

# Comparing different siRNA delivery systems to target *Diuraphis noxia*

by

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## Declaration

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## Abstract

*Diuraphis noxia*, also known as the Russian wheat aphid, is a major pest of wheat. Breeding for resistance against *D. noxia* has been relatively successful in wheat as there has been many resistance genes incorporated into wheat in the past. However, this resistance has more often than not been counteracted by *D. noxia* through the development of a new biotype. The mechanism with which *D. noxia* is able to do this is not well understood. Previously, a highly virulent, laboratory generated biotype, known as SAM (South African Mutant), was compared to its avirulent progenitor, SA1, through proteome analysis of the salivary glands and complete genome sequence analysis. It was found that, among other differences, the cuticle protein, *Dncpr1-8*, containing a Rebers and Riddiford consensus was present in the salivary gland of SAM but not SA1. The gene also contained single nucleotide polymorphisms (SNPs) between the biotypes. In this study the function of *Dncpr1-8* was investigated through RNA interference (RNAi). As RNAi has never been performed in *D. noxia*, several methods of siRNA delivery to this organism were compared. Injection of siRNA into the aphid haemolymph and ingestion of siRNA through artificial feeding medium was not successful. Allowing *D. noxia* to feed on wheat inoculated with a virus-induced gene silencing (VIGS) vector modified to contain *D. noxia* transcript sequence was partly effective, but overall had variable results. Finally, siRNA delivery through injection into wheat and allowing *D. noxia* to feed around the injection site, proved to be the most effective. Delivery of *Dncpr1-8*-siRNA using this method resulted in reduced survival and fecundity of biotype SAM while feeding on resistant wheat. The phenotypic responses were then compared to that of another aphid species, *Myzus persicae*, feeding on *Arabidopsis thaliana* injected siRNA targeting the same gene. *M. persicae* did not display reduced survival, but did produce fewer nymphs. Collectively, the results were then used to draw conclusions on the putative function of *Dncpr1-8* in the plant-aphid interaction.

## Uittreksel

*Diuraphis noxia*, ook bekend as die Russiese koringluis, is 'n belangrike plaag van koring. Koring wat weerstandig is teen *D. noxia* is met relatiewe sukses geteel omdat vele weerstandbiedende gene al voorheen in koring geïnkorporeer is. Hierdie weerstand word dikwels afgebreek deur *D. noxia* deur die ontwikkeling van 'n nuwe biotipe. Die meganisme waardeur *D. noxia* nuwe biotipes vorm word nog nie goed verstaan nie. 'n Hoogs virulente laboratorium-gegenereerde biotipe, bekend as SAM (Suid-Afrikaanse Mutant), was

voorheen vergelyk met sy stamvader, SA1, deur middel van proteïoomanalise van die speekselkliere asook deur volledige genoömanalise. Onder andere was daar gevind dat die kutikula-proteïen, *Dncpr1-8* (wat 'n Rebers en Riddiford konsensusvolgorde bevat), teenwoordig was in die speekselklier van SAM, maar nie in SA1 nie. 'n Enkel-nukleotiedpolimorfisme was ook tussen die twee biotipes opgemerk. Die funksie van *Dncpr1-8* was deur middel van RNS-inmenging (RNSi) in hierdie studie ondersoek. Verskeie klein inmengende-RNS (kiRNS)-toediendingsmetodes was met mekaar vergelyk, aangesien RNSi nog nie van tevore in *D. noxia* uitgevoer is nie. Toediening via die inspuit van kiRNS direk in die hemolimf van die plantluis en inname van kiRNS deur kunsmatige voeding was nie suksesvol nie. *D. noxia* wat voed op koring wat geïnkuleer is met 'n virus-geïnduseerde geen onderdrukingsvektor wat gemodifiseer is om 'n *D. noxia*-transkripsievolgorde te bevat was gedeeltelik suksesvol, maar die resultate was inkonsekwent. Laastens was kiRNS in koringblare ingespuit en *D. noxia* toegelaat om rondom die inspuitingsarea te voed – hierdie metode was die effektiëste. Toediening van *Dncpr1-8*-siRNS deur middel van hierdie metode het tot 'n verminderde oorlewing en vrugbaarheid van biotipe SAM gelei terwyl dit op weerstandige koring gevoed het. Hierdie fenotipiese reaksies was met 'n ander plantluispesie, *Myzus persicae*, vergelyk. Dit het op *Arabidopsis thaliana* gevoed, wat ingespuit is met kiRNS wat dieselfde geen, *cprr1-8*, teiken. *M. persicae* het nie verminderde oorlewing getoon nie, maar het wel minder nimfe produseer. Gesamentlik was die resultate gebruik om gevolgtrekkings oor die vermeende funksie van *Dncpr1-8* in plant-plantluis interaksies te formuleer.

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## List of Abbreviations

<b>5caC</b>	5-carboxylcytosine
<b>5fC</b>	5-formylcytosine
<b>5hmC</b>	5-hydroxymethylcytosine
<b>5mC</b>	5-methylcytosine
<b>AM</b>	Active modification
<b>ANOVA</b>	Analysis of variance
<b>AR</b>	Active restoration
<b>BER</b>	Base excision repair enzymes
<b>bp</b>	Base pair(s)
<b>BSMV</b>	Barely stripe mosaic virus
<b>CAF</b>	Central Analytical Facility
<b>CAT</b>	Catalase
<b>cDNA</b>	Complementary DNA
<b>CDS</b>	Coding domain sequence
<b>CpG</b>	Cytosine followed by a guanine base
<b>dATP</b>	Deoxyadenosine triphosphate
<b>dH<sub>2</sub>O</b>	Distilled water
<b>Dn</b>	<i>Diuraphis noxia</i>
<b>Dnmts</b>	DNA methyltransferases
<b>dNTP</b>	Equal volumes of deoxyadenosine triphosphate, deoxyguanosine triphosphate, deoxythymidine triphosphate and deoxycytidine triphosphate
<b>dsRNA</b>	Double stranded RNA
<b>GamR</b>	Gamtoos-R wheat cultivar containing <i>Dn7</i>
<b>GamS</b>	Gamtoos-S wheat cultivar lacking <i>Dn7</i>
<b>H</b>	A, C or T
<b>hpi</b>	Hours post introduction
<b>IPTG</b>	Isopropyl $\beta$ -D-1-thiogalactopyranoside
<b>Kb</b>	Kilobase pair(s)
<b>LB</b>	Luria Broth
<b>MOPS</b>	4-morpholinepropanesulfonic acid
<b>Mp</b>	<i>Myzus persicae</i>
<b>NGS</b>	Next-generation sequencing
<b>NiLs</b>	Near isogeneic wheat lines
<b>nt</b>	Nucleotide
<b>PCR</b>	Polymerase chain reaction
<b>PDS</b>	Phytoene desaturase
<b>POX</b>	Peroxidase
<b>RISC</b>	RNA-induced silencing complex
<b>RNAi</b>	RNA interference
<b>ROS</b>	Reactive oxygen species

<b>RT-qPCR</b>	Reverse transcription quantitative PCR
<b>SD</b>	Standard deviation
<b>SEM</b>	Standard error of the mean
<b>siRNA</b>	Small interfering ribonucleic acid
<b>TAE</b>	2-amino-2-(hydroxymethyl)-1,3-propanediol, acetic acid and ethylenediaminetetraacetic acid
<b>TDG</b>	Thymine DNA glycosylase
<b>TE</b>	Transposable elements
<b>TET</b>	Ten-eleven translocase
<b>Tris</b>	2-Amino-2-(hydroxymethyl)propane-1,3-diol
<b>TSS</b>	Transcription start site
<b>UK</b>	United Kingdom
<b>USA</b>	United States of America
<b>VIGS</b>	Virus-induced gene silencing
<b>X-gal</b>	5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside

# **Chapter 1**

## **Introduction**

## 1.1 Introduction

Wheat is one of the most important food crops in the world. Compared to other crops, wheat is planted on the most land area in the world. Over 220 million hectares of wheat was planted in 2016, followed by maize at almost 188 million hectares. Wheat was the 3<sup>rd</sup> most-produced crop in the world, at almost 750 million tons generated in 2016 (Food and Agriculture Organization of the United Nations 2018). Wheat is not only an important source of carbohydrates, but also of protein, B vitamins and dietary fiber. It is mostly consumed in the processed form as bread, biscuits and breakfast cereals and is becoming more popular as people are converting to a more westernized diet (Shewry and Hey 2015). In South Africa, more wheat is consumed than what is produced, but it is nonetheless a large industry producing an average of between 1.3 to 2 million tons from 2004 to 2015. The Western Cape Province is the largest area of wheat production followed by the Northern Cape and the Free State (Department of Agriculture, Forestry and Fisheries, South Africa 2010).

*Diuraphis noxia* (Kurdjumov, Hemiptera: Aphididae), also known as the Russian wheat aphid, is a major pest of wheat, responsible for large economic losses (Burd and Burton 1992). It occurs in all major wheat producing countries with a preference for a dry environment compared to other aphid pests of wheat. *D. noxia* infestation of wheat result in chlorotic streaking, stunted growth and plant death in severe cases (Goggin 2007). Like other aphid species, *D. noxia* is capable of a high reproduction rate, resulting in large scale damage to wheat fields if left uncontrolled (Davis 2012). Although *D. noxia* can be controlled with pesticides, breeding wheat that has resistance to this pest is preferred from an economic and environmental perspective.

Breeding for *D. noxia* resistance in wheat has been fairly successful as a number of resistance genes have been incorporated into wheat varieties. These resistance genes are however frequently overcome by *D. noxia* through the development of a new biotype. A new biotype is defined by an aphid's ability to feed on a wheat cultivar previously considered to be resistant. This biotype is now considered virulent to the previously resistant cultivar it is able to feed on (Botha 2013). Of the seventeen *D. noxia* resistance genes present in wheat cultivars, only *Dn7* (and possibly a new gene, *Dn10*) confers resistance to all the biotypes currently found in the USA and South Africa (Li *et al.* 2018). The remaining resistance genes have been broken down by biotypes with varying virulence levels, 4 of which are found in South Africa and 8 in the USA (Jankielsohn 2016). Breeding for pest resistance is a process that takes time and considerable financial investment. In order to allow informed breeding and sustainable management of the available resistance, a better understanding of the

interaction between plant and aphid, with specific focus on the formation of virulence, is required.

Previously, a highly virulent laboratory generated *D. noxia* biotype, South African Mutant (SAM), was compared to its relatively avirulent progenitor, SA1. Specifically, a proteome comparison of the salivary glands as well as a whole genome sequence analysis was performed (Van Zyl 2007; Cloete 2015). A few differences between the biotypes were observed during these analyses, among which *Dncpr1-8* was found to be present in the salivary gland of SAM, but not SA1. The gene encoding this protein also contained SNPs observed during sequence comparison.

It is believed that the interaction between wheat and RWA is based on the gene-for-gene principle wherein the wheat plants' R gene product recognizes the product of the aphid's effectors, likely a salivary protein (Lapitan *et al.* 2007), to induce its defense response. Virulent aphids, like SAM, have the ability to avoid this recognition (Botha 2013; Botha *et al.* 2014) and feed on the host unhindered. These aphids may also have proteins that protect them from products produced by the host during its defense response. It is further hypothesized that *Dncpr1-8* may be such a protein.

## 1.2 Aim and objectives

The aim of this study was to identify or develop a method of siRNA delivery to *Diuraphis noxia* to investigate the putative function of *Dncpr1-8* in the *D. noxia*-wheat model through RNA interference. It was also to investigate the function of this protein in another aphid species, *Myzus persicae*.

The following objectives were set to achieve this aim:

1. Validation of SAM genome sequence and *in silico* gene prediction of *Dncpr1-8* with Sanger sequencing of DNA and cDNA.
2. Compare the efficiency of the different siRNA delivery methods by evaluating the phenotypic effect seen in *D. noxia* using the well-studied gene, *c002* as reference. These siRNA delivery methods include:
  - 2.1. aphid feeding on artificial medium containing siRNA,
  - 2.2. injection of siRNA into the aphid haemolymph,
  - 2.3. aphid feeding on wheat inoculated with a virus-induced gene silencing vector modified to contain *D. noxia* transcript sequence and
  - 2.4. aphid feeding on wheat injected with siRNA.

3. Functional characterization of *Dncprr1-8* in *D. noxia* by delivery of *Dncprr1-8*-siRNA using the most effective method determined in Objective 2.
  - 3.1. Determine survival rate and fecundity
  - 3.2. Perform biochemical measurements of aphid and plant
  - 3.3. Perform RT-qPCR to confirm gene silencing
  
4. Functional characterization, of *Dncprr1-8* in *D. noxia* by delivery of *Dncprr1-8*-siRNA in an environment free of plant defense compounds, i.e. through artificial feeding medium
  - 4.1. Determine survival rate and fecundity
  - 4.2. Perform RT-qPCR to confirm gene silencing
  
5. To investigated DNA methylation patterns in an attempt to explain *Dncprr1-8* regulation
  
6. Functional characterization of *cpr1-8* in another aphid species, *M. persicae* through *Mpcprr1-8*-siRNA delivery to *M. persicae* using the most efficient method determined in Objective 2.
  - 6.1. Determine survival and fecundity

## 1.2 Thesis layout

Firstly, in **Chapter 2**, literature on *D. noxia* and the interaction with its host is reviewed. An overview of genes believed to be associated with virulence in *D. noxia* based on previous studies is also given. Information from literature on RNAi and the different methods to deliver siRNA and dsRNA to aphids and other insects is presented. A gene commonly used in aphid RNAi studies, *c002*, is investigated next. DNA methylation in insects is also reviewed and finally a different aphid species, *M. persicae* is introduced.

In **Chapter 3**, the DNA and transcript sequences of *Dncprr1-8* are characterized. Thereafter, four methods of siRNA delivery to *D. noxia* are compared and a novel method developed. Using the most effective delivery method, the putative function of *Dncprr1-8* was studied through RNA interference. The potential effect of DNA methylation patterns on *Dncprr1-8* expression was also investigated.

**Chapter 4** describes the investigation into the putative function of *cpr1-8* in another aphid species, namely *Myzus persicae*. This was done using the siRNA delivery method developed in Chapter 3.

**Chapter 5** summarizes the major findings of this study.

## 1.3 Research outputs

### Participation at conferences or symposia

Swiegers, H. W. and A-M. Botha (2016) Comparing different siRNA delivery systems to target *Diuraphis noxia*. 22nd International Plant Resistance to Insects Symposium, 6-8 March, Stellenbosch, South Africa (Presentation).

Botha, A-M. and H. W. Swiegers (2016) Studying host-insect interactions using viral induced gene silencing and siRNA. 4th International Conference on Plant Genomics, 14-15 July, Brisbane, Australia (Presentation – On invitation).

Swiegers, H. W. and A-M. Botha (2017) Using dsRNA production in wheat to induce RNA interference in *Diuraphis noxia* RR1. 13th International Wheat Genetics Symposium, 23-28 April, Tulln, Austria (Poster).

Swiegers, H. W. and A-M. Botha (2017) A novel method: dsRNA production in wheat to induce RNA interference in *Diuraphis noxia* RR1. 3rd Hemipteran-Plant Interaction Symposium (HPIS 2017), 4-8 June, Madrid, Spain (Poster).

Swiegers, H. W. and A-M. Botha (2018) Ingestion of *cprr1-8*-siRNA reduce virulence in both *Diuraphis noxia* and *Myzus persicae*. 23rd International Plant Resistance to Insects Symposium, 7-9 March, Harpenden, United Kingdom (Presentation).

### Chapter 3 submitted for review

Swiegers, H. W. and A-M. Botha Silencing of cuticle protein (RR-1) containing the chitin-binding Rebers and Riddiford consensus decrease virulence in *Diuraphis noxia* biotype SAM. Journal of Experimental Biology (submitted) (MS ID#: JEXBIO/2018/179614)

### Patent

Botha-Oberholster, A-M, H. W. Swiegers and N. F. V Burger. "siRNA FOR CONTROLLING PEST INFESTATIONS AND METHOD OF USE". South African application number: P3482ZA00. Filed: 2018/03/05. **Appendix 1.**

## 1.4 References

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## **Chapter 2**

### **Literature review**

## 2.1 *Diuraphis noxia*

*Diuraphis noxia* (Kurdjumov) (Homoptera: Aphididae), also known as the Russian wheat aphid, is an aphid with a green elongated body. It feeds mainly on the phloem of cereal grasses like bread wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.). Since its introduction to South Africa in 1987, *D. noxia* has been responsible for massive economic losses (Burd and Burton 1992). Currently, *D. noxia* also occurs in other wheat producing countries like Canada, Argentina, Chile, USA and recently Australia (Yazdani *et al.* 2017).

*Diuraphis noxia* has the ability to reproduce sexually, as well as asexually through parthenogenesis. A high multiplication rate is observed during asexual reproduction, which contributes to the spread of *D. noxia*, causing major damage to crop fields (Burd and Burton 1992). The annual alternation between sexual and asexual reproduction is known as cyclical parthenogenesis (Davis 2012). Sexual reproduction is rarely seen in *D. noxia*, which is supported by the fact that a male *D. noxia* has never been found in South Africa (Botha 2013). Parthenogenic females are also viviparous, meaning that they give birth to live young, which further contributes to the rapid reproduction rate of *D. noxia*. These two characteristics ensure for the very short generation cycle of *D. noxia* (Goggin 2007).

Typical symptoms of a *D. noxia* infestation seen in wheat include purple discoloration, white streaking, stunted growth, leaf rolling and plant death under severe infestation (Burd and Burton 1992; Goggin 2007) (Figure 2.1). *Diuraphis noxia* and its main host, bread wheat, are in a continuous evolutionary arms race as stated by Botha (2013). *D. noxia*, unlike many other aphids, is a specialist aphid species that has a small plant host range. Thus, as wheat evolves a defense mechanism against *D. noxia*, the aphid must counter-adapt by evading the defense mechanism in order to survive. This phenomenon would cause the appearance of a new *D. noxia* biotype. A biotype is defined as a population with the capability to damage a plant variety containing genes previously resistant to the biotypes present at that time (Shufran and Payton 2009; Liu *et al.* 2010; Botha 2013), where the new biotype is said to be virulent to the previously resistant (now susceptible) wheat.



**Figure 2.1** Typical symptoms of a *Diuraphis noxia* infestation of wheat include leaf rolling, purple streaking and chlorosis. Figure sourced from Botha *et al.* (2014b).

In South Africa, currently four ecological *D. noxia* biotypes are found, namely: SA1, SA2, SA3 and SA4 (Jankielsohn 2016). Out of the 17 *D. noxia* resistance genes found in wheat, *Dn1 – Dn10*, *Dnx*, *Dny*, *Dn626580*, *Dn2401*, *Dn2414*, *Dn1818* and *Dn100695* (Li *et al.* 2018), biotype SA1 is able to feed on *dn3*. SA2 is virulent towards *Dn1*, *Dn2*, *dn3*, *Dn8* and *Dn9*, while SA3 was found to be virulent to the same wheat cultivars as SA2 but, also to *Dn4* and *Dny*. SA4 is virulent to *Dn5* in addition to all the genes that SA3 is virulent towards (Jankielsohn 2016, Burger *et al.* 2017).

From the four biotypes in South Africa, SA4 is the most prevalent and SA2 the least prevalent overall. In the western Free State, South Africa, SA2 was the most frequently sampled, in the eastern Free State it was SA3, while SA1 was most frequently sampled both the Northern Cape and Western Cape (Jankielsohn 2016). It is interesting to note that although SA3 is resistant to the *Dn4* gene, *Dn4* cultivars have not yet been used in South Africa at the time SA3 was discovered. This finding indicated that the presence of a certain resistance gene in wheat is not required for an aphid population to acquire resistance (Jankielsohn 2011). Unique biotypes with different virulence characteristics are found in the United States, namely US-RWA1 – 8 (Puterka *et al.* 2014). Biotype US-RWA8 is the least virulent in contrast with US-RWA2 being the most virulent. Biotypes US-RWA3 – 7 are virulent to all the resistance genes except *Dn6* and *Dn7*, apart from US-RWA6 being avirulent towards *Dn4*. *Dn7* is the only resistance gene in wheat which still provides a highly resistant phenotype (Jankielsohn 2011, 2016) as none of the South African or American

wild-type *D. noxia* biotypes confers virulence to cultivars containing *Dn7*. While *Dnx* does provide resistance to all the wildtype South African biotypes, it has not been tested against the US biotypes. The rye 1RS chromosome arm, containing *Dn7*, tends to result in poor dough processing quality when incorporated into wheat (Lelley *et al.* 2004). The reluctance to use this resistance gene in commercial cultivars could explain why it is still effective against *D. noxia* biotypes. One exception to this rule is the mutant *D. noxia* biotype, SAM (South African Mutant). Biotype SAM was developed in the laboratory from SA1 under selection pressure and is virulent to all the resistance genes against which it has been screened including *Dn1*, *Dn2*, *Dn4*, *Dn5*, *Dn7*, *Dn8* and *Dn9* (Botha *et al.* 2014a; Burger *et al.* 2017). It is thus a useful model to use in studies to elucidate the mechanism of virulence against resistance genes, especially when compared to the other *D. noxia* biotypes.

