PEER REVIEWED PUBLICATIONS

1) **Taning, Clauvis NT**, Olivier Christiaens, XiuXia Li, Luc Swevers, Hans Casteels, Martine Maes, and Guy Smagghe. “Engineered Flock House Virus for targeted gene suppression through RNAi in fruit flies (Drosophila melanogaster) in vitro and in vivo.” Frontiers in physiology (2018). (Submitted).

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ORAL PRESENTATIONS

1) **Taning, Clauvis NT**. Control of *Drosophila suzukii* using RNA interference technology." In The 1st Han Hong Science and technology forum of Southwest University. Chongqing, China (2017)
2) **Taning, Clauvis NT**. "*Drosophila suzukii*: An invasive pest with the potential to attack wild berries in the North." In Sustainability of Wild Berry Production in the North. Helsinki, Finland (2016).
3) **Taning, Clauvis NT**. "RNA Interference (RNAi) Technology: Possible Applications and Predictive Risks." In Dual use of innovative technologies. Poznan, Poland (2014)

CONFERENCE POSTER

1) Ericmar Avila dos Santos, **Taning Clauvis NT ***, Olivier Christiaens, Moises Zotti, Guy Smagghe. Engineering of the *Halyomorpha halys virus* for RNAi in *Euschistus heros*. 70th International Symposium on Crop Protection. Ghent, Belgium (2018). *Equal first author
2) Colmenarez Ortiz, Claudia, and **Taning Clauvis NT**. "Consequences of Coexistence Policies on the Environment: A Different View." In The Sixth International Conference on Coexistence between Genetically Modified (GM) and non-GM based Agricultural Supply Chains. Lisbon, Portugal (2013).

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I could keep going, but the list would never end. However, I will have to end somewhere……

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