### **2.1.1 Resistance in host plant**

Resistance in plants towards their insect pests can be classified into three categories. Firstly, antibiosis, which is defined as the ability of plants to harm the insect, was shown to be associated with the hypersensitive response (i.e. an oxygen burst) (Botha *et al.* 2010; Botha *et al.* 2014b). Secondly, antixenosis will deter the insect from feeding or using the plant for reproductive or protective purposes. It is accompanied by the release of volatile organic compounds and the use of the ethylene pathway after aphid feeding is detected. Lastly, a tolerant plant would rather manage the physiological effects of aphid feeding than influence the aphid, likely by managing photosynthetic flux (Botha *et al.* 2006; Botha 2013). Tolerant plants respond quickly by releasing reactive oxygen species and causing an influx of  $\text{Ca}^{2+}$  ions into the cells (Botha *et al.* 2010; Smith *et al.* 2010). The aforementioned responses of wheat to an aphid infestation is mediated by the different resistance genes present. This concept is based on the gene-for-gene model (Keen 1990) where the host plant contains *R* genes and the aphid contains *Avr* genes. This hypothesis is supported by the fact that the resistance genes in wheat only confers resistance to *D. noxia* and no other aphid species (Botha *et al.* 2005). Wheat that does not contain specific *R* genes reacts in a non-specific manner and thus relies solely on its innate resistance.

### **2.1.2 *Diuraphis noxia* feeding and saliva**

Aphids feed on plant phloem by inserting its mouthpart, called a stylet, into the sieve elements of its host. The stylet is comprised of two outer mandibles and two inner maxillae. The feeding procedure of an aphid starts with it secreting a tiny amount of gelling saliva on the feeding site. Next, the aphid inserts its flexible stylet through the apoplasm in between

neighboring cells towards the sieve elements. During this process, the stylet is also inserted into cells to analyze its internal chemistry (Giordanengo *et al.* 2010).

A study done by Tjallingii and Esch (1993) using an electrical penetration graph technique showed that many cells are penetrated, including sieve elements and only thereafter a particular sieve element was chosen to feed from. Throughout this initial process, gelling saliva is secreted and by doing so, the punctured cells are resealed again. The gelling saliva lines the entire puncture wound forming a canal that envelopes the stylet, called the salivary sheath. Watery saliva is also momentarily secreted during this phase, specifically when cells are punctured (Martin *et al.* 1997). Once an appropriate sieve element has been found, watery saliva is secreted after which it is only intermittently secreted during feeding (Tjallingii 2006). The mechanical damage of sieve elements should activate the release of proteins which would subsequently block the downstream sieve plate (Knoblauch and Van Bel 1998). However, when aphids are feeding, sometimes for hours on end, this is not the case. This is because the proteins in the watery saliva of aphids stop this phenomenon by interacting with the host proteins and avoiding defense responses (Will *et al.* 2009). The sheath formed by gelling saliva also aids the aphid in avoiding the host plant's defense responses by creating a barrier between the stylet and the plant. It also ensures a leak proof seal between the aphid stylet and its host, as well as closing the sieve element and salivary sheath after the stylet is removed (Miles 1999).

The composition of the watery saliva and the gelling saliva differs from one another, however the exact composition of neither is well understood. Results were obtained from experiments that involved artificial media, but the validity of these experiments is questionable, as aphid species possibly change the relative concentration of different components of its saliva depending on its diet (Habibi *et al.* 2001). These studies could however be used as an indication of the qualitative constituents of aphid saliva. The watery saliva is thought to contain amino acids, pectinases, cellulose and other carbohydrate depolarizing enzymes, phenolic glycoside hydrolyzing enzymes, oxidases and possibly amylases and enzymes that hydrolyze sucrose (Miles 1999). The function of watery saliva is expected to include the suppression of wound responses, reduce clotting of sieve plates and the stylet, cause a change in the physiology of the host and assist in the breakdown of ingested phloem components. Interestingly, the response seen in susceptible wheat upon *D. noxia* infestation, for example leaf curling, was shown in a study done by Lapitan *et al.* (2007) to be elicited by a protein found in the aphid, as proven by injecting susceptible and resistant wheat with *D. noxia* protein homogenate. The susceptible symptoms, i.e. leaf rolling and

induction of pathogen related proteins, were only seen in the susceptible wheat. Considering that only the saliva of *D. noxia* enters the plant, this result substantiates the notion that the eliciting agent of resistant wheat is a salivary protein of *D. noxia*.

## **2.2 *Myzus persicae***

While not the main aphid model in this study, *Myzus persicae* (also known by many common names including green peach aphid) is likely the most studied aphid species by the scientific community apart from the pea aphid, *Acyrtosiphon pisum*. Whereas the pea aphid is convenient to study because of its large size, *M. persicae* is studied because of its pest status. It is able to feed on the most diverse array of plant species of any aphid and thus serves as a generalist aphid model. While it is restricted to *Prunus sp.* (usually, *P. persicae*) in winter during its oviparous stage, during summer (parthenogenic viviparous stage) its hosts include plants in over 40 different families. In areas with mild winter temperatures it is able to remain in the parthenogenic stage and thus has a less restricted host range throughout the year. It is furthermore distributed all over the world apart from areas with extremely high or low humidity or temperature (Centre for Agriculture and Biosciences International 2018). Secondly, it is capable of spreading numerous plant viruses and is considered by some to be the most prolific vector of plant viruses. Kennedy *et al.* (1962) recorded over one hundred plant viruses that *M. persicae* is able to spread. Damage to plants as result of a viral infection is often greater compared to feeding by the aphid alone. To add to the abovementioned, *M. persicae* is also highly resistant to organophosphates, carbamates, pyrethroids and more recently to neonicotinoids. Resistance to the neonicotinoid, imidacloprid in a *M. persicae* strain, French Clone C, is achieved through increased cytochrome P450 expression as well as a reduction in the binding affinity of the nicotinic acetylcholine receptor to neonicotinoids because of a point mutation in a subunit of the receptor (Bass *et al.* 2011). This resistant strain was further able to detect leaf areas that are treated with neonicotinoids, preferring to feed on untreated areas (Fray *et al.* 2013). It appears thus that *M. persicae* is able to acquire multiple modes of insecticide resistance.

The availability of a genome sequence for *M. persicae* greatly assists a genetic investigation into this organism. Mathers *et al.* (2017) used the genome sequence to determine that the polyphagous ability of *M. persicae* is not a result of an increase in paralogues. Although *M. persicae* experienced gene family expansion after divergence from the specialist feeder, *A. pisum*, it was significantly less than *A. pisum*. Furthermore, a significantly greater amount of ancestral gene families lost one or more paralogue in *M. persicae* compared to *A. pisum*, resulting in a more condensed *M. persicae* genome. Transcriptome sequencing revealed

that gene families specific to or expanded in aphids were mostly differentially expressed as a whole when *M. persicae* were fed on different plant families. Genes within a family were also more likely to be regulated in the same direction. The RR-2 protein family contain the most genes differentially expressed. This gene family was upregulated when *M. persicae* fed on *Nicotiana benthamiana* compared to *Brassica rapa* (Chinese cabbage). Reverse transcription quantitative PCR showed that the cathepsin B and RR-2 gene families were differentially regulated within two days after a host shift, indicating quick adaptation to a different host.

*M. persicae* mainly feeds on dicotyledonous plants, is able to feed on a diverse array of plant families, is a vector of numerous plant viruses, and has been reported to be resistant to different classes of insecticides. *D. noxia* on the other hand feeds on monocotyledonous plants, has a small host range, i.e. a specialist and has not been reported to spread any virus or to be resistant to insecticides. In terms of economically important aphids, these two species differ quite substantially. The cause of these differences is still unknown.

### **2.3 c002**

Mutti *et al.* (2006) first discovered *c002* in *A. pisum*. Interest in this transcript was initiated by the fact that it is one of the most prevalent transcripts in the salivary gland of *A. pisum* and that it appears to be unique to Aphididae. The authors reported that aphids injected with *c002*-siRNA showed a higher mortality rate when fed on fava bean. In a subsequent study, it was reported that C002 is only present in a few cells of the principle salivary gland and it was also found to be present in fava bean leaves after aphid feeding, suggesting that C002 is produced in the salivary glands and secreted into the plant during aphid feeding. Furthermore, *A. pisum*'s feeding behavior was significantly affected by *c002* transcript knockdown. Using electrical penetration graph analysis, it was determined that the knockdown-aphids spent much less time probing the leaves. When probing began, less than half of the epidermal and mesophyll cells were punctured compared to the control-injected aphids. The knockdown aphids were also mostly unable to find a sieve element to feed on and when it did, the feeding duration was much shorter (Mutti *et al.* 2008).

Bos *et al.* (2010) proved that the reverse of the experiment above is true in *M. persicae*. When *M. persicae* was allowed to feed on *N. benthamiana* leaf disks transiently overexpressing *M. persicae* C002 (MpC002) an increase in fecundity was observed, but it had no effect on survival compared to the control. Silencing of *Mpc002* in *M. persicae* again did not have an effect on survival but, had a negative effect on the reproduction rate when

aphids fed on *N. benthamiana* leaf disks expressing *MpC002*-dsRNA. The same phenomenon was also observed when aphids fed on *Arabidopsis thaliana* stably expressing *MpC002*-dsRNA. This phenotype is a result of a 30-40% and 60% decrease in *MpC002* expression by *M. persicae* when feeding on *N. benthamiana* leaf disks and transgenic *A. thaliana* expressing *MpC002*-dsRNA, respectively (Pitino *et al.* 2011).

*MpC002* contains five repeats of seven amino acids that is not found in *A. pisum* C002, therefore Pitino and Hogenhout (2012) expressed *MpC002*, *A. pisum* C002 and *MpC002* without the repeat region in *A. thaliana* to elucidate the importance of the repeat. When *M. persicae* fed on *MpC002* expressing plants, roughly 20% increase in fecundity was observed compared to the control. However, when *M. persicae* fed on plants expressing either *A. pisum* C002 or *MpC002* without the repeat, the fecundity observed was not different from the control.

Zhang *et al.* (2015a) found that *Schizaphis graminum* (greenbug) also only expressed C002 in the salivary gland, corresponding to previous findings. When *c002* was silenced in *S. graminum*, the survival rate dropped to below 40% after feeding on susceptible wheat. However, when *c002*-knockdown aphids fed on artificial media containing siRNA, the survival rate increased more than 80%.

Together, these results indicate the importance of C002 in an aphid's ability to feed on plants, especially since this has been observed in more than one species of aphid. It also appears to primarily promote fecundity and is species-specific. To date, there is no evidence that the plant is harmed by the presence of the protein and it could thus be hypothesized that it only influences the aphid, perhaps as a stimulant to reproduce.

## 2.4 Genes associated with virulence

Using tandem mass spectrometry, proteins of the excised salivary glands were compared between *D. noxia* biotype SA1 and SAM. Among other differences, a cuticle protein with a Rebers & Riddiford consensus sequence (CPRR1-8) and a protein kinase C  $\delta$ , TPA-1, was found to be present in the salivary gland of SAM but not SA1 (Cloete 2015). It was also reported that SNPs were found between biotype SA1 and SAM in the coding domain sequence (CDS) of afore mentioned genes (Burger and Botha 2017). As biotypes SA1 and SAM have shared genealogy with a relatively short evolutionary history, differences between the biotypes may be the cause of virulence seen in biotype SAM. However, the exact mode of involvement requires investigation. The following information from literature will assist in this endeavor.

### 2.4.1 *cpr1-8*

The cuticle is arguably the cause of success of the phylum, Euarthropoda. Terrestrial arthropods make up approximately 78% of all species on earth (Mora *et al.* 2011; Stork *et al.* 2015). The arthropod cuticle is connected by joints and acts as an exoskeleton, protecting the organism from water loss, xenobiotics and physical damage as well as against pathogenic microorganisms. It is mainly composed of chitin and cuticular proteins, but also contains lipids, catecholamines (a benzene ring with two hydroxyl groups at carbons 1 and 2 and an amine side-chain) and minerals (Zhu *et al.* 2016). Chitin is a polymer composed of *N*-acetylglucosamine linked with  $\beta$ -1,4 glycosidic bonds. It also contains a small amount of glucosamine. The monomers are linked by the same bond as cellulose, but in chitin the 2-hydroxyl group is replaced with an acetyl amino group. This modification allows for strong hydrogen bonds between the chitin strands, enhancing its tensile strength (Sawada *et al.* 2012). Approximately twenty chitin strands are arranged in an antiparallel fashion to form an  $\alpha$ -chitin microfibril, primarily found in the arthropod cuticle. A cuticle's physical properties can be altered by a change in composition. Beetle wings are, for example, much more flexible and lightweight than a beetle's outer wings cases (elytra) which is much stronger (Vincent and Wegst 2004). The specific properties of the cuticle are in large determined by the cuticular proteins present in the cuticle. The cuticular proteins could furthermore be sclerotized to form an even harder cuticle (Arakane *et al.* 2012). This occurs when quinones and quinone methides react with the nucleophilic side chains of cuticle proteins, cross linking the proteins (Arakane *et al.* 2005). Thirteen families of cuticle proteins have been described to date with the largest group containing the R&R consensus (Victor *et al.* 2018), originally described by Rebers and Riddiford (1988). Proteins containing the R&R consensus are further divided into three subgroups: RR1, -2 and -3. RR1 and -2 proteins have been associated with different types of cuticle. While RR1 proteins are mainly found in cuticles that are more elastic and soft, RR2 proteins are found in hard and even sclerotized cuticles (Andersen 1998). A third group namely RR3, is much smaller than the previously mentioned and differs in the N-terminal compared to RR1 and -2 (Andersen 2000).

The exoskeleton of insects is not the only structure where a chitin matrix embedded with cuticular proteins is found. The peritrophic matrix is found in the alimentary canal of insects protecting the insect from physical damage, damage from its own enzymes and even other xenobiotics (Hegedus *et al.* 2009). Besides a chitin matrix fixed with chitin binding proteins, glycoproteins are also found in the peritrophic matrix. Although most insects appear to have a peritrophic matrix that lines the entire alimentary canal, phloem feeding Hemiptera is an

exception (Lehane 1997). Instead a double microvillar membrane (that does not contain chitin) was found to line the gut of *Rhodnius prolixus* (Lane and Harrison 1979). It has however been reported that the aphid stylet is composed of chitin embedded with chitin binding proteins (Uzest *et al.* 2007). As mentioned previously *cpr1-8* was found in the salivary gland of *D. noxia* biotype SAM. Considering that an aphid's stylet is directly connected to the salivary glands, the presence of a cuticular protein in a salivary gland protein extract can be explained. It is also possible that the glands are enclosed in a chitin and cuticle protein matrix.

#### **2.4.2 *tpa-1***

In *Caenorhabditis elegans*, TPA-1 is involved in the regulation of innate immunity in response to various stimuli found in the intestinal lumen or cuticle (Pujol *et al.* 2008; Ren *et al.* 2009; Ziegler *et al.* 2009; Lamitina and Chevet 2012; Van der Hoeven *et al.* 2012). More specifically, it is an important component in the network that relays the detection of a pathogen to the p38 mitogen-activated protein kinase (MAPK) cascade-mediated immune response. TPA-1 activates TIR-1, which in turn activates the p38 MAPK pathway (Liberati *et al.* 2004; Pujol *et al.* 2008). TPA-1 is homologous to the human protein kinase C  $\delta$  (Ziegler *et al.* 2009) and is therefore activated by diacylglycerol. This is produced together with inositol 1,4,5-trisphosphate as a product of the hydrolysis of the membrane lipid phosphatidylinositol 4,5-bisphosphate, catalyzed by phospholipase C  $\beta$  (*egl-8*). Phospholipase C  $\beta$  is in turn activated by the release of a  $G_{\alpha q}$  subunit (*egl-30*) from a G protein-coupled receptor that is presumed to interact with a pathogen derived ligand (Van der Hoeven *et al.* 2012). These findings are supported by the fact that *egl-30* and *egl-8* knockdown in the intestine results in increased susceptibility to pathogens in *C. elegans* (Kawli *et al.* 2010).

The *C. elegans* NADPH dual oxidase, Ce-Duox1/BLI-3, has been shown to generate reactive oxygen species (ROS) as a form of immunity to protect the worm against pathogens. In doing so, the p38 MAPK cascade is activated, resulting in the phosphorylation and localization of the SKN-1 transcription factor in the nucleus (Van der Hoeven *et al.* 2011). SKN-1 responds to ROS induced by xenobiotics or chemically via the regulation of phase II detoxification (An and Blackwell 2003). As a result of discoveries in *Drosophila melanogaster* by van der Hoeven *et al.* (2012), it was proposed that Ce-Duox1/BLI-3 is also activated by the release of  $G_{\alpha q}$  and phospholipase C  $\beta$ . This time, the inositol 1,4,5-trisphosphate produced by phospholipase C  $\beta$  binds to the inositol 1,4,5-trisphosphate p3

receptor on the endoplasmic reticulum. This induces the release of  $\text{Ca}^{2+}$  from the endoplasmic reticulum to adjust activity of Ce-Duox1/BLI-3 via the EF hands.

The evidence above leads to the hypothesis that the modifications in *D. noxia* biotype SAM *tpa* gives it the ability to tolerate the oxidative stress response or xenobiotics produced by a resistant wheat cultivar.

## 2.5 RNAi

RNA interference (RNAi) can be used to determine gene function *in vivo* by specific knockdown of a target gene. This is done by observing the phenotypic effects when a specific gene is said to be silenced through RNAi. With the *D. noxia* genome recently made available (Burger and Botha 2017), many genes with unknown function were identified and predicted function could be confirmed using this technique. Gaining this information will aid in the understanding of the aphid-plant interaction and could thus result in the breeding of crop cultivars with lasting resistance towards insect pests. A total of three Hemipteran genomes are available illustrating the need for methods to determine gene function *in vivo* in these organisms.

RNAi was first observed in *C. elegans* (Fire *et al.* 1998) with the first insect showing the same phenomenon being *Drosophila melanogaster* (Kennerdell and Carthew 1998). Subsequently, RNAi have been observed in many insect species including the pea aphid, *A. pisum* (Mutti *et al.* 2006). The use of RNAi allows the investigation of gene function through transient knockout of specific genes. This is done by delivery of small interfering RNA (siRNA) or double stranded RNA (dsRNA) that cause sequence specific degradation of targeted mRNA (Fire *et al.* 1998). When dsRNA enters the cell, it is cleaved into small siRNAs by an enzyme, namely Dicer (Bernstein *et al.* 2001; Hannon 2002). Dicer forms part of the RNase III family that produce a 5'-phosphorylated termini after cleavage of dsRNA (Hannon 2002). The siRNA formed by Dicer, is about 21–25 nucleotides in length, contains a 5'-phosphorylated termini and a 3' overhang of 2 nucleotides. The siRNA is then incorporated into the multiprotein RNA-induced silencing complex (RISC). One of the siRNA strands is released after ATP activation, while the other is used to guide the enzyme to RNA molecules complementary to the retained strand. When a complementary strand is found, it is endonucleolytically cleaved by RISC. If this cleaved complementary strand is the mRNA of a specific gene, the expression of said gene is effectively silenced, but only where mRNA is complementary to the siRNA (Hannon 2002). The simplest example of RISC would be an

Argonaute protein associated with a short RNA strand (like a strand from siRNAs or micro RNAs), but it may also form complexes with many other proteins (Pratt and MacRae 2009).

The correct design of the RNAi molecule is very important in a gene silencing experiment. It has to be sequence-specific as it has been known that only one nucleotide difference could cause the silencing effect to diminish (Joseph and Osman 2012). This high specificity can be used to one's advantage when many genes with high similarity are located in the target organism. The results may also be influenced by the size of the siRNA molecule.

### **2.5.1 RNAi in insects**

In insects, the effectiveness differs from species to species, but a dsRNA molecule of about 50 to 200 nucleotides long seems to give the best results (Huvenne and Smaghe 2010). The mode of dsRNA or siRNA transfer into the insect is mostly done through microinjection into the haemolymph or through feeding on artificial media (Scott *et al.* 2013). When using microinjection, one should consider how the insect is immobilized together with the volume and concentration of RNAi it is injected with. Feeding experiments often have lower silencing success especially because it is difficult to regulate how much RNAi molecules the insect ingests (Scott *et al.* 2013).

## **2.6 siRNA delivery methods to aphids**

To perform gene-knockdown through RNAi, siRNA or dsRNA needs to be delivered to the aphid in a manner that would allow effective uptake of siRNA/dsRNA by the cells. RNAi was attempted by Cloete (2015) on *D. noxia* but with limited success. It has been successfully executed in closely related *A. pisum*, *M. persicae* and *S. graminum* (Mutti *et al.* 2006; Pitino *et al.* 2011; Zhang *et al.* 2015a). Mutti *et al.* (2006) was the first to induce RNAi in *A. pisum*. In this experiment, a salivary protein transcript, namely C002, was silenced which resulted in the premature death of the aphids. In the case at hand, RNAi was accomplished with the injection of siRNAs, but dsRNA has also been shown to work effectively in gene silencing of *A. pisum* with a reduction of about 40% in gene expression seen using dsRNA (Jaubert-Possamai *et al.* 2007). Further, it was reported that an injected volume of less than 46 nanolitre dramatically decreased the mortality rate (Jaubert-Possamai *et al.* 2007). If attempted in *D. noxia*, this volume would have to be even lower, as *D. noxia* is much smaller in size. Below, alternative methods of siRNA/dsRNA delivery are examined.

### 2.6.1 Artificial feeding media

To silence the *ApAQP1* gene of *A. pisum*, dsRNA was delivered orally through artificial feeding medium. Using a dsRNA concentration of 1 µg/µl, more than a twofold reduction in expression of *ApAQP1* was observed after 24 h. As this involved only a single dose of dsRNA, the silencing effect observed was transient. Nevertheless, a phenotypic response was still observed in this study (Shakesby *et al.* 2009).

Whyard *et al.* (2009) determined that 0.0034 mg *vATPase*-dsRNA per gram of diet caused the death of 50% of aphids after a period of one week. In this experiment a 31.2% downregulation of gene expression was observed for *A. pisum vATPase*. The time of sampling was not reported. Christiaens *et al.* (2014) repeated this experiment using the same primers and other experimental parameters but could not report concurring results. A survival rate of 90% was observed for both *vATPase*-dsRNA fed and control aphids when the same concentration of dsRNA was used as reported by Whyard *et al.* (2009). The authors also targeted the hormone receptor (*EcR*) of *A. pisum* by feeding *EcR*-dsRNA via artificial medium to *A. pisum*. Here, 200 ng/µl dsRNA was fed to the aphids and no phenotypic change was reported compared to the control. *EcR* expression was not determined. Lastly, the authors showed that dsRNA degrades in artificial media on which aphids are fed, but not while suspended in media on which aphids did not feed. The validity of these results is disputable, as RNA sampled from media after 48 h of feeding still indicated a clear band of the correct size on an agarose gel. The RNA is thus only partially degraded and could therefore still initiate an RNAi effect.

If one assumes, like Christiaens *et al.* (2014), that the entities responsible for dsRNA degradation is at a high concentration in the gut of the aphid, the results of the aforementioned authors would fit. It could also explain why using only 200 ng/µl dsRNA Christiaens *et al.* (2014) failed to observe RNAi, but Shakesby *et al.* (2009) did using 1 µg/µl dsRNA.

In another study, Mao and Zeng (2012) successfully silenced the *hunchback* gene of *A. pisum* (*Aphb*) using artificial feeding media. Two-day old *A. pisum* was fed on artificial diet containing 750 ng/µl *Aphb*- and *EGFP* dsRNA. This resulted in 45% and 20% mortality after 7 days of feeding on *Aphb*- and *EGFP* dsRNA, respectively. At this time, *A. pisum* feeding on *Aphb*-dsRNA expressed *Aphb* at only 54% of the expression observed after control dsRNA feeding.

### 2.6.1.1 Composition of *D. noxia* feeding media

In order to perform gene silencing in *D. noxia* through siRNA ingestion via an artificial feeding medium, a feeding medium specific to *D. noxia* is required. Bahlmann (2005) compared different artificial feeding media and also determined the optimal sucrose concentration to use for *D. noxia* by evaluating the survival and reproductive success when the aphids were placed on the different media. The optimal sucrose concentration was determined to be 20% and the ideal composition, in terms of amino acids as well as salts, was determined and named “Diet A,” as shown in Table 2.1. The essential amino acids increased the number of nymphs produced per day, while the salts increased the lifespan of the aphids. Using this composition, 90% of aphids became reproductively active. As the effect of this medium on aphid reproduction is minimal, the phenotypic effect of RNAi should be clearly noticeable. It should therefore be suitable to use as a mechanism of siRNA delivery to *D. noxia* and subsequent phenotypic analysis as a result of RNAi.

**Table 2.1.** Composition of artificial feeding medium, Diet A‡ (Bahlmann 2005)

Component	Mass (g) to make 100 ml medium:
Methionine	0.10
Leucine	0.20
Tryptophan	0.10
Sucrose	20.00
MgCl <sub>2</sub> .6H <sub>2</sub> O	0.20
K <sub>3</sub> PO <sub>4</sub>	0.25

‡pH adjusted to 7.0 using 100 mM K<sub>2</sub>HPO<sub>4</sub> and dH<sub>2</sub>O added to reach the final volume.

### 2.6.2 Virus-induced gene silencing

The transformation of host plant to generate siRNA can also be used as a mechanism to induce RNAi especially in phloem feeding insects (Pitino *et al.* 2011; Zha *et al.* 2011). The transformation could either be permanent by creating transgenic plants, or transiently induced with the use of a virus. The latter is known as virus-induced gene silencing (VIGS), a mechanism based on the immune response of plants towards a virus infection (Waterhouse *et al.* 2001). The dsRNA virus genome is recognized and cleaved by Dicer into siRNA which RISC use to cleave any remaining RNA particles of viral sequence. When sequence identical to a plant open reading frame is inserted into the virus vector, the plant mRNA (produced from the endogenous open reading frame) will also be cleaved along with

the viral RNA. In this process the expression of said gene is silenced (Ruiz *et al.* 1998). VIGS has been applied in many dicot plants (Waterhouse *et al.* 2001), but only more recently has it been implemented in monocots. In a study done by Holzberg *et al.* (2002), Barley stripe mosaic virus (BSMV) was used as a vector to silence the gene encoding phytoene desaturase which resulted in photo-bleaching of barley. Phytoene desaturase is a common target to visually validate if a VIGS experiment was successful.

BSMV is able to infect other grasses, a property that Scofield *et al.* (2005) exploited to perform VIGS in wheat. Firstly, it was noted that the symptoms of a BSMV infection in wheat is less apparent than in barley, but the silencing efficiency was the same in terms of the amount of affected leaves when a young plant was inoculated. The effects of silencing seen in wheat were however delayed by three days compared to barley. Furthermore, it was found that homologous inserts smaller than 120 bp resulted in a reduction of silencing. Lastly the expression of the targeted gene, *PDS* (phytoene desaturase), decreased at least by 60% with maximum silencing occurring at day 13 after injection (Scofield *et al.* 2005). Similar results were found by Van Eck *et al.* (2010) and Schultz *et al.* (2015) using other genes (*WRKY53*, *PAL* and *GST*). Collectively, these results prove BSMV to be an effective vector for VIGS in wheat.

For the previously mentioned application of plant-mediated RNAi in phloem feeding insects, the inserted sequence will not be of plant origin, but will rather be a sequence homologous to a gene of interest in the insect. Accordingly, if the insect feeds on the plant infected with the manipulated virus, the representative gene in the insect will be silenced (Araujo *et al.* 2006; Zha *et al.* 2011). BSMV could therefore be used to induce RNAi in *D. noxia* through wheat-mediated delivery of siRNA.

### **2.6.3 RNAi through siRNA injection *in planta***

To the author's knowledge, injection of siRNA or dsRNA into the host plant of a plant-feeding insect to induce the RNAi pathway in the insect during feeding has not been reported. Inspiration for the abovementioned was however obtained from Lapitan *et al.* (2007), who injected wheat with *D. noxia* protein extracts which resulted in the formation of susceptible symptoms in some wheat varieties.

## **2.7 DNA methylation**

The addition of a methyl group (-CH<sub>3</sub>) to the adenosine and cytosine bases of DNA can be observed in both eukaryotes and prokaryotes. Specifically, 5-methylcytosine (5mC) and N6-methyl-adenosine can be found in many fungi, bacteria and protists, while N4-methyl-

cytosine is exclusive to bacteria. Although a small amount of adenosine methylation was found in *Drosophila* (Zhang *et al.* 2015b), it is an uncommon occurrence in eukaryotes where the main type of methylation observed being 5mC. In mammals, DNA methylation occur almost exclusively on the cytosine of CG dinucleotides (Law and Jacobsen 2010), where it is involved in important biological processes such as gene regulation, chromatin organisation, genomic imprinting and X-chromosome inactivation (Li *et al.* 1993). The most important of these functions in the context of this study would be the regulation of gene expression. DNA methylation was first connected to gene expression when methylated DNA, injected into *Xenopus laevis* oocytes was shown not to be transcribed (Vardimon *et al.* 1982).

DNA methyltransferases (Dnmts) are responsible for the transfer of methyl groups to cytosine using S-adenosyl methionine as methyl donor. In animals three groups of Dnmts are present namely, Dnmt1, Dnmt2 and Dnmt3. Dnmt3 is responsible for *de novo* cytosine methylation that is thereafter maintained by Dnmt1. Although Dnmt2 is structurally very similar to Dnmt1 and Dnmt3, it does not methylate DNA, but RNA. The human Dnmt2 was specifically shown to methylate the aspartic acid transfer RNA (Goll *et al.* 2006). *A. pisum* contains two paralogues of Dnmt1 and one copy Dnmt2 and Dnmt3, respectively. A Methyl-CpG-binding protein is also present in the aphid's genome as well as a Dnmt1-associated protein, both involved in gene regulation via DNA methylation (Walsh *et al.* 2010).

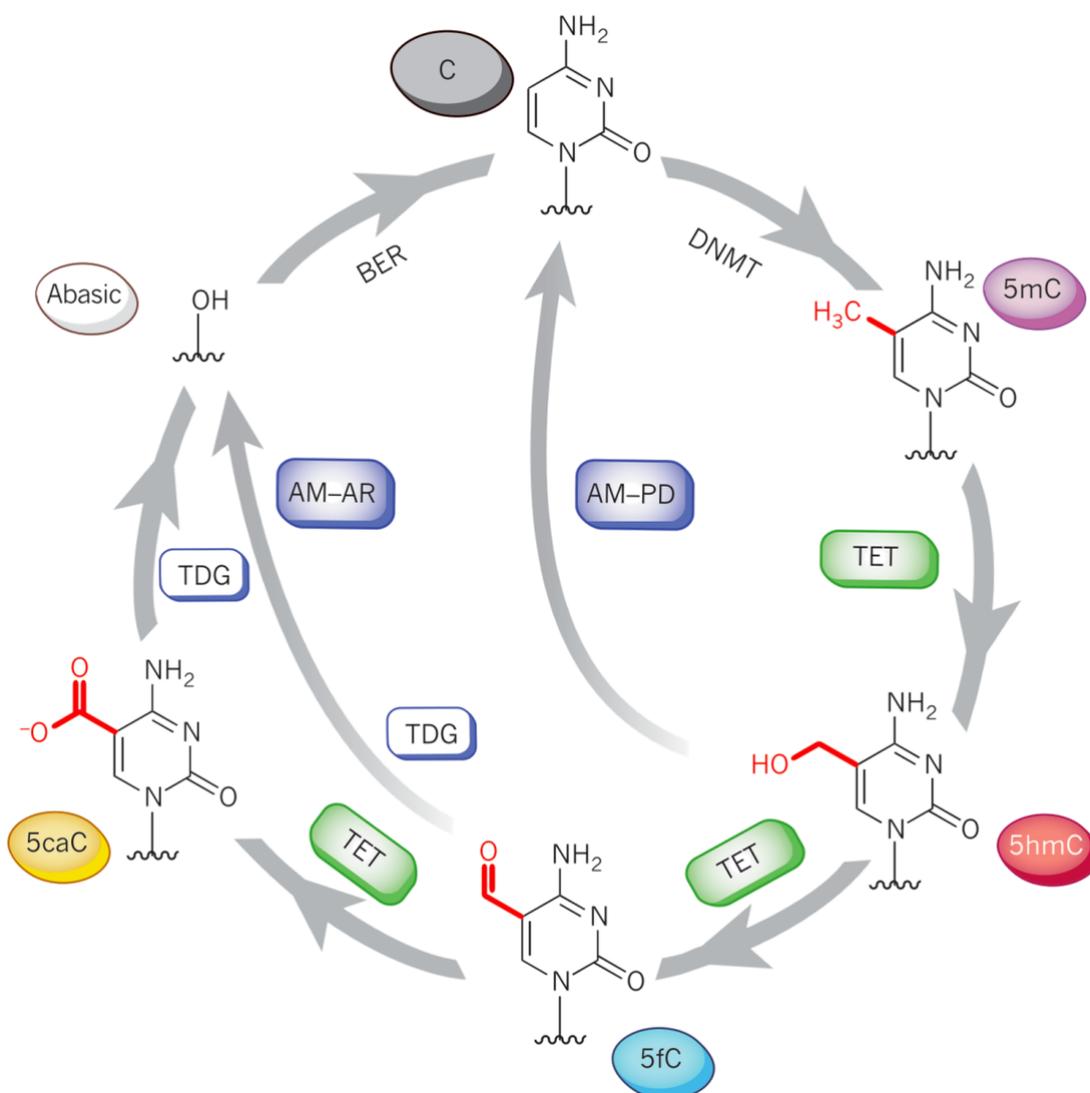
Methylation patterns vary across the genomes of different clades which indicate that the function of DNA methylations is not always the same. In the genomes of land plants (*Arabidopsis thaliana* and rice), green algae (*Chlorella* sp. NC64A and *Volvox carteri*) and vertebrates (puffer fish), Zemach *et al.* (2010) found an increase in methylation of transposable elements (TEs). Genic methylation was also observed, but to a lesser extent and methylation proximal to the transcription start sites (TSS) was found to be much lower. Moderately expressed genes contain the highest amount of methylation, while genes either highly expressed or expressed at a low degree being methylated the least. Furthermore, an increase in methylation around the TSS is associated with decreased gene expression. In contrast, invertebrates (*Ciona intestinalis* [vase tunicate], *Apis mellifera*, *Bombyx mori*, *Nematostella vectensis* [anemone]) do not hypermethylate TEs and there is also not correlation between methylation of the TSS and transcription. In these groups DNA methylation is mainly found in open reading frames. There are some exceptions to the invertebrate group: *Drosophila melanogaster* and *Tribolium castaneum* (flour beetle) have methylation levels that are hardly detectable.

Hunt *et al.* (2010) reported that genes in the insects *A. mellifera*, *A. pisum* and *B. mori*, that are ubiquitously expressed in different tissues are more likely to be densely methylated compared to genes that are differentially expressed between tissues. Genes that are differentially expressed between genetically identical phenotypic morphs of *A. pisum* (e.g. winged or wingless) also exhibit lower levels of methylation. The differential expression between phenotypic morphs should therefore be a result of another form of gene regulation. This argument is in contrast to a result found by Walsh *et al.* (2010) when a specific juvenile hormone regulating gene, *ApJHBP*, was examined. At a specific CpG site, a 50% reduction in methylation was observed for winged versus wingless. The global view taken by Hunt *et al.* (2010) would not have taken such small details into account. It asks the question whether methylation of a specific site would have a larger effect on gene regulation than the density of methylation of the entire open reading frame.

Pasquier *et al.* (2014) investigated green-orange polyphenism in *A. pisum* through methylated DNA fragment enrichment and subsequent sequencing. The authors found that scaffolds from the green phenotype was more methylated, and that most of these scaffolds produced less transcripts compared to the orange phenotype. Using bisulfide sequencing, Gong *et al.* (2012) compared four genes believed to be expressed in the salivary gland of *D. noxia*. The less virulent biotype US-RWA1 displayed more methylation than the more virulent US-RWA2. This finding concurs to that of Breeds *et al.* (2018) who examined the global methylation patterns of South African *D. noxia* biotypes using a technique called restriction site-specific fluorescent labeling. It was found that the most virulent biotype, SAM, was methylated the least and interestingly in the order of decreasing virulence the biotypes were methylated more. Using an antibody-based approach, it was also found that biotype SAM contained the most 5-hydroxymethylcytosine (5hmC), which is the first step in an active demethylation pathway, explained in more detail below. This finding again corresponds to the lower levels of methylation found in virulent biotypes.

The reversal of cytosine methylation requires more enzymatic steps to complete than the initial methylation (Kohli and Zhang 2013) (Figure 2.2). The ten-eleven translocase (TET) enzyme family is responsible for the active oxidation of 5mC to 5hmC. Accumulation of 5hmC occurs in most mammalian cell types and up to 40% as abundant as 5mC in neuronal cells. This accumulation could indicate that 5hmC also has a regulatory effect, perhaps distinct to 5mC. 5hmC can then be diluted through DNA replication or actively removed and restored to unmodified cytosine. Active removal begins with repetitive oxidation by TET which results in 5-formylcytosine (5fC) and thereafter in 5-carboxylcytosine (5caC). Thymine

DNA glycosylase (TDG) will then excise 5fC or 5caC from duplex DNA and the resulting abasic site will then be excised by base excision repair enzymes and replaced with an unmodified cytosine. The 5fC and 5caC excision activity of TDG was discovered when its deletion led to accumulation of 5caC in mouse embryonic stem cells (He *et al.* 2011). TDG is structurally specific to 5fC and 5caC thus does not excise 5mC and 5hmC (Zhang *et al.* 2012). In the honey bee 5hmC levels are variable, but generally low. It is however higher in ovaries and testes. The honey bee TET also has 5mC to 5hmC oxidation activity as in mammalian cells. The expression of honey bee TET is relatively high throughout development and in the adults, with the highest expression observed in adult brain tissue (Wojciechowski *et al.* 2014).



**Figure 2.2.** The complete pathway for the modification of cytosine. Active modification (AM) of 5mC can lead to passive dilution to an unmodified cytosine through DNA replication. Through active restoration (AR) 5hmC could also be further oxidized by TET and the oxidation product removed by TDG. The resulting abasic site will then be replaced by an unmodified cytosine by base excision repair enzymes (BER). Figure sourced from Kohli and Zhang (2013).

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## Chapter 3

### **Ingestion of siRNA targeting the cuticle protein, CPRR1-8, in *Diuraphis noxia* cause a reduction in fitness**

### 3.1 Introduction

Various wheat cultivars exist that demonstrate resistance to *Diuraphis noxia*, but this is often counteracted by the emergence of a new *D. noxia* biotype that is virulent to the now previously resistant wheat (Botha 2013). Of the 17 resistance genes in wheat *Dn7*, *Dn2401* and likely *Dn10* (has only been screened against the most virulent US biotype, US-RWA2) remains effective to *D. noxia* biotypes in the USA (Puterka *et al.* 2015; Li *et al.* 2018). In South Africa, of the resistance gene screened, *Dn7*, *Dn6* and *Dnx* confers effective resistance against the four biotypes present (Jankielsohn 2016). One exception is the laboratory generated biotype, SAM (South African Mutant) (Botha *et al.* 2014). This biotype was developed from biotype SA1 (the least virulent SA biotype and only virulent to *dn3*) by placing it under continuous selection pressure from resistant germplasm (*Dn1* and later *Dn5*) and has been shown to express virulence to all the resistance genes against which it has been screened, including *Dn1*, *Dn2*, *Dn4*, *Dn5*, *Dn7*, *Dn8* and *Dn9* (Botha *et al.* 2014; Burger *et al.* 2017). The rapid biotypification observed in *D. noxia* emphasizes the necessity to better our understanding of the plant-aphid interaction in order to breed wheat with durable resistance to not only *D. noxia*, but other aphid species as well.

When comparing biotypes SA1 (least virulent SA biotype, Jankielsohn 2014, 2016) to SAM (most virulent SA biotype, Botha *et al.* 2014; Burger and Botha 2017), genetic mechanisms associated with the development of virulence, and eventually new biotypes can be elucidated. In a direct comparison between the salivary proteome of biotypes SA1 and SAM (Cloete 2015) several unique peptides were found. As the RR1 cuticle protein, *cpr1-8*, was unique to and isolated from the salivary gland of the most virulent biotype SAM, it may contribute to virulence in this biotype. *cpr1-8* also contained SNPs when biotype SAM was compared to SA1 (Burger and Botha 2017). As the evolutionary distance between these two biotypes are relatively small, it can again be concluded that any difference may likely be associated with virulence. To test this hypothesis, the function of RR1 during aphid feeding was investigated. This was done using RNA interference (RNAi) via a novel small interference RNA (siRNA) delivery method after determining the most effective method of siRNA delivery.

In aphids, RNAi-mediated gene silencing or knockdown can be achieved through direct injection of dsRNA or siRNA into aphid haemolymph (Mutti *et al.* 2006, 2008; Jaubert-Possamai *et al.* 2007) or feeding of dsRNA from an artificial diet (Shakesby *et al.* 2009; Whyard *et al.* 2009) as previously demonstrated. Plant-mediated RNAi (transgenic plant

producing dsRNA) to initiate down-regulation of aphid gene targets has also been shown (Bhatia *et al.* 2012; Guo *et al.* 2014; Coleman *et al.* 2014; Pitino *et al.* 2011; Pitino and Hogenhout 2013). However, working with a small aphid (1.4-2.6 mm in size, Centre for Agriculture and Biosciences International 2018) microinjections requires specialized equipment. Furthermore, wheat, the host of *D. noxia*, has a large genome (17 000 MB) (Gill *et al.* 2004) and unlike *Arabidopsis*, proved cumbersome to transform (Le Roux *et al.* 2015), necessitating alternative strategies to achieve RNAi-mediated gene silencing in *D. noxia*. Thus, the objective of this chapter is to elucidate the role of this gene in awarding virulence to biotype SAM against its host, wheat. To this end, the *Dncpr1-8* gene and its transcript were sequenced, three siRNA delivery techniques were compared, a novel siRNA delivery method developed and lastly, the methylation patterns in *Dncpr1-8* were investigated as it has been reported that the environment has an effect on DNA methylation in aphids (Pasquier *et al.* 2014). As several studies have been conducted on *c002* (Mutti *et al.* 2006, 2008; Coleman *et al.* 2014; Visser 2017), this gene from *D. noxia* was included as reference in the present study.

## 3.2 Materials and Methods

### 3.2.1 Aphid populations

Colonies of parthenogenetic (apterous) female aphids of South African *D. noxia* biotypes SA1, and SAM, expressing different levels of virulence, were separately established in BugDorm cages (MegaView Science Education Services Co. Ltd., Taiwan) in an insectary with the following conditions:  $22.5 \pm 2.5^\circ\text{C}$ , 40% relative humidity, and continuous artificial lighting from high pressure sodium lamps. The fact that *D. noxia* biotypes SA1 and SAM share genealogy, and that SAM is virulent to all resistance genes against which it has been screen to date, while SA1 is the most avirulent biotype in South Africa, makes biotype SAM a useful model to elucidate the mechanism of virulence against resistance genes.

The aphid colonies are maintained on near isogenic wheat lines, Tugela (*D. noxia* susceptible, biotype SA1) (Hewitt *et al.* 1984), while SAM was maintained on Tugela*Dn1*, a wheat cultivar containing the *Dn1* resistance gene. All cultivars were planted in sand-filled pots and watered daily with a fertilizer that consisted of 2 g Microplex (Ocean Agriculture, South Africa), 164 g Sol-u-fert (Kynoch Fertilizers, South Africa) and 77 ml potassium nitrate per 100 liters of water.

### 3.2.3 Plant material and growth conditions

Near-isogenic wheat lines, Gamtoos-R (GamR; resistant) and Gamtoos-S (GamS; susceptible) were used for feeding experiments. These plants were grown under King Plus 800W LED lights for a 12h photoperiod and at a temperature of 20°C ± 1°C. The resistance in GamR was obtained after a 1RS/1BL translocation from rye (*Secale cereale* L.) (Marais *et al.* 1994), is denoted *Dn7*, and is known to express antixenosis and antibiosis against aphids during feeding (Zaayman *et al.* 2009; Lapitan *et al.* 2007a, 2007b; Botha *et al.* 2010). Antibiosis is observed when the plant reduces the reproductive fitness of aphids feeding on it, while antixenosis is the non-preference of a cultivar as host (Painter 1951, 1958).

### 3.2.4 Sample preparation, RNA extraction and DNA extraction

For RNA extraction of both aphid and plant material, samples were immediately flash-frozen in liquid nitrogen and grounded in 1.5 ml Eppendorf tubes using a micropestle. RNA from aphids was extracted using a RNeasy Mini Kit (Qiagen, Germany) and on-column DNase I treatment (Qiagen) following the manufacturer's protocol. While in the case of wheat, RNA was extracted by adding 600 µl TRI Reagent® (Zymo Research, USA) to the ground material after which the Direct-zol™ RNA MiniPrep Plus kit (Zymo Research) was used by following the manufacturer's protocol and stored at -80°C until further use.

Complementary DNA (cDNA) synthesis was conducted using an iScript cDNA Synthesis Kit (Bio-Rad, USA). Two microliters of 5X iScript reaction mix and 0.5 µl iScript reverse transcriptase was added to 5 µl RNA. Thereafter it was placed in a T100 Thermal Cycler (Bio-Rad) set to 25°C for 5 min, 42°C for 30 min and 85°C for 5 min. The cDNA was then stored at -20°C until further use.

DNA was extracted from about 20 mg of aphids by grinding it while cooled with liquid nitrogen, using a micropestle and a reaction tube (Greiner Bio-One, Austria). The DNeasy Blood & Tissue Kit (Qiagen) was used after this step following the manufacturer's protocol exactly. DNA was stored at -20°C.

### 3.2.5 Sequence characterization of *Dncprr1-8* that encodes the protein RR1 from *Diuraphis noxia*

Using the available sequenced SAM genome, SAM v1.0 (GenBank assembly accession: GCA\_001465515.1 and BioProject PRJNA29716; Burger and Botha 2017), PCR primers were designed to amplify mRNA transcripts of *Dncprr1-8* and *Dnc002* as well as the genic area of *Dncprr1-8* (Table 3.1). Synthesized cDNA was used as template for the amplification of transcript sequence and extracted DNA as template for the amplification of genes. PCR

reactions consisted of 0.125 µl template (DNA or cDNA), 5 µl Phusion HF Buffer (New England Biolabs, USA), 0.2 mM dNTPs (New England Biolabs), 0.25 µl Phusion High-Fidelity DNA polymerase (New England Biolabs), 0.5 µM forward and reverse primers and 0.75 µl dimethyl sulfoxide (New England Biolabs) in a total volume of 25 µl. PCR cycling profile consisted of an initial step of 98°C for 30 s, followed by 30 cycles at 98°C for 10 s, 30 s at the annealing temperature indicated in Table 3.1 and 72°C for 30 s per Kb of PCR amplicon length (Table 3.1) followed by a final step of 72°C for 10 min. The PCR products were purified using the MinElute Reaction Cleanup Kit (Qiagen) and Sanger sequenced at Central Analytical Services (CAF), Stellenbosch University.

**Table 3.1.** Primers used for sequence verification, Sanger sequencing, gene expression analysis or determination of siRNA concentration.

Name of primer	Sequences of primers (5'-3')	Template	Amplicon length (bp)	Annealing temp. (°C)
<i>c002_qPCR F</i>	CCCGTATGAGAAGCCGACTG	cDNA	123	60
<i>c002_qPCR R</i>	CCATCTTGGTGGGAGCTCTG			
<i>cprr1-8_CDS F</i>	CGGCAATTTTCTGATCACGG	cDNA	434	59
<i>cprr1-8_CDS R</i>	CTGTGGTTGGGAGGCGATTA			
<i>cprr1-8_qPCR F</i>	CCCATCCAACCAAGCCTA	cDNA	123	56
<i>cprr1-8_qPCR R</i>	TAGTATCCTTGTTGTCCCGG			
<i>cprr1-8_gene_1 F</i>	GCATCAGTTGTGTCATTTGTCCA	DNA	1765	57
<i>cprr1-8_gene_1 R</i>	GTTTGGGCCGTTTCAGCG			
<i>cprr1-8_gene_2 F</i>	TCGTACTTTATCATACACTTATGAATT	DNA	1141	58
<i>cprr1-8_gene_2 R</i>	GCGGGTCTCTATTTCTCAAT			

The PCR products of primers *cprr1-8\_gene\_2 F* and *cprr1-8\_gene\_2 R* (Table 3.1) were cloned before plasmids were Sanger sequenced. In order to clone the PCR products generated using high fidelity DNA polymerase into the p-GEM T-easy vector (Promega, USA), 3' adenine overhangs were added using *Taq* DNA polymerase (Knoche 1999). This reaction consisted of 15 µl PCR product purified as mentioned above, 2 µl 10X Standard *Taq* Reaction Buffer (New England Biolabs), 0.2 mM dATP (Roche, Switzerland) and 5 units/µl *Taq* DNA polymerase (New England Biolabs) in a total volume of 20 µl. Of this reaction mixture, 1.5 µl was added to 2.5 µl 2X Rapid Ligation Buffer (Promega), 50 ng pGEM-T Easy Vector (Promega), and 0.5 µl T4 DNA ligase (Promega) which was then incubated overnight at 4°C for the ligation to take place.  $\alpha$ -Select Bronze Competent Cells (Bioline, UK) were transformed by firstly adding 2 µl of the ligation reaction to 50 µl of thawed competent cells and incubating it on ice for 20 min with periodic mixing. The cells were then

heat shocked for 45 s at 42°C, returned to ice for 2 min after which 950 µl Luria Broth (LB) (Sigma-Aldrich) was added and it was incubated for 90 min at 37°C while shaking at 15 rpm. One hundred microliters of the cell suspension were then spread on a LB (Sigma-Aldrich) agar (Merck, Germany) plate which also contained 100 µg/ml ampicillin (Sigma-Aldrich), 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma-Aldrich) and 40 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Sigma-Aldrich). After overnight incubation at 37°C, white colonies were selected and inoculated in 5 ml LB medium with 100 µg/ml ampicillin and incubated overnight as mentioned before. Of the overnight cultures a volume of 0.5 µl was added to a PCR reaction that consisted of 2 µl 10X NEB Standard *Taq* Reaction Buffer (New England Biolabs), 0.2 mM dNTPs (New England Biolabs), 0.1 µl NEB *Taq* DNA polymerase (New England Biolabs) and 0.5 mM of the reverse and forward insert-specific primers in a total reaction volume of 20 µl. The PCR products were analysed on a 1% (m/v) TAE (40 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol, 20 mM acetic acid and 1 mM ethylenediaminetetraacetic acid, pH 8) agarose gel using a 1 Kb ladder (New England Biolabs) as a size reference and GelRed (Biotium, USA) to stain the DNA samples. A miniprep was then performed on the liquid cultures of colonies that tested positive using the QIAprep Spin Miniprep Kit (Qiagen), following the manufacturer's protocol. The obtained plasmids were Sanger sequenced using SP6 and T7 primers at CAF, Stellenbosch University.

Both ends of the raw reads were then trimmed in Geneious 9.1.8 (Biomatters, New Zealand) using the Trim Ends function which trims vector sequence (not required in the case of direct sequencing of PCR products), primer sequence and low quality sequence (error probability set 0.01). The Geneious assembler was used to assemble the trimmed reads *de novo* at the highest sensitivity, after which it was aligned to the next-generation sequencing (NGS) reference genome sequence of biotype SAM (Burger and Botha 2017) using MUSCLE 3.8 (Edgar 2004) (Geneious 9.1.8, Biomatters, Kearse *et al.* 2012).

The obtained sequences were furthermore submitted to the basic local alignment search tool (BLASTn and BLASTx, Altschul *et al.* 1990, 1997) at the National Centre for Biotechnology Information (NCBI) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to confirm the identity of genes *Dncprr1-8* and *Dnc002*. Geneious 9.1.8 (Biomatters) was used to translate the obtained *Dncprr1-8* sequence to amino acid sequence based on a *in silico* prediction (Burger and Botha 2017) of the CDS of *Dncprr1-8*. CutProtFam-Pred, based on profile Hidden Markov Models was applied to confirm the RR1 protein identity (<http://bioinformatics.biol.uoa.gr/CutProtFam-Pred>) *Dncprr1-8* (Ioannidou *et al.* 2014). The

InterProScan 1.1.4 Geneious (version 9.1.8, Biomatters) plugin was used to identify protein domains (Quevillon *et al.* 2005; Kearse *et al.* 2012). Secondary protein structure was determined using the EMBOSS 6.5.7 tool garnier (Garnier *et al.* 1978). To predict tertiary protein structure the Phyre2 web portal was used (Kelly *et al.* 2015).

### **3.2.6 Construction of virus-induced gene silencing vector of wheat to silence genes in *Diuraphis noxia***

To induce gene silencing in *D. noxia*, a virus-induced gene silencing (VIGS) vector of wheat, *barley stripe mosaic virus* (BSMV) was modified to contain *D. noxia* transcript sequence. The plasmid containing the BSMV  $\gamma$ -genome segment, p $\gamma$ SL038-1, was modified and the inoculation of wheat was performed as described by Scofield *et al.* (2005). In short, primers were designed to amplify 434 bp and 441 bp segments of *Dncpr1-8* and *Dnc002*, respectively (Supplementary Table S3.1). *PacI* recognition sites were added to the forward while the reverse primers contained *NotI* restriction sites at the 3' end to allow directional cloning into the  $\gamma$ -genome segment. The  $\gamma$ -genome segment is one of three genome segments that constitutes BSMV. Once the orientation and presence of the *Dncpr1-8* and *Dnc002* fragments in p $\gamma$ SL038-1 were verified, plasmids containing BSMV  $\alpha$  (p $\alpha$ 46)-,  $\beta$  (p $\beta$ 42sp1)- and the various  $\gamma$ -genome segments were isolated and linearized. The  $\gamma$ -genome plasmids included: p $\gamma$ SL038-1 (unmodified), p $\gamma$ SL039B-1 (BSMV  $\gamma$  containing the barley phytoene desaturase [PDS]), p $\gamma$ SL038-1:*Dnc002* and p $\gamma$ SL038-1:*Dncpr1-8*. *MluI* was used to linearize the plasmids, apart from p $\beta$ 42sp1, which was linearized using *SpeI*. Following verification of linearization and purification of the linear fragments, it was used as template to synthesize capped RNA transcripts using the mMMESSAGE mMACHINE® T7 Ultra Kit (Ambion, USA). One microliter (about 1-1.5  $\mu$ g) of a specific  $\gamma$  genome transcript with equal volumes of the  $\alpha$ - and  $\beta$ -genome transcripts was added to 23  $\mu$ l FES buffer (0.1 M glycine [Sigma-Aldrich, USA], 0.06 M K<sub>2</sub>HPO<sub>4</sub> [Sigma-Aldrich], 1% w/v tetrasodium pyrophosphate [Sigma-Aldrich], 1% w/v bentonite [Sigma-Aldrich], 1% w/v celite [Sigma-Aldrich], pH 8.5) and used to rub inoculate the 3<sup>rd</sup> leaf of 10 GamR wheat plants (Scofield *et al.* 2005).

One apterous adult aphid was contained in a cage on the 4<sup>th</sup> leaf of each of the 10 plants inoculated per experiment. A cage comprised of inverted 15 ml polypropylene tubes (Greiner Bio-One) modified to allow the leaf to be threaded through a slit in the lid. Any opening was closed with cotton wool (Supplementary Figure S3.1). Fecundity and survival were determined daily. The mean of 10 biological repeats were calculated and compared using a logistic regression to determine if significant difference as present (XLstat, Addinsoft).

### 3.2.7 Design of siRNA

The Custom RNAi Design Tool (Owczarzy *et al.* 2008) was used to design siRNAs targeting *Dncpr1-8* and *Dnc002*. Sanger sequence of the *Dncpr1-8* transcript was used as template for *Dncpr1-8*-siRNA and the SAM v1.0 genome sequence (GenBank assembly accession: GCA\_001465515.1 and BioProject PRJNA29716, Burger and Botha 2017) as template for *Dnc002*-siRNA design. The standard parameters were changed to select siRNAs with a 19 nt duplex region and a 2 nt 3'-overhang. siRNA sequences returned were screened to verify its theoretical efficiency (Table 3.2). According to Khvorova *et al.* (2003), siRNA has to cohere to the 'asymmetric thermodynamic rule' which is based on the principle that the siRNA strand that is bound less stably at the 5'-end is preferentially incorporated into the RNA-induced silencing complex (RISC). The siRNAs were also designed to contain a GC content of 30-52%, as well as the absence of internal repeats (Horn and Boutros 2013) (Table 3.2). The synthesized siRNA was obtained from IDT (<https://www.idtdna.com/>).

**Table 3.2.** *Dncpr1-8*- and *Dnc002*-siRNA sequences. siRNAs were designed to contain a 19 bp duplex region and a 2 nt 3'-overhang on either side.

Sequence target	Duplex siRNA sequence
<i>Dncpr1-8</i>	5' -UAAACAAUCGCAAGAAGCUGA-3'       3' -GAAUUUGUUAGCGUUCUUCGA-5'
<i>Dnc002</i>	5' -AUUUCAGAGAGACAUCGGAGG-3'       3' -GUUAAAGUCUCUCUGUAGCCU-5'

### 3.2.8 Aphid feeding on siRNA-containing artificial media

An artificial feeding media developed by Bahlmann (2005) specifically for *D. noxia* was used for aphid feeding. It was modified to contain the following: 0.10 g L-methionine (Merck), 0.20 g L-leucine (Sigma-Aldrich), 0.10 g L-tryptophan (Merck), 20.00 g sucrose (Merck), 0.20 g MgCl<sub>2</sub>.6H<sub>2</sub>O (Merck), 0.25 g K<sub>2</sub>HPO<sub>4</sub> (Sigma-Aldrich), pH was adjusted to 7.0 using KOH (Merck) and dH<sub>2</sub>O added to a final volume of 100 ml. The media was then filter sterilized (0.2 µm pore size) and stored at 4°C.

Adult apterous *D. noxia* was placed individually inside a glass test tube with a 14 mm outside diameter. Parafilm M (Bemis, USA) was stretched close to its maximum capacity and placed over the opening. One microliter of siRNA (25 µg/µl) dissolved in RNase-free water (Ambion), or 1 µl RNase-free water (Ambion) for the control, was added to 24 µl artificial feeding media and placed on the stretched Parafilm M. Another layer of Parafilm M was then placed over the artificial media, spreading the media between the two layers. The test tubes

were placed vertically in a stand with the open end at the bottom. Yellow tape was placed below the parafilm sachet to encourage the aphids to feed (Supplementary figure S3.2) (Kieckhefer *et al.* 1976). The experiment was repeated ten times (n=10) for each siRNA (i.e., *Dnc002* and *Dncpr1-8*) and control (only media). The survival rate and the number of nymphs produced by each foundress was determined daily for four days as described by Van Eck *et al.* (2010).

### 3.2.9 Aphid feeding on siRNA-injected wheat

The leaves of 30-day-old wheat plants were injected with 1  $\mu$ l of 1  $\mu$ g/ $\mu$ l siRNA dissolved in 10 mM 2-Amino-2-(hydroxymethyl)propane-1,3-diol (Tris)-HCl (Sigma-Aldrich) pH 7.0, at two locations in the midvein  $\pm$ 5 mm apart resulting in a total of 2  $\mu$ g siRNA injected into each leaf. For the control 10 mM Tris (Sigma-Aldrich) at pH 7.0 was injected. A 10  $\mu$ l, model 1701 Hamilton syringe with a 25.4 mm needle of 34 gauge, and 45° tip (Hamilton, USA) was used for the injections.

To contain aphids at the injection site, 15 ml polypropylene tubes (Greiner Bio-One) were cut 45 mm from the opening after which the closed end of the bottom tube was also removed to produce two tubes of  $\pm$ 45 mm in length (Supplementary Figure S3.3). After 15 adult aphids were placed on the leaf between the injection sites, the leaf was threaded through the modified polypropylene tube which was then held in place by cotton wool inserted at the top and bottom of the leaf at each end of the tube to form a cage. The cotton wool was adjusted to allow aphids to move around freely within a  $\pm$ 25 mm<sup>2</sup> area centered around the injection sites. The cages were supported by wire wrapped around the tubes and anchored to a wooden rod. The foundress aphids were then allowed to feed for a period of 6h or 48h before the survivors were counted and sampled for further analysis. Fertility was determined by calculating the mean number of nymphs between three biological repeats as described by Van Eck *et al.* (2010). Leaf samples were taken at the same time points (6h and 48h), as well as directly after injection (0h). The experiment was performed in triplicate for every time point and repeated twice over time (n = 18).

### 3.2.10 Gene expression analysis in aphids

Silencing of candidate genes were confirmed via reverse transcription quantitative PCR (RT-qPCR). Primers were all designed to be 20 bp in length, to amplify a product of 123 bp in size and bind to the coding domain sequences of the *Dnc002* and *Dncpr1-8* genes. cDNA was synthesized as described whereafter the concentration was determined through Qubit analysis (Thermo Fisher Scientific, USA; CAF, Stellenbosch University). The RT-qPCR

setup comprised of 0.5 ng cDNA, 5 µl SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad) and one of the following primer sets: 0.5 µM of *c002\_qPCR* F and *c002\_qPCR* R or *cpr1-8\_qPCR* F and *cpr1-8\_qPCR* R, 0.4 µM *L32* F and 0.6 µM *L32* R or 0.6 µM of *L27* F and *L27* R in 10 µl total reaction volumes (Table 3.1). The PCR cycling profile consisted of two initial steps of 50 °C for 1 min and 95 °C for 5 min, followed by 40 cycles at 95°C for 10 s, 20 s at annealing temperatures specified in Table 3.1 and 72°C for 20 s. A melt curve was also performed at 0.5°C increments every 5 s from 65°C to 95°C (Supplementary Figures S3.4, S3.5, S3.6 and S3.7). Relative expression was calculated by means of the mathematical model by Pfaffl (2001) using untreated aphids sampled at day 0 as the calibrator and the ribosomal proteins *L27* (Sinha and Smith 2014) and *L32* (Shakesby *et al.* 2009; De Jager *et al.* 2014) were used as normalizers.

### 3.2.11 siRNA concentration in wheat

A section of leaf material 10 mm in length, which included the two injection sites in the center, was used for RNA extraction as described above. Stem-loop primers specific to the synthetic siRNAs were designed as described by Varkonyi-Gasic *et al.* (2007) (Supplementary Table S3.1). Each 20 µl cDNA synthesis reaction contained 3 mM MgCl<sub>2</sub>, 0.5 mM of each dNTP, 30 µM random hexamer primers, 0.5 µM specific stem-loop primer, 150 ng RNA template, 1 µl ImProm-II™ Reverse Transcriptase (Promega) and 4 µl ImProm-II™ 5X Reaction Buffer (Promega). Five nanogram cDNA was used in each 10 µl RT-qPCR reaction as well as 5 µl SsoAdvanced universal SYBR® Green supermix (Bio-Rad), 1 µM universal stem-loop reverse primer (Varkonyi-Gasic *et al.* 2007) and 1 µM specific forward primer (*DnC002-siRNA* F or *Dncpr1-8-siRNA* F, Supplementary Table S3.1). The samples were incubated at 95°C for 5 min, followed by 35 cycles of 95°C for 5 s, 60°C for 10 s, and 72°C for 1 s. *18S* expression levels for each sample was determined in a 10 µl RT-qPCR reaction consisting of 0.2 ng cDNA, 5 µl SsoAdvanced Universal SYBR® Green Supermix (Bio Rad) and 0.4 µM of both the forward and reverse *18S* primers (Supplementary Table S3.1). After an initial 3 min step at 95 °C, 40 cycles of 95°C for 10 s, 54°C for 30 s and 72°C for 30 s were followed to amplify the product. A melt curve was also performed at 0.5°C increments every 5 s from 65°C to 95°C (Supplementary Figures S3.8, S3.9 and S3.10). The concentration of the siRNAs was calculated relative to *18S* expression using the mathematical model by Pfaffl (2001).

### 3.2.12 Aphid protein assays

Aphids were ground in ice-cold 50 mM phosphate buffer (pH of 7.5), 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich) and 1 mM dithiothreitol (Sigma-Aldrich). The extract was centrifuged at 4°C for 15 min at 17 200 rpm. The supernatant was removed and kept on ice until protein assays were performed.

Catalase activity was determined colometrically by adding an aliquot of protein extract to 0.2 M phosphate buffer (pH 6.5) and 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> (Sigma-Aldrich) as previously described (Johansson and Borg 1988) and the degradation of H<sub>2</sub>O<sub>2</sub> was observed at 260 nm. Enzyme activity was expressed as  $\mu$ mol H<sub>2</sub>O<sub>2</sub>.mg protein<sup>-1</sup>.min<sup>-1</sup>.

Peroxidase activity was measured by adding an aliquot of protein extract to 0.2 M phosphate buffer (pH 5.0), 100 mM H<sub>2</sub>O<sub>2</sub> (Sigma-Aldrich) and 30 mM guaiacol (Sigma-Aldrich) (Zieslin and Benzaken 1991). The formation of tetraguaiacol was a linear function of enzyme concentration and peroxidase activity was expressed as mmol tetraguaiacol min<sup>-1</sup>. mg<sup>-1</sup> protein.

### 3.2.13 Wheat protein assays

Liquid nitrogen was used to freeze wheat leaf material while it was ground. To this, 100 mM potassium phosphate at pH 7.5 (Sigma-Aldrich), 1 mM ethylenediaminetetraacetic acid (Merck) and 1% polyvinylpyrrolidone (Sigma-Aldrich) was added to further homogenize the leaf material using a micropestle. The supernatant was collected after centrifugation at 4°C for 15 min at 17 200 rpm (Rao *et al.* 1997).

Peroxidase activity was determined as previously described (Hildebrand *et al.* 1986) and horse radish peroxidase (New England Biolabs) was used as a standard. Hydrogen peroxide (0.06%) was added into a mixture containing 2  $\mu$ g of leaf extract, 6 mM guaiacol (Sigma-Aldrich), 25 mM potassium phosphate buffer (pH 6.0) and 24% distilled water. The formation of tetraguaiacol was a linear function of enzyme concentration and peroxidase activity was expressed as mmol tetraguaiacol min<sup>-1</sup>.mg<sup>-1</sup> protein.

### 3.2.14 Aphid and plant protein concentrations

All protein concentrations were determined following a method described by Bradford (1976) using bovine serum albumin (Bio-Rad) as standard. The Glomax spectrophotometer (Promega) was used for this purpose as described by Rybutt and Parish (1982).

### 3.2.15 DNA methylation of *Dncpr1-8*

In our laboratory Du Preez, Burger, Truter, Swiegers and Botha (unpublished data) determined the DNA methylation state of *D. noxia* biotype SA1 and SAM through whole genome bisulfite sequencing. In short the authors firstly extracted DNA from at least 150 *D. noxia* biotype SA1 and SAM, respectively, as mentioned here, in section 3.2.4. The quality of DNA was assessed through Qubit 2.0 (Thermo Fisher Scientific) and a 2% (m/v) TAE agarose gel. The DNA samples were sent to Macrogen (South Korea) where the library preparation and sequencing was performed. After trimming and filtering the HiSeq X (Illumina, USA) reads obtained, Bismark (Krueger and Andrews, 2011) was used to determine the methylation status of SA1 and SAM using the SAM genome as reference (GenBank assembly accession: GCA\_001465515.1 and BioProject PRJNA29716, Burger and Botha 2017). For every cytosine in the reference genome Bismark outputs the amount of times to was methylated or unmethylated based on the bisulfite reads mapped to that position. It also distinguishes between the different contexts of cytosine (CpG, CHG and CHH) and the DNA strand that was methylated. In the present study this data set was manipulated to determine the proportion of methylation at every cytosine present in *Dncpr1-8* which was then used to determine the total methylation in this gene and to graph the proportion of methylation at every site using Microsoft Excel (Microsoft).

### 3.2.16 Statistical analysis

An analysis of variance (ANOVA) with Tukey's HSD (honestly significant difference) test was used to determine if statistically significant differences exist between means of parametric data. Data for aphid survival on BSMV inoculated wheat was binary and thus a logistic regression was performed to determine if statistically significant differences were present. All statistical analyses were conducted by using GraphPad Prism 7 (GraphPad Software) or Xlstat (Addinsoft) with significance set at  $\alpha = 0.05$ .

## 3.3 Results

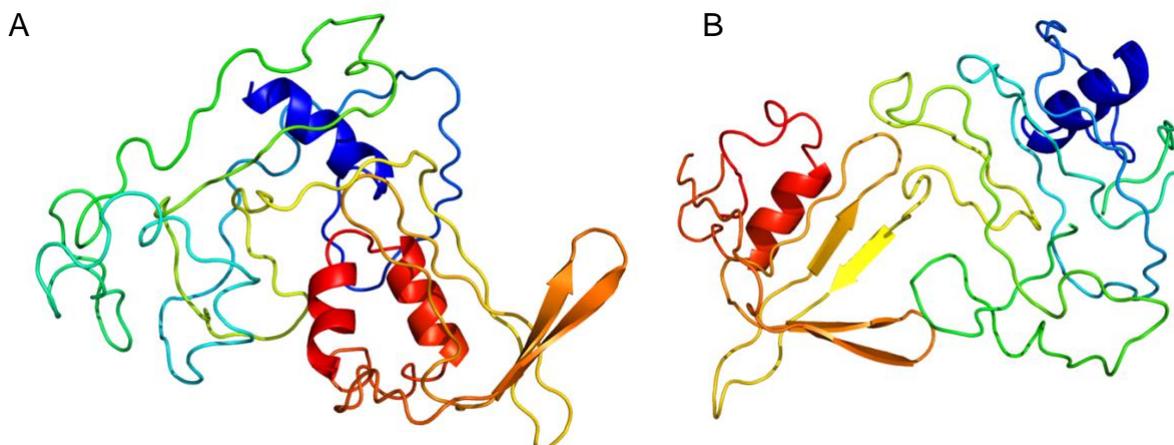
### 3.3.1 Sequence characterization of *Diuraphis noxia Dncpr1-8*

#### 3.3.1.1 DNA sequencing

Of the 4197 bp that make up *Dncpr1-8*, 1663 bp of biotype SA1 and 1689 bp of biotype SAM was Sanger sequenced. The initial Sanger reads of *Dncpr1-8*, generated using primers *cpr1-8\_gene\_1* F and *cpr1-8\_gene\_1* R, did not align properly to the reference

SAM genome sequence as there was a sequence gap in this reference sequence (Supplementary Figures S3.11 and S3.12). Primers *cprr1-8\_gene\_2* F and *cprr1-8\_gene\_2* R were then designed on the initial Sanger reads obtained to close the sequence gap. As direct PCR sequencing was not possible, the fragments from primers *cprr1-8\_gene\_2* F and *cprr1-8\_gene\_2* R were cloned before it was sequenced.

Analysis of the available sequences revealed that the *Dncprr1-8* gene is 4197 bp long, contains 5 exons and a non-cytoplasmic domain which includes a 35-36 amino acid motif known as the chitin-binding Rebers and Riddiford (R&R) consensus (Willis 1999; Rebers and Willis 2001). SAM clone #2 was found to be different to the SA1 clones. At base position 160 of the CDS SAM clone #2 had a thymine instead of the guanine found in the other SA1 and SAM clones. This should result in glycine to cysteine amino acid change. When predicted secondary structures of both G and T alleles were compared, the length of beta-strands was different and an additional coil was observed on the original G allele around the single nucleotide polymorphism (SNP) (Supplementary Figure S3.13). The predicted tertiary structures also differed between alleles (Figure 3.1).



**Figure 3.1.** Predicted tertiary structure of the G- (A) and T-allele (B) of CPRR1-8. Predicted models were obtained with Phyre2 web portal (Kelly *et al.* 2015).

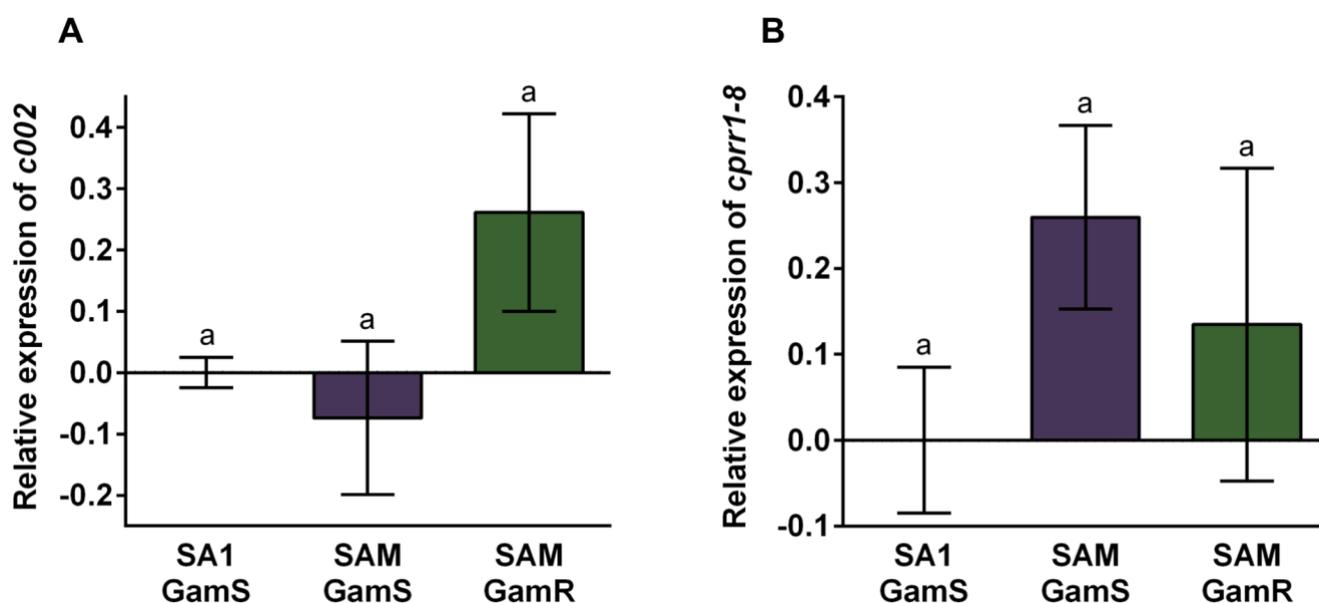
### 3.3.1.2 Complementary DNA sequencing

The Sanger sequencing reads of biotype SA1 and SAM *Dncprr1-8* transcripts (cDNA) almost covered the entire CDS. From the 826 bp *in silico* predicted *Dncprr1-8* transcript, 718 bp from biotype SA1 and 730 bp from biotype SAM were Sanger sequenced directly from PCR products (Supplementary Figures S3.14 and S3.15). A C/T polymorphism was found at position 218 from the *in silico* predicted transcription start site. This polymorphism was

present in both SA1 and in SAM and had no effect on amino acid sequence. No other polymorphisms were found between the two biotypes, based on cDNA sequencing.

### 3.3.2 Relative expression of *Dnc002* and *Dncpr1-8* in *Diuraphis noxia* biotypes SA1 and SAM.

In order to assess whether there is any difference in the inherent expression of *Dncpr1-8* and *Dnc002* in biotypes SA1 and SAM when feeding on resistant (GamR) and susceptible (GamS) near-isogenic wheat lines, the biotypes were fed on these lines for 10 days. Thereafter, RT-qPCR expression analyses were conducted for *Dncpr1-8* and *Dnc002* and relative expression calculated (Figure 3.2). The obtained results revealed that even though the level of *Dncpr1-8* expression was higher in SAM relative to its parent SA1, it was not statistically significant (Figure 3.2B).



**Figure 3.2.** Relative expression of *Dnc002* (A) and *Dncpr1-8* (B) in *Diuraphis noxia* biotype SA1 and SAM after feeding on susceptible wheat cultivar Gamtoos-S (GamS) or resistant Gamtoos-R (GamR) for at least 10 days. An ANOVA with Tukey's HSD test was used to determine if statistically significant differences exist between means.

While the expression of *Dnc002* did not differ between the aphid biotypes when feeding on GamS, it was higher in SAM when feeding on GamR, but this was also not statistically significant (Figure 3.2A).

### 3.3.3 Optimizing siRNA delivery to *Diuraphis noxia*

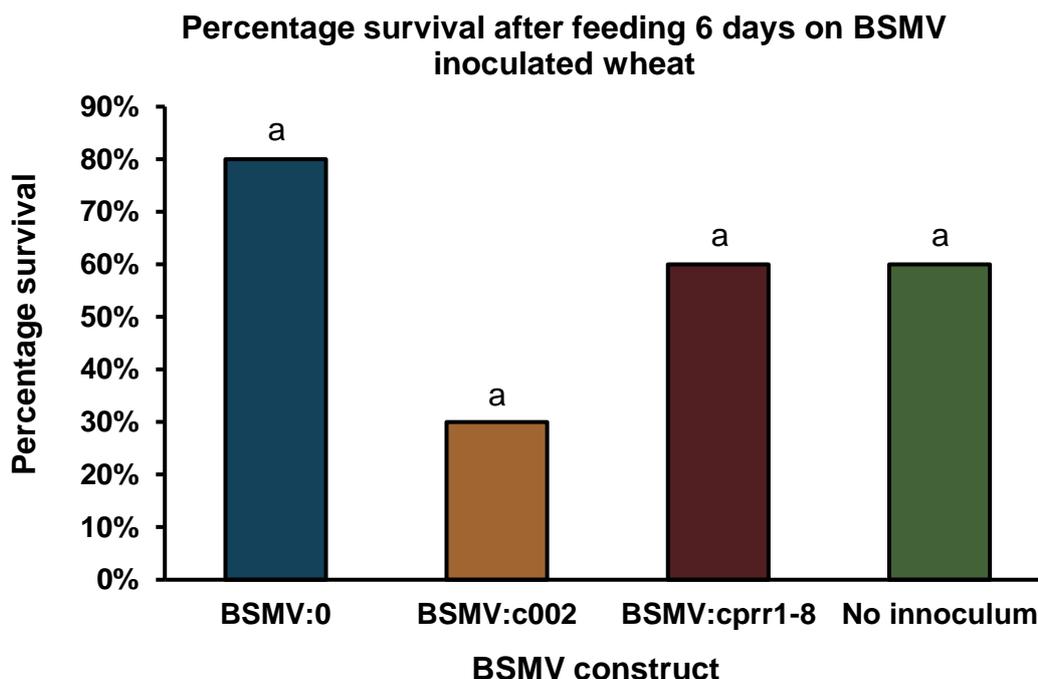
Four different methods of siRNA delivery to *D. noxia* were compared. Firstly, siRNA was delivered through direct injection into the insect haemolymph (Mutti *et al.* 2006, 2008; Jaubert-Possamai *et al.* 2007). This technique proved impossible due to the size of the

aphids, as all the aphids died shortly after injection, irrespective of being injected with no fluid, buffer or siRNA (data not shown).

siRNA was then delivered to the aphid by adding the siRNA to an artificial feeding medium (Shakesby *et al.* 2009; Whyard *et al.* 2009) and *in planta*. *In planta* delivery was firstly performed by injecting *Dnc002*- or *Dncpr1-8*-siRNA into wheat cultivar GamR and containing the aphids on the leaf area around the injection site. Secondly, *in planta* delivery was performed by inserting *Dnc002* or *Dncpr1-8* sequences in a VIGS vector of wheat. Inoculation of wheat by this modified virus would result in dsRNA of *Dnc002* or *Dncpr1-8* sequence to accumulate in wheat before aphids are allowed to feed and taking up the dsRNA in the process.

Feeding on artificial media containing *Dncpr1-8*- and *Dnc002*-siRNA had no effect on the survival of biotype SAM. A 100% survival rate was observed regardless of feeding on siRNA or on a water control for a period of 4 days (data not shown). After feeding for a period of 6 days on wheat inoculated with BSMV:*Dnc002*, a greater decrease in survival was observed compared to feeding on wheat inoculated with unmodified BSMV. The survival rate of SAM feeding on BSMV:*Dncpr1-8* was slightly lower at 60% and comparable to GamR that received a mock inoculation with FES buffer (Figure 3.3). Finally, the only siRNA delivery method that resulted in significant reduction in survival rate compared to the control was by feeding SAM GamR injected with *Dncpr1-8*- or *Dnc002*-siRNA (Supplementary Figure S3.16, two-sided Dunnett test,  $P \leq 0.05$ ).

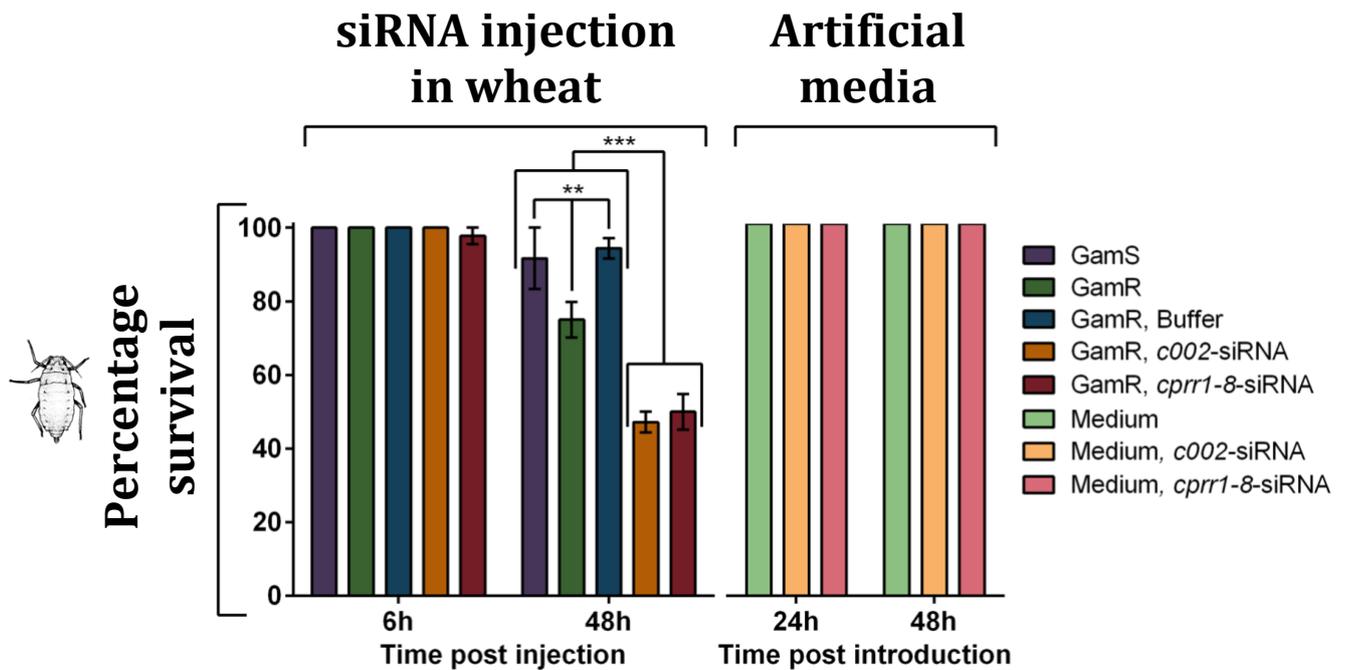
When feeding SAM on artificial media containing siRNA, nymph production, like survival, did not differ significantly (Supplementary Figure S3.17, Tukey's HSD test,  $P \leq 0.05$ ). Nymph production also did not differ significantly when biotype SAM fed on BSMV inoculated wheat (Supplementary Figure S3.18, Tukey's HSD test,  $P \leq 0.05$ ). Although the average nymph production did differ slightly between treatments using this siRNA delivery method, large variability was observed compared to other siRNA delivery methods. Nymph production of aphids feeding on GamR was also relatively variable, although the average production was less when feeding on *Dnc002*- or *Dncpr1-8*-siRNA injected wheat.



**Figure 3.3.** *Diuraphis noxia* biotype SAM feeding on wheat (Gamtoos-R) inoculated with modified *barley stripe mosaic virus* (BSMV). BSMV:0, unmodified BSMV virus-induced gene silencing vector; BSMV:c002, BSMV containing *Dnc002* transcript sequence; BSMV:cprr1-8, BSMV containing *Dncpr1-8* transcript sequence. Ten biological replicates were performed for every experimental condition. A logistic regression confirmed that the survival rates did not differ significantly. Equal alphabets indicate no significant difference.

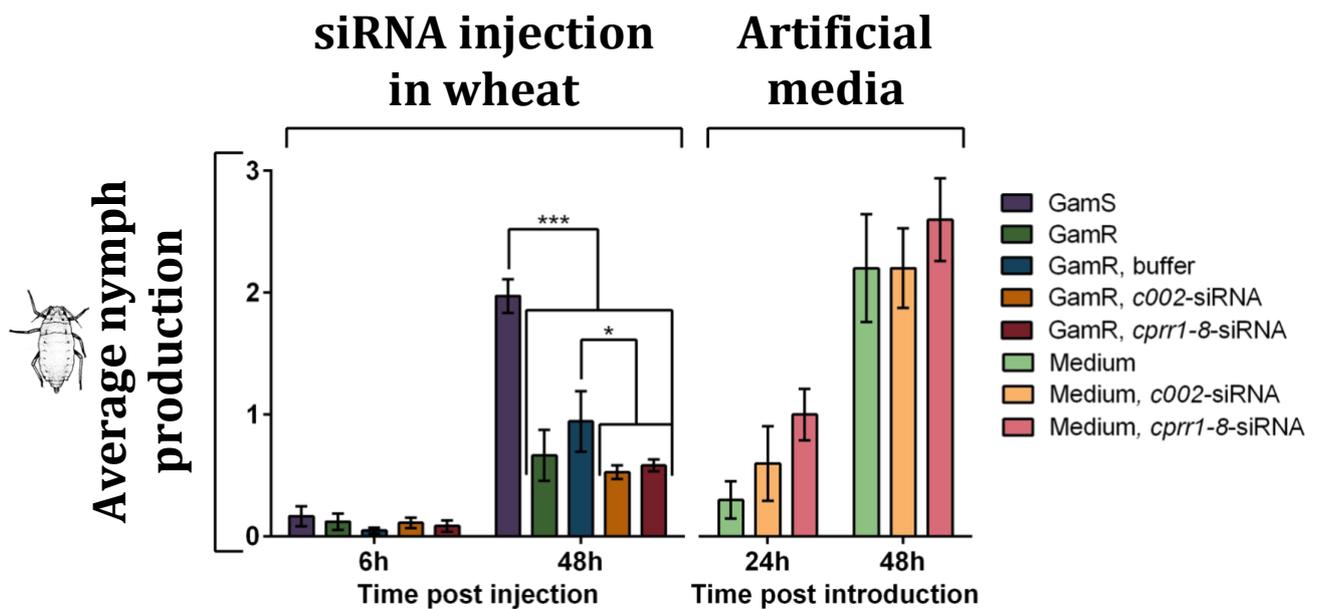
### 3.3.4 Investigating the function of *Dncpr1-8*

Based on the preliminary results, siRNA delivery through injection into wheat was used for further study. The functions of *Dnc002* and *Dncpr1-8* in the salivary glands of virulent biotype SAM while feeding on one of the most *D. noxia* resistant wheat varieties, GamR (containing *Dn7*), were investigated through gene-silencing. Biotype SAM was allowed to feed on uninjected, buffer-injected and plants injected with either 2 µg *Dnc002*-siRNA or 2 µg *Dncpr1-8*-siRNA, where after it was phenotypically characterized and relative gene expression measured. The method of siRNA delivery through artificial media was also included here as it represents an environment that does not include any plant defense molecules. GamS was also included here as the effect of the *Dn7* resistance gene on biotype SAM can be observed when compared to GamR while SAM is feeding on these cultivars.



**Figure 3.4.** Percentage survival of *Diuraphis noxia* biotype SAM after feeding on wheat leaves injected with siRNA that targets the *D. noxia* genes *c002* or *cpr1-8*. The percentage survival of SAM feeding on artificial media supplemented with the same siRNAs is also presented. GamS, *D. noxia* susceptible wheat cultivar 'Gamtoos-S'; GamR, *D. noxia* resistant wheat cultivar 'Gamtoos-R'; Buffer, 10mM Tris (pH 7); *c002*-siRNA, siRNA targeting *c002* dissolved in 10mM Tris (pH 7); *cpr1-8*-siRNA, siRNA targeting *cpr1-8* dissolved in 10mM Tris (pH 7); Medium, *D. noxia* artificial feeding medium (composition described in Materials and Methods); \*\*, significantly different at  $P \leq 0.01$ ; \*\*\*, significantly different at  $P \leq 0.001$ . Significant differences determined using Tukey's HSD test. Error bars represent SEM.

As expected, survival (Figure 3.4) and nymph production (Figure 3.5) of SAM was affected by feeding on wheat injected with *Dnc002*- and *Dncpr1-8*-siRNA in the same manner as the preliminary study. Likely as a result of an increased sample size, a significant difference was observed here between the buffer injection and *Dnc002*- and *Dncpr1-8*-siRNA injection. Feeding on GamS did result in a much higher nymph production than feeding on GamR, but not as high as feeding on artificial media.

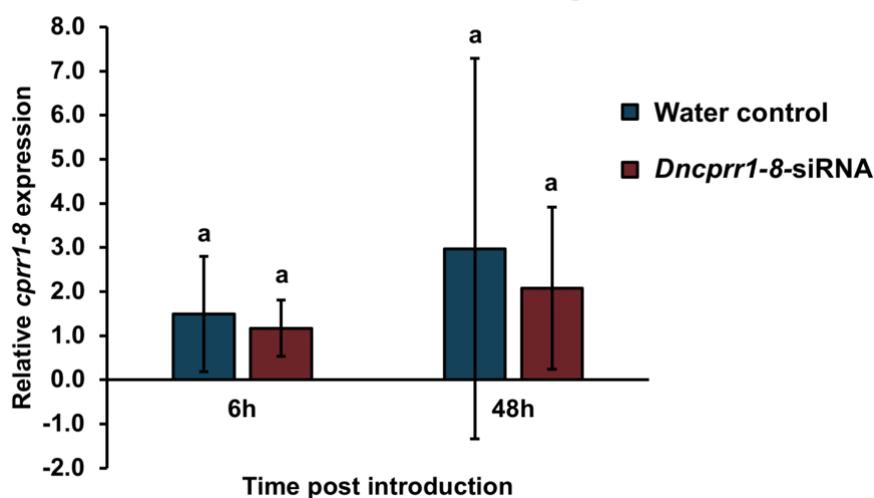


**Figure 3.5.** Average nymph production of *Diuraphis noxia* biotype SAM after feeding on wheat leaves injected with siRNA (19 nt duplex region and a 2 nt 3'-overhang) that targets the genes *c002* or *cpr1-8*. siRNA was dissolved in 10 mM Tris (pH 7.0) before injection. GamS, susceptible wheat cultivar 'Gamtoos-S'; GamR, resistant wheat cultivar 'Gamtoos-R'; No injection, wheat leaves without injection; Buffer, 10 mM Tris (pH 7); *c002*-siRNA, siRNA targeting *c002* dissolved in 10 mM Tris (pH 7); *cpr1-8*-siRNA, siRNA targeting *cpr1-8* dissolved in 10 mM Tris (pH 7); Medium, *D. noxia* artificial feeding medium (composition described in Materials and Methods); \*, significantly different at  $P \leq 0.05$ ; \*\*\*, significantly different at  $P \leq 0.001$ . Significant differences determined using Tukey's HSD test. Error bars represent SEM.

Feeding on *Dnc002*- or *Dncpr1-8*-siRNA supplemented artificial media did not result in gene silencing of *Dnc002* (Figure 3.6) or *Dncpr1-8* (Figure 3.7) in SAM, respectively.

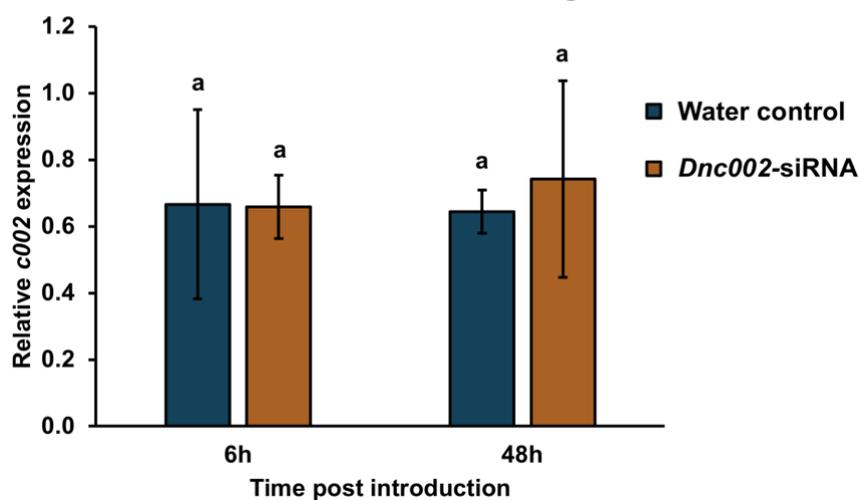
When biotype SAM fed on *Dnc002*-siRNA for 48h, overexpression of *Dnc002* was observed relative to untreated and buffer injected leaves at 6 and 48 hours post introduction (hpi) of aphids. Upregulation of *Dnc002* was also observed when SAM fed on uninjected GamR compared to GamS (Figure 3.8 A).

***Diuraphis noxia* *cprr1-8* expression after feeding artificial media containing siRNA**

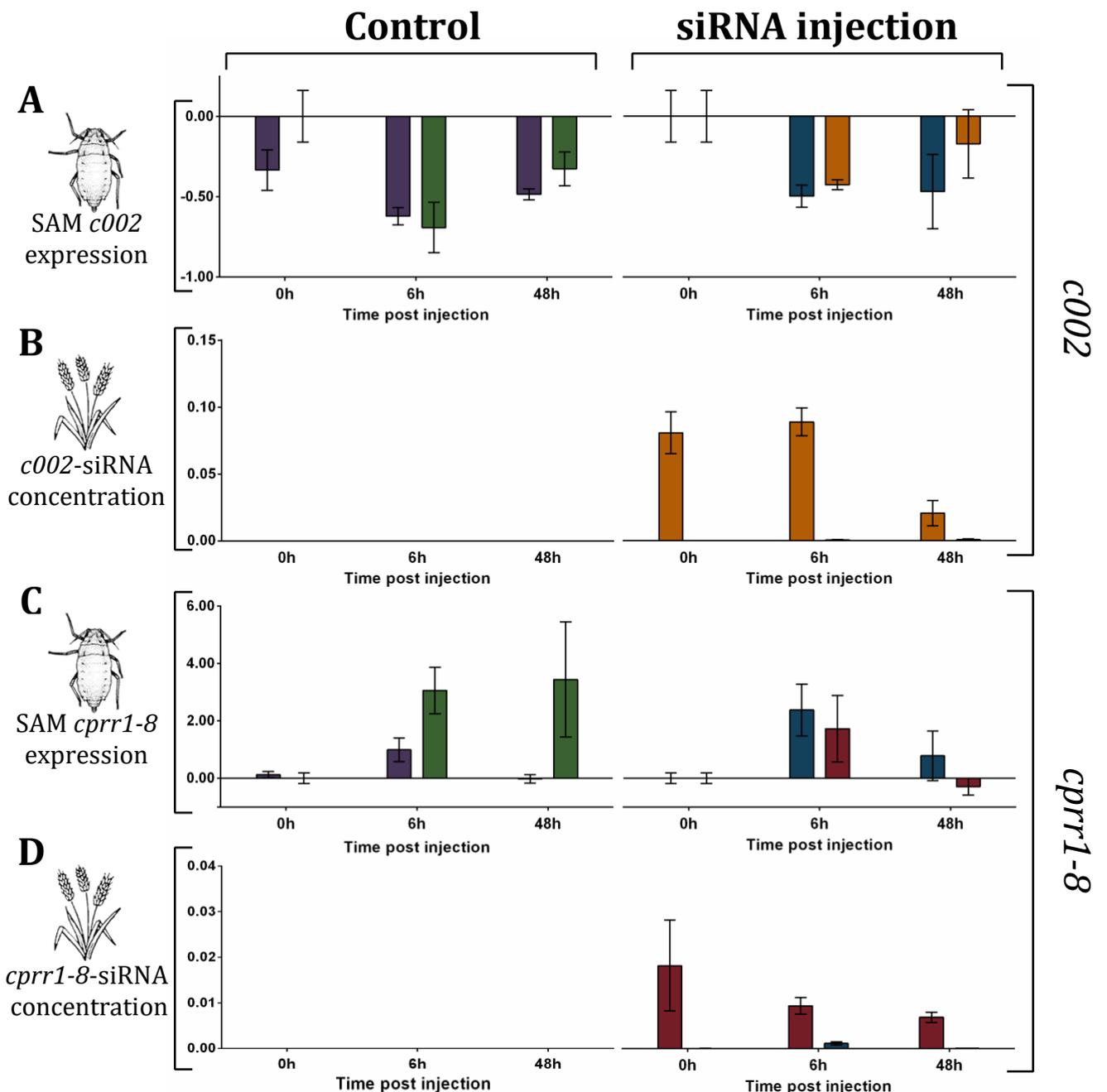


**Figure 3.6.** Relative *Dnc002* expression after *Diuraphis noxia* biotype SAM fed on artificial feeding media supplemented with *Dnc002*-siRNA or water as control. The ribosomal proteins *L27* and *L32* were used as normalizers. Means were tested for statistically significant differences using Tukey's HSD test. Equal alphabets indicate no significant difference.

***Diuraphis noxia* *c002* expression after feeding artificial media containing siRNA**



**Figure 3.7.** Relative *Dncprr1-8* expression after *Diuraphis noxia* biotype SAM fed on artificial feeding media supplemented with *Dncprr1-8*-siRNA or water as control. The ribosomal proteins *L27* and *L32* were used as normalizers. Means were tested for statistically significant differences using Tukey's HSD test. Equal alphabets indicate no significant difference.



**Figure 3.8.** Relative expression of *Dnc002* and *Dncpr1-8* and concentration of siRNA in wheat leaf after injection. *Diuraphis noxia* biotype SAM was allowed to feed on wheat cultivar Gamtoos-R injected with *Dnc002*-siRNA, *Dncpr1-8*-siRNA or buffer (control, no siRNA). *D. noxia* biotype SAM was also allowed to feed on wheat cultivar Gamtoos-R and Gamtoos-S that was not injected (control). Relative expression of *D. noxia* biotype SAM *c002* (A) *cpr1* (B) was determined with RT-qPCR while SAM fed on wheat injected with siRNA using the ribosomal proteins *L27* and *L32* as normalizers. RT-qPCR was also used to determine the *c002*-siRNA (C) or *cpr1-8*-siRNA (D) concentration in the injected wheat leaf on which *D. noxia* biotype SAM fed. Wheat 18S was used as normalizer in this case. ■, Gamtoos-S, no injection; ■, Gamtoos-R, no injection; ■, Gamtoos-R, buffer injection; ■, Gamtoos-R, *Dnc002*-siRNA injection; ■, Gamtoos-R, *Dncpr1-8*-siRNA injection. Error bars represent SEM.

At 6h after aphid introduction, *Dncpr1-8* expression measured in SAM differ according to the plants they fed on and was in the following order (from highest): uninjected GamR > buffer injected GamR > *Dncpr1-8*-siRNA > GamS (Figure 3.8 C). However, the *Dncpr1-8* expression differed only statistically between aphids that fed on GamS, GamR and buffer injected plants ( $P \leq 0.05$ ). At 48 hpi the expression of *Dncpr1-8* in aphids feeding on GamS was significantly lower than that measured after 6 hpi, and also lower than in aphids feeding on *Dncpr1-8*-siRNA injected GamR plants. In fact, the levels of *Dncpr1-8* of the latter aphids were comparable to those feeding on GamS and much lower than those feeding on uninjected GamR plants.

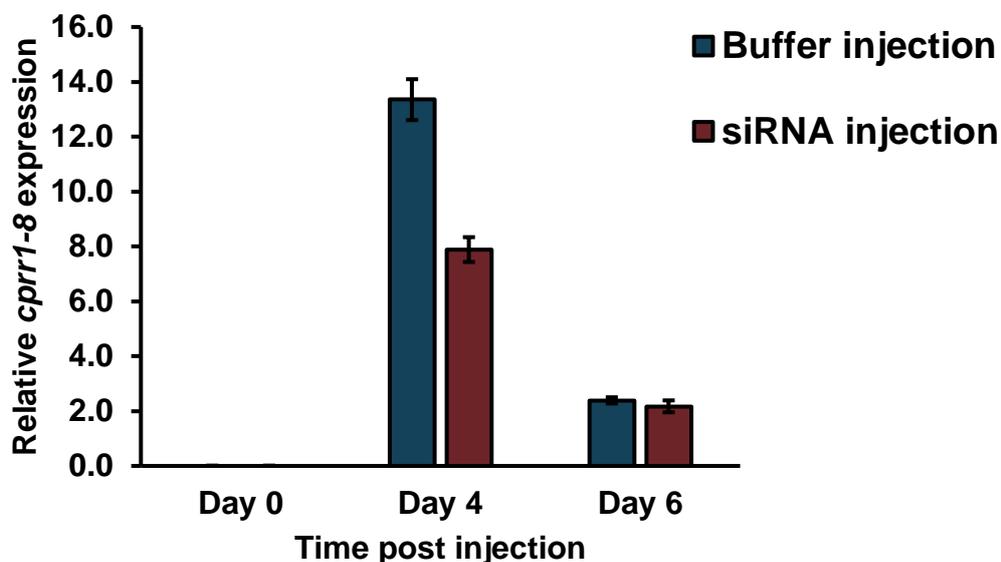
At 48 hpi the expression of *Dncpr1-8* was the lowest in aphids feeding on *Dncpr1-8*-siRNA followed by GamS, buffer injected GamR and the highest expression was observed in aphids feeding on GamR. Although not significant, *Dncpr1-8* expression was lower in aphids that fed on both *Dncpr1-8*-siRNA injected GamR and GamS at 48 hpi compared to 6 hpi. Between the same time points *Dncpr1-8* expression of aphids that fed on buffer injected GamR also decreased, but not to the same extent as *Dncpr1-8*-siRNA injected plants and GamS. When feeding on GamR, *Dncpr1-8* expression stayed roughly the same.

To validate that the response measured in the feeding aphids can be directly correlated to siRNA present in the plants, the levels of *Dncpr1-8*-siRNA and *Dnc002*-siRNA was quantified using stem-loop primers and RT-qPCR analyses to reveal the siRNA concentration relative to wheat *18S* expression (Figure 3.8 B and D). These results confirmed that *Dnc002*-siRNA and *Dncpr1-8*-siRNA was present in the siRNA injected leaves and absent from the untreated leaves. Six hours after injection the siRNA was still present at levels equivalent to levels measured directly after injection. After 48h a decrease in siRNA was observed, although it was still present in significant quantities indicating relative stability within the plant. The measured levels of siRNA were significantly higher ( $P \leq 0.05$ ) in the *Dncpr1-8*-siRNA and *Dnc002*-siRNA injected plants, when compared to all other plants.

### 3.3.5 Transgenerational effect of siRNA

To validate whether the interference also affects the embryos of the feeding foundresses, newly born nymphs were sampled on 0, 96 and 144 hpi and assayed for the expression of *Dncpr1-8* (Figure 3.9). Interestingly, the effect of knockdown was most severe in newly born nymphs produced 96 hpi and differed from that in nymphs produced from foundresses feeding on uninjected GamR plants.

***Dncpr1-8* expression in nymphs born from mothers that fed on wheat injected with *cprr1-8*-siRNA**



**Figure 3.9.** Relative expression of *Dncpr1-8* in nymphs born from siRNA fed adults. *Diuraphis noxia* biotype SAM was allowed to feed on wheat (Gamtoos-R) injected with *cprr1-8*-siRNA dissolved in 10 mM Tris (pH 7) or buffer (10 mM Tris, pH 7). At day 0, 4 and 6, a minimum of 6 nymphs were sampled and combined to determine relative *Dncpr1-8* expression in the nymphs. The ribosomal proteins *L27* and *L32* were used as normalizers. Error bars represent SEM of the RT-qPCR technical repeats (multiple biological samples could not be taken owing to the small amount of biological material).

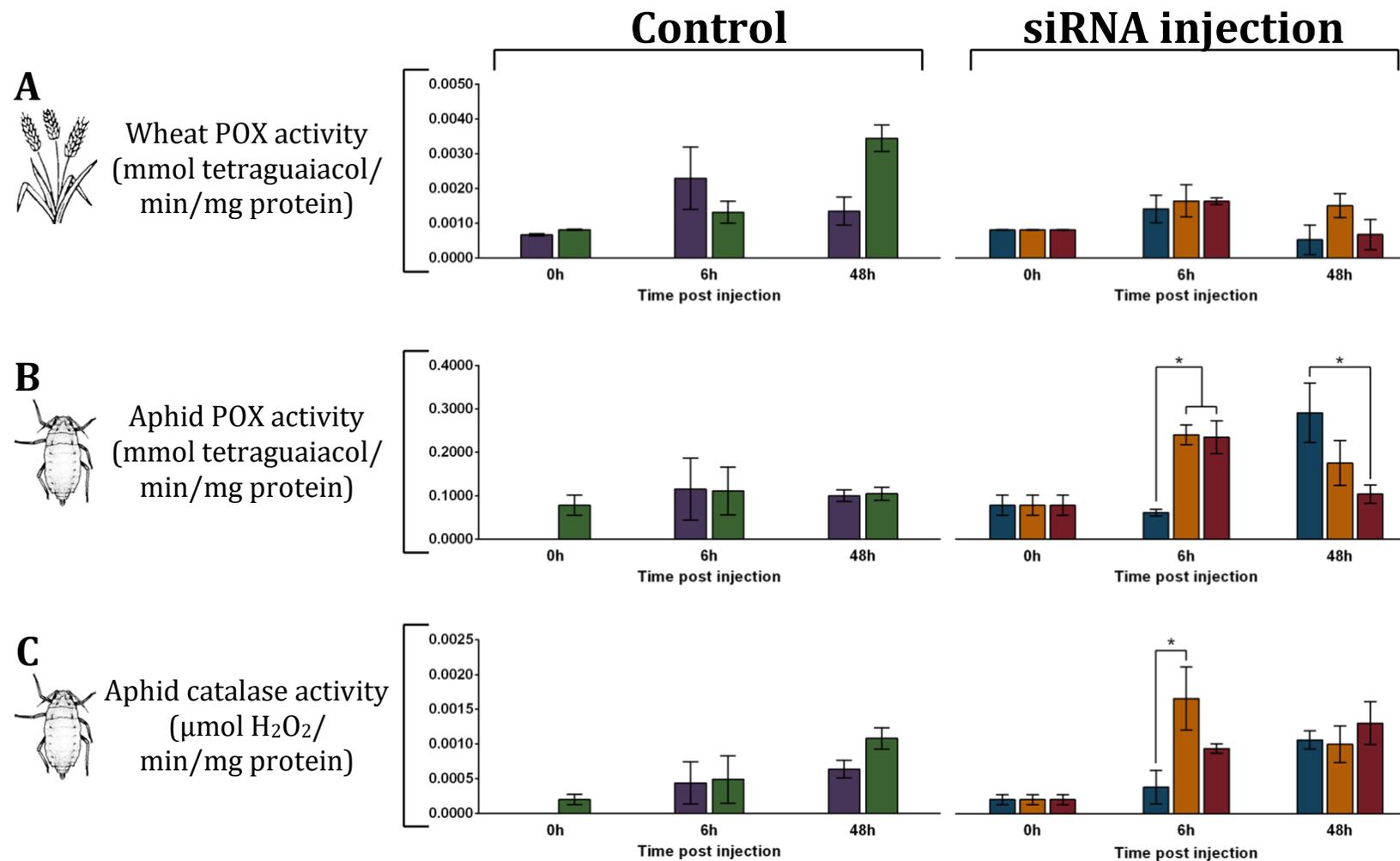
### 3.3.6 Peroxidase and catalase activity

To elucidate the functions of *Dnc002* and *Dncpr1-8* in the salivary glands of virulent biotype SAM during feeding on GamR (a wheat cultivar expressing antibiosis and antixenosis) (Painter 1951, 1958), biotype SAM was allowed to feed on uninjected, buffer injected and plants injected with either 2  $\mu$ g *Dnc002*-siRNA or 2  $\mu$ g *Dncpr1-8*-siRNA, where after the peroxidase activities in the host and feeding aphids and catalase in the aphids were assayed (Figure 3.10). SAM was also allowed to feed in GamS to allow a comparison to uninjected GamR.

As peroxidase (POX) is a ROS enzyme and a marker of the oxidative burst during the host defense throughout the interaction of wheat and *D. noxia* (Van der Westhuizen *et al.* 1998), it was assayed at 0, 6 and 48 hpi (Figure 3.10 A). When comparing the POX activity between uninjected, infested GamS and GamR plants, higher POX activity was measured in the plants after infestation, with the highest POX activity assayed in the GamR 48 hpi ( $P \geq 0.05$ ), which is indicative of the induction of the host defense response (Van der Westhuizen *et al.* 1998; Botha *et al.* 2010, Botha 2013). However, even though POX activity increased slightly

in the *Dnc002*-siRNA and *Dncpr1-8*-siRNA injected plants after 6 hpi, it decreased after 48 hpi to the same level as 0h.

Although, aphid survival rate was still unaffected 6h after feeding on siRNA (Figure 3.4), catalase (CAT) and POX activity increased in aphids feeding on uninjected GamS and GamR, buffer injected and *Dnc002*-siRNA or *Dncpr1-8*-siRNA injected plants over the 48h period (Figure 3.10 B and C). However, POX activity was only significantly higher in aphids feeding on *Dnc002*-siRNA or *Dncpr1-8*-siRNA injected plants ( $P \geq 0.05$ ) 6 hpi (Figure 3.10 B). CAT activity was also elevated at 6 hpi in aphids that fed on *Dncpr1-8*- and *Dnc002*-siRNA injected plants, with CAT activity in *Dnc002*-siRNA fed aphids being significantly higher than in aphids that fed on buffer injected plants (Figure 3.10 C,  $P \geq 0.05$ ).



**Figure 3.10.** Peroxidase and catalase activity. Peroxidase activity of wheat (A) and peroxidase (B) and catalase (C) activity of *Diuraphis noxia* biotype SAM after feeding on wheat leaves not injected, injected with buffer, *c002*-siRNA or *cpr1-8*-siRNA respectively. siRNA was dissolved in 10 mM Tris (pH 7.0) before injection. ■, Gamtoos-S, no injection; ■, Gamtoos-R, no injection; ■, buffer (10 mM Tris, pH 7) injection; ■, *c002*-siRNA, siRNA targeting *c002* dissolved in 10 mM Tris (pH 7); ■, *cpr1-8*-siRNA, siRNA targeting *cpr1-8* dissolved in 10 mM Tris (pH 7). Significant differences determined using Tukey's HSD test. Error bars represent SEM.

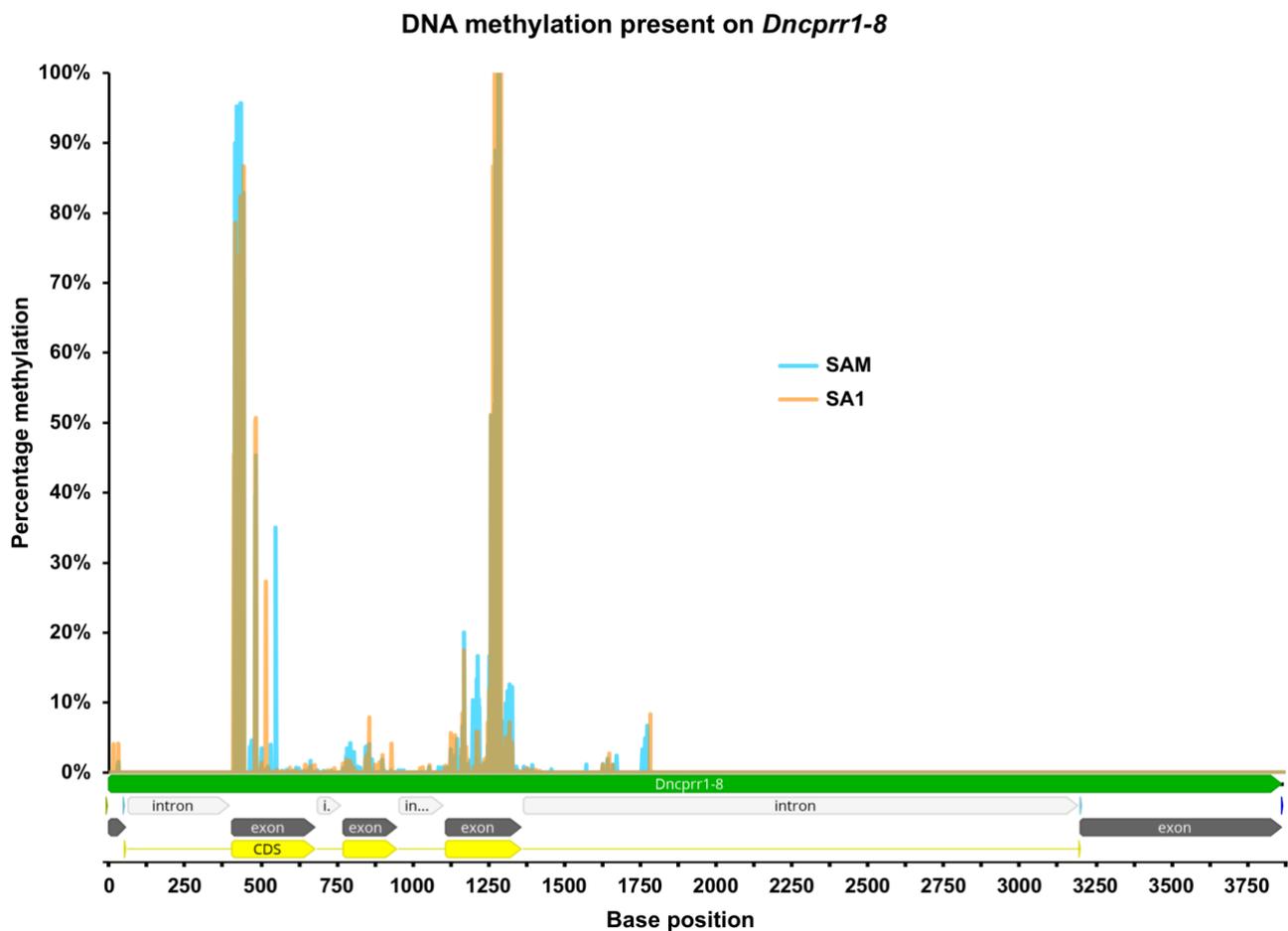
### 3.3.7 DNA methylation of *Dncpr1-8*

In order to determine if DNA methylation is involved in the differential expression of *Dncpr1-8* between biotype SAM and SA1, whole genome sequence data of bisulfite treated DNA from least one hundred and fifty aphids from each biotype, was analyzed (Du Preez, Burger, Truter, Swiegers and Botha, unpublished data). In doing so the proportion of DNA methylation at every cytosine of *Dncpr1-8* was determined for the two biotypes. Methylation was observed at more sites and at a higher frequency in SAM compared to SA1 in the CpG, CHG and CHH contexts (Table 3.3).

**Table 3.3.** DNA methylation of *Dncpr1-8*. The amount of cytosine sites and total proportion of methylation (5-methylcytosine) in the contexts of CpG, CHG and CHH is compared between SA1 and SAM *Dncpr1-8*.

<i>Dncpr1-8</i> methylation								
	Amount of sites methylated				Percentage methylation			
	CpG	CHG	CHH	Total	CpG	CHG	CHH	Total
<b>SA1</b>	38	34	58	130	1.83%	1.13%	0.76%	1.08%
<b>SAM</b>	54	42	67	163	2.58%	1.31%	1.04%	1.45%

Methylation was mainly observed in the exonic areas of *Dncpr1-8* (Figure 3.11). At 416 bp, 422 bp, 433 bp and 436 bp after the TSS, SAM is methylated 90.00%, 95.24%, 95.65% and 95.65% while SA1 was methylated at 78.57%, 73.68%, 82.35% and 82.35%, respectively. That amounts to a difference in the average methylation for those sites of 14.90%. At 518 bp after the TSS of *Dncpr1-8* (second exon), SA1 is methylated at 27.3% while no methylation was observed in SAM even though 26 reads were mapped at that position. In the area of *Dncpr1-8* that translates to the chitin-binding domain (1197-1274 bp from the TSS) the following observations were made: from 1,200 bp to 1,225 bp from the TSS (just left from the highest peak on the graph on the 4<sup>th</sup> exon as seen in Figure 3.11), SAM is 2.13% more methylated than SA1. Furthermore, the highest frequency of DNA methylation in both biotypes was observed at 1268-1294 bp from the TSS.



**Figure 3.11.** Methylation patterns of *Dncpr1-8*. Bisulfite sequencing was used to determine the position and amount of 5-methylcytosine compared to unmethylated cytosine of *D. noxia* biotype SAM and SA1. Coverage of the 3' region of the gene was low and thus presence of methylation could not be determined. A higher frequency of methylation is observed in the exonic regions. Annotations for *Dncpr1-8* obtained from Burger and Botha (2017).

### 3.4 Discussion

Arthropod cuticle is a composite, bipartite system made of chitin filaments embedded in a proteinaceous matrix, which serves as a protective barrier and provides structural and mechanical support (Neville 1993). The physical properties of cuticle are determined by the structure and the interactions of its two major components, which are cuticular proteins (CPs) and chitin (Neville 1993). The proteinaceous matrix consists mainly of structural CPs, while the majority of these belonging to the CPR family, containing a conserved R&R region (Rebers and Riddiford Consensus) (Willis 1999; Rebers and Willis 2001). Two major subfamilies of the CPR family (i.e., RR-1 and RR-2) (Karouzou *et al.* 2007) have been identified from conservation at sequence level and some correlation with the cuticle type (Ioannidou *et al.* 2014). Some proteins containing the RR-1 motif were found in soft (flexible) cuticles, while the proteins containing the RR-2 motif were found in hard (rigid) cuticles, but this distinction is not firmly established (Andersen 2000). While Rebers and Riddiford (1988)

suggested that the original consensus would turn out to be a region of structural importance, Andersen *et al.* (1995) postulated that this motif might be involved in protein/chitin interaction.

In the present study, the function of *Dncpr1-8* is being investigated as it has been shown to be expressed in the more virulent biotype SAM, but not in SA1 when feeding on *D. noxia* resistant wheat plants (Cloete 2015). The possible involvement of the protein product of *Dncpr1-8* in protection against plant defense molecules, especially when feeding on resistant wheat cultivars is discussed here.

In the study of Burger and Botha (2017), the authors found two SNPs in between the biotypes SA1 and SAM *Dncpr1-8*, based on NGS. In the current study, Sanger sequencing of *Dncpr1-8* did not exhibit these SNPs, however a novel SNP was revealed. It was present in one clone from the virulent biotype SAM and results in a change in predicted protein structure. Although an alteration in protein structure could result in a change in protein function and therefore perhaps explain the fact that SAM is able to feed on *D. noxia* resistant wheat, the low frequency (1/11) of the polymorphism indicate that this cannot be the only explanation.

The cause of the presence of *Dncpr1-8* in the salivary gland of SAM, but not SA1 (Cloete 2015) could be explained by the elevated *Dncpr1-8* expression (0.25 fold) in SAM compared to SA1 feeding on GamS. Although this difference is not statistically significant a larger sample size could lead to a significant result. Furthermore, gene expression was measured after 10 days of aphid feeding, while a greater change in gene expression is expected directly after a host shift. This should be investigated in the future.

Based on the preliminary results, the novel method of injecting siRNA into wheat proved to be the most effective method of siRNA delivery to *D. noxia*. It had the greatest phenotypic effect in terms of survival and nymph production and this was shown through gene expression analysis likely to be a result of RNAi mediated gene silencing. In terms of an experimental method, it also has the benefits of being the most cost effective and the quickest method to perform compared to the other methods investigated. siRNA was also present in the host plant for a surprising amount of time. It was at most less than 4-fold lower at 48h compared to 6h post injection in the case of *Dnc002*-siRNA.

Using a viral vector to produce long dsRNA of aphid sequence in its host was a promising method as in some insects long dsRNA is more effective at inducing RNAi than short dsRNA (Huvenne and Smaghe 2010). This is in part because an siRNA molecule is very specific

and thus any change in sequence identity or the occurrence of mRNA secondary structure will have a larger impact on its effectiveness. In an experimental setting this can be negated with some success through proper design of the siRNA molecule, but in a pest control application long dsRNA would be preferred as it should be less susceptible to the development of resistance by the target insect.

The fact that feeding on siRNA supplemented artificial media by SAM did not result in death of any aphids could be as a result of an ideal environment for the aphid, lacking any antagonism by its host. This was however proven not likely to be the case as gene expression analysis demonstrated no gene silencing using this method of siRNA delivery. The lack of phenotypic response is thus more likely simply because siRNAs are not effectively delivered to aphids through artificial media. Although most reports of siRNA delivery to aphids through artificial media result in successful RNAi, Christiaens *et al.* (2014) reported that aphid saliva is able to degrade RNA. It could be that aphid saliva is transported elsewhere by the phloem while the aphid is feeding on a plant, whereas the saliva accumulates while feeding on artificial media.

Feeding on *Dncprr1-8*-siRNA not only resulted in gene silencing of adult aphids, but also in the nymphs produced by these adults. A proportion of the reduction of nymphs observed after ingestion of *Dncprr1-8*-siRNA could thus be the result of increased nymph death, instead of a lower nymph production by the adults. Gene silencing in nymphs is in accord to earlier findings in *Myzus persicae* (Coleman *et al.* 2014), where the *Mpc002* was down-regulated in nymphs born from mothers exposed to *c002*-dsRNA-producing transgenic *Arabidopsis* plants. In their study, they followed the impact of RNAi over three generations of aphids and revealed that aphids reared on *c002*-dsRNA producing transgenic plants experienced a 60% decline in aphid reproduction levels compared with a 40% decline of aphids reared on *Rack1*-dsRNA- and *MpPIntO2*-dsRNA producing plants.

In higher organisms, reactive oxygen species (ROS) are regularly generated by mitochondrial electron transport, when partially reduced and highly reactive metabolites of O<sub>2</sub> such as superoxide anion (O<sub>2</sub><sup>-·</sup>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) are formed during cellular respiration. Excessive release of ROS damages lipids, proteins, and DNA; which leads to oxidative stress, loss of cell function, and programmed cell death (Freeman and Crapo 1982; Starkov 2008). ROS are also actively released by hosts, in response to cellular invasion by pathogens as first line of defense and occurs in all eukaryotic cells. To regulate oxidative stress, the eukaryotic cell produces different ROS-scavenging enzymes, such as superoxide

dismutase (which reduces  $O_2^-$  to  $H_2O_2$ ), glutathione peroxidase and catalase (which reduces  $H_2O_2$  to  $H_2O$ ) (Thannickal and Franburg 2000).

In the context of this study, it is deemed to happen in both the host plant (Fahnenstich *et al.* 2008) and insect species (Miller *et al.* 2000; Molina-Cruz *et al.* 2008). An increase in peroxidase activity also occurs in wheat after *D. noxia* infestation which is indicative of the activation of systemic acquired resistance (SAR) (Van der Westhuizen *et al.* 1998; Smith *et al.* 2005, 2010; Botha *et al.* 2010, Botha 2013), albeit the induction is delayed in susceptible varieties (Van der Westhuizen *et al.* 1998). However, virulent *D. noxia* biotype SAM avoids detection by its host plant during feeding and a limited increase in peroxidase activity and SAR is measured (Botha *et al.* 2014).

POX activity in GamR injected with *Dnc002*- or *Dncpr1-8*-siRNA decreased at 48h hpi of SAM compared to 6 hpi, in contrast to uninjected GamR where the highest induction of POX was measured at 48 hpi of SAM. This observation suggests that unlike aphids feeding on uninjected GamR plants, the aphids feeding on the *Dnc002*-siRNA and *Dncpr1-8*-siRNA injected plants were not perceived as invasive. Hence the decrease in the transcription of host defense proteins like POX, as these are expected to increase as part of the systemic acquired resistance pathway in the resistant GamR plants and remained elevated to provide prolonged basal resistance (Van der Westhuizen *et al.* 1998; Smith *et al.* 2005, 2010; Lapitan *et al.* 2007b; Botha *et al.* 2010, Botha 2013).

ROS metabolism influences critical parameters of insect physiology, including fecundity (DeJong *et al.* 2007; Diaz-Albiter *et al.* 2011), and immune response (Ha *et al.* 2005a, 2005b). The increase of POX activity at 6 hpi in biotype SAM that fed on *Dnc002*- and *Dncpr1-8*-siRNA and CAT activity in SAM that fed on *Dnc002*-siRNA injected GamR at 6 hpi compared to buffer injection is indicative of cellular stress experienced in response to the aphids' feeding environment. These results suggest that both genes afford the aphids some level of "protection" while feeding on the antixenotic and antibiotic GamR, as partial knockdown of these genes also decreased foundress survival by approximately 50%, and affected nymph production significantly during *in planta* feeding experiments. In a field setting, a reduction of the aphid reproduction by 40–60% would dramatically decrease aphid population growth, contributing to a substantial reduction in agricultural losses (Coleman *et al.* 2014).

As analysis of DNA sequence could not explain the difference in expression of *Dncpr1-8*, methylation patterns of this gene were compared between biotypes SA1 and SAM. The

differences in the methylation patterns between SA1 and SAM are likely large enough to be able to result in a change in gene expression. Walsh *et al.* (2010) correlated a 50% decrease in methylation at a specific site with the formation of a winged aphid morph. This was found in the juvenile hormone binding protein of *Acyrtosiphon pisum*. The elevated *Dncpr1-8* expression found here could therefore possibly be explained by the difference in DNA methylation of *Dncpr1-8*.

A general trend exists that more virulent biotypes are less methylated on a genome scale (Gong *et al.* 2012; Breeds *et al.* 2018), which implies that specific genes involved in virulence should also be less methylated, on average. Furthermore, lower levels of methylation result in variable levels of expression between genes and also of the same gene between different tissues or phenotypic morphs, while a higher proportion of methylation, as observed in SAM, is usually linked to ubiquitous expression (Hunt *et al.* 2010). Thus, it can be hypothesized that virulent aphid biotypes contain less methylation in order to be able to be more responsive in terms of gene expression when confronted with a resistant host. Clearly this is not the case with *Dncpr1-8* as there is a definite increase in methylation in the virulent biotype, SAM. Even though bisulfite sequencing does not distinguish between 5mC and 5hmC (Huang *et al.* 2010) and thus a proportion of the methylation in SAM may be 5hmC, higher initial levels of 5mC is required for high 5hmC to be observed. Thus, at least in some point in the past the 5mC levels in SAM had to be higher than SA1. However, *Dncpr1-8* is still expected to be involved in virulence of SAM as it has been implicated before (Cloete 2015) and here it was shown to be expressed at a higher level than in SA1 combined with the fact that reduced expression results in a decrease in survival rate and nymph production. The increase in methylation in SAM is thus rather expected to result in stable and increased expression of *Dncpr1-8* to possibly protect it from xenobiotics produced by some resistant wheat cultivars.

### 3.4. Conclusion

RNA interference (RNAi) has been successfully used as a tool to study gene function in aphids, and in this study it was applied to demonstrate the importance of *Dncpr1-8* in *D. noxia* fecundity. As demonstrated, partial knockdown of these genes decreased foundress survival by approximately 50%, and affected nymph production significantly during feeding on siRNA injected host plant. This study also describes the first report of gene silencing in *D. noxia* and a novel method of siRNA delivery to aphids and perhaps other plant feeding insects. This method allows one to observe the interaction of plant and aphid during a gene silencing experiment, while this is not the case for siRNA/dsRNA delivery through artificial

media, a popular method for aphid-gene silencing studies; it is also non-invasive unlike direct injection of siRNA or dsRNA into the aphid hemolymph and less laborious than plant transformation if elucidating gene function is the immediate goal. In the present experiment, it is also demonstrated that transgenerational knockdown as decreased expression of the *Dncpr1-8* gene in the newly born nymphs, making this method highly useful and feasible for aphid-plant interaction studies.

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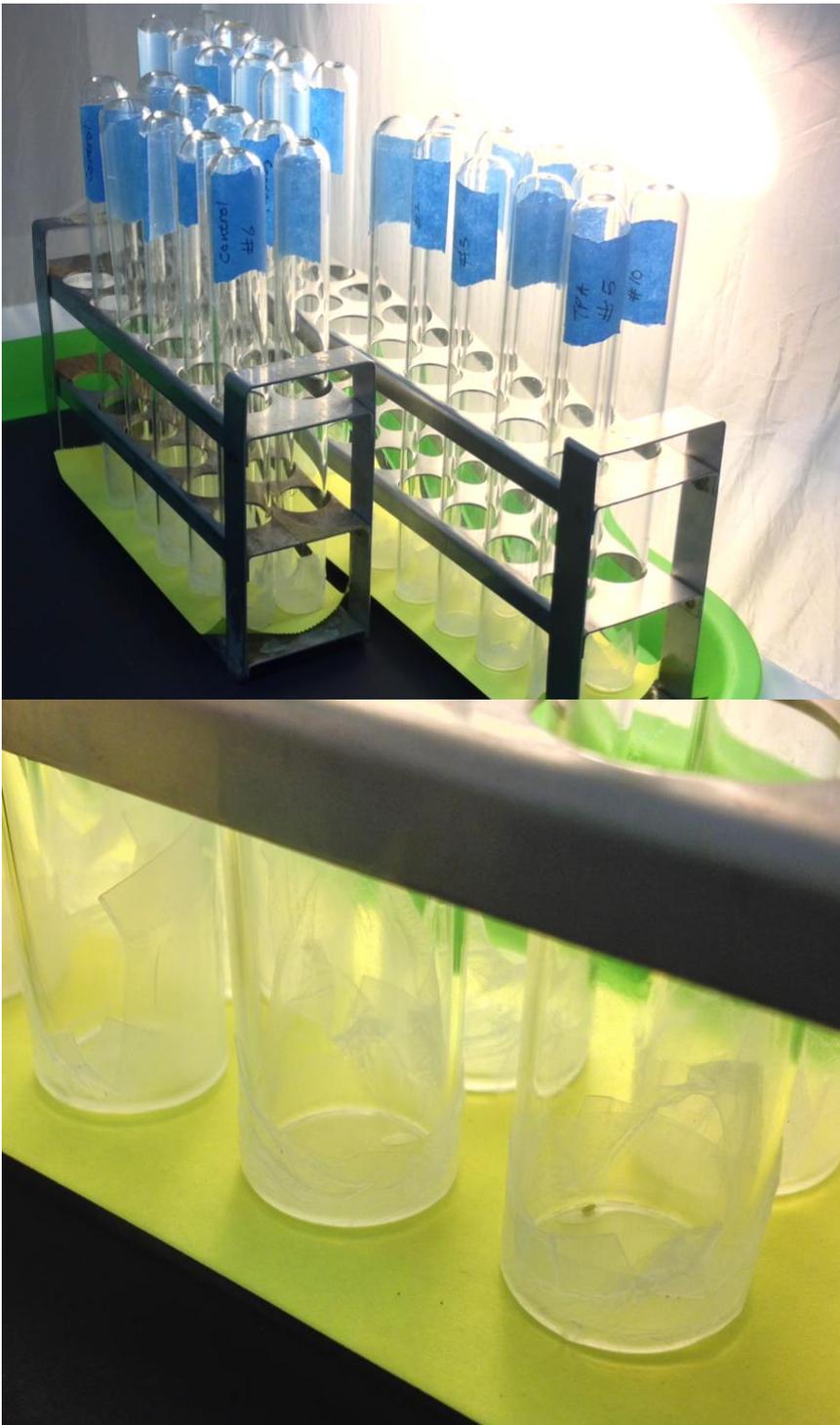
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Centre for Agriculture and Biosciences International 2018 Invasive Species Compendium  
*Diuraphis noxia* (Russian wheat aphid). (Available at: <https://www.cabi.org/isc/datasheet/9887>, accessed on 8 September 2018).

### 3.6 Supplementary material



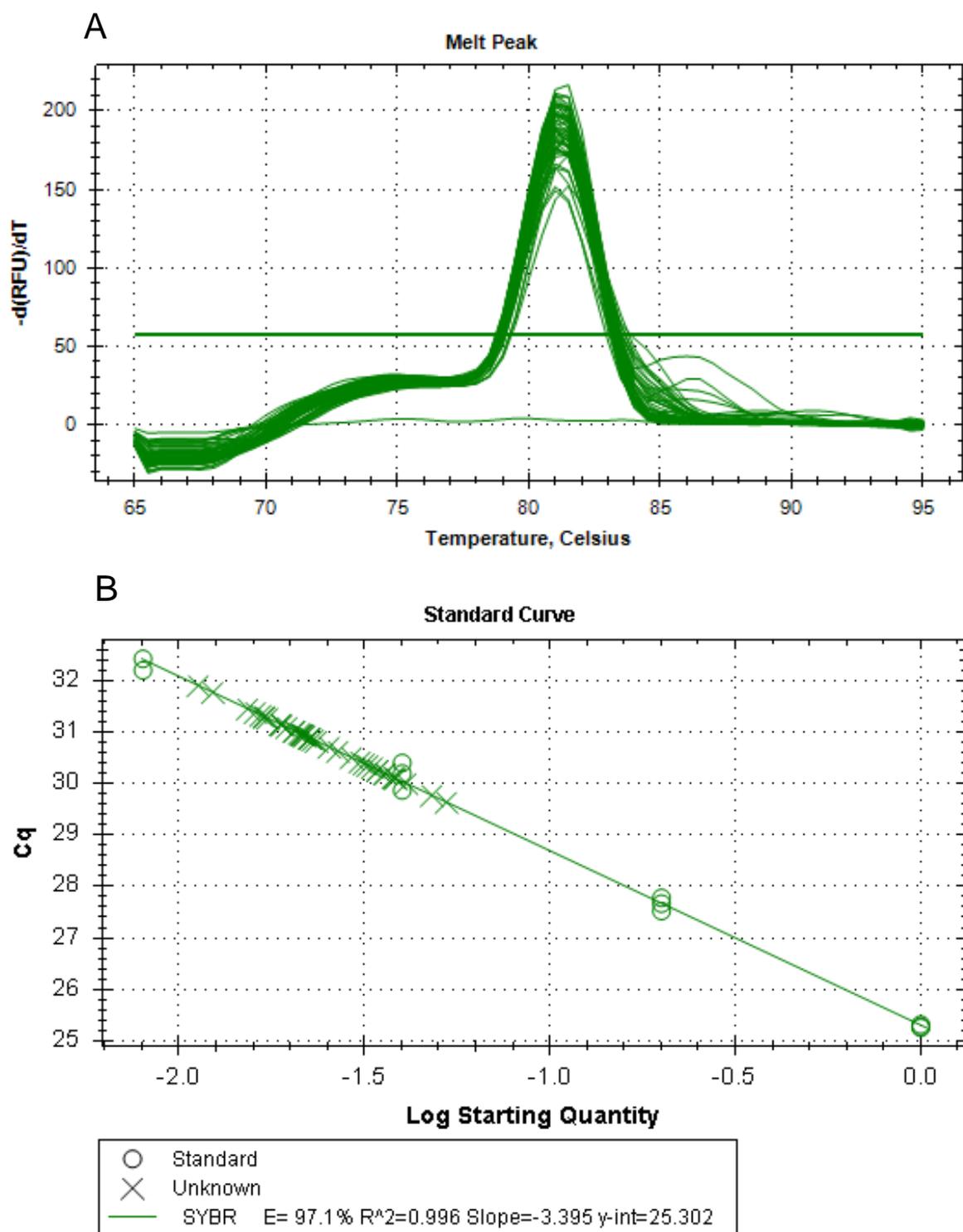
**Supplementary Figure S3.1.** Aphid cages: virus mediated dsRNA delivery. An example of the cages used to contain *Diuraphis noxia* on modified *barley stripe mosaic virus*-inoculated wheat (Gamtoos-R, containing *Dn7*). A cage comprised of inverted 15 ml polypropylene tubes (Greiner Bio-One) modified to allow the leaf to be threaded through a slit in the lid. Any opening was closed with cotton wool.



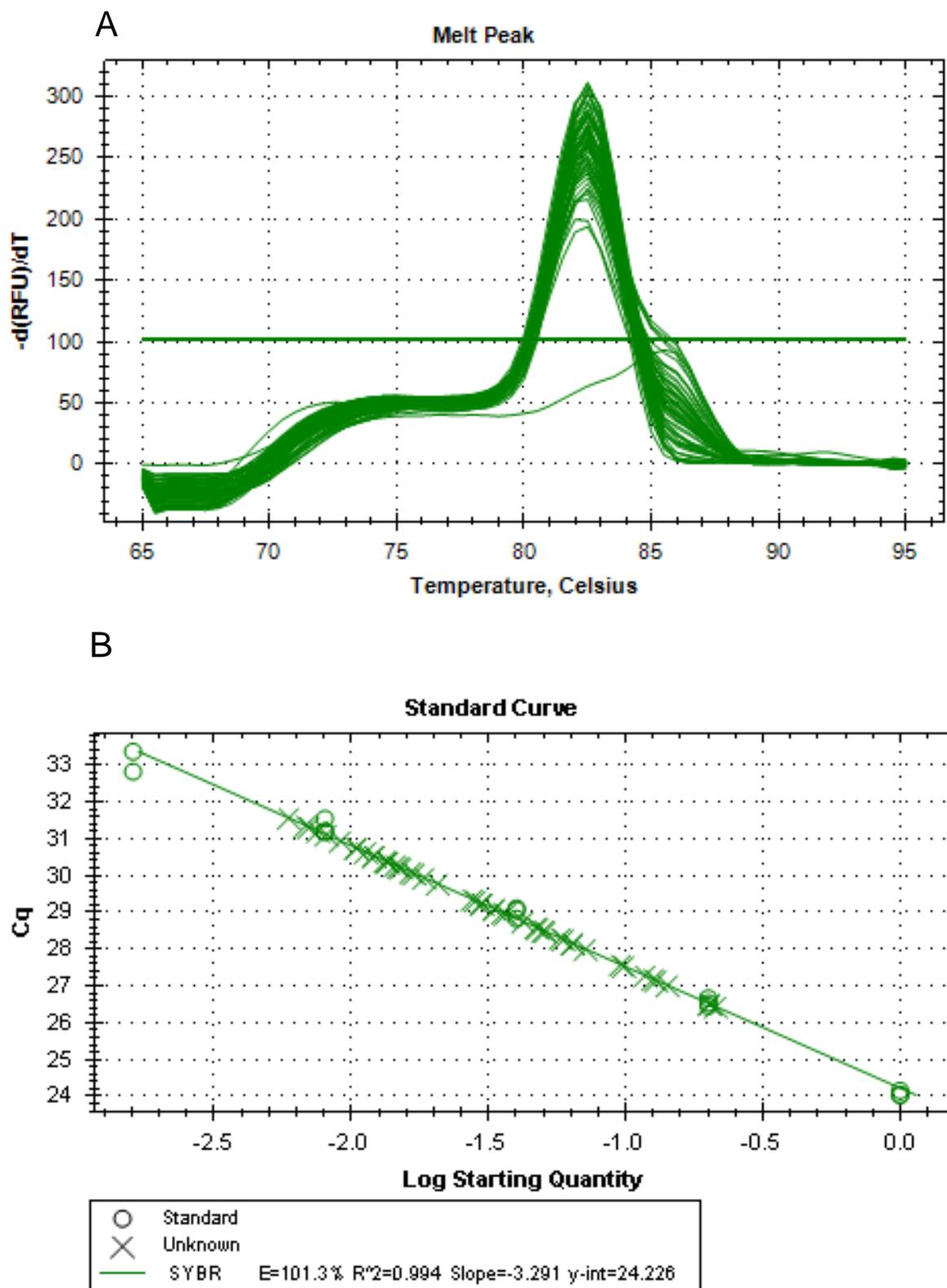
**Supplementary Figure S3.2.** Containment and exposure of *Diuraphis noxia* to artificial feeding medium. An example of the setup used to contain and allow feeding of *D. noxia* on artificial feeding medium supplemented with siRNA. The medium contained 0.10 g L-methionine (Merck), 0.20 g L-leucine (Sigma–Aldrich), 0.10 g L-tryptophan (Merck), 20.00 g sucrose (Merck), 0.20 g  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (Merck), 0.25 g  $\text{K}_2\text{HPO}_4$  (Sigma–Aldrich), pH 7.0 (KOH, Merck) in a final volume of 100 ml. After an aphid was placed a test tube Parafilm M (Bemis, USA) was stretched close to its maximum capacity and placed over the opening of a test tube (14 mm outside diameter). A volume of 25  $\mu\text{l}$  siRNA-supplemented artificial medium was placed on the stretched Parafilm M. Another layer of Parafilm M was then placed over the artificial media, spreading the media between the two layers.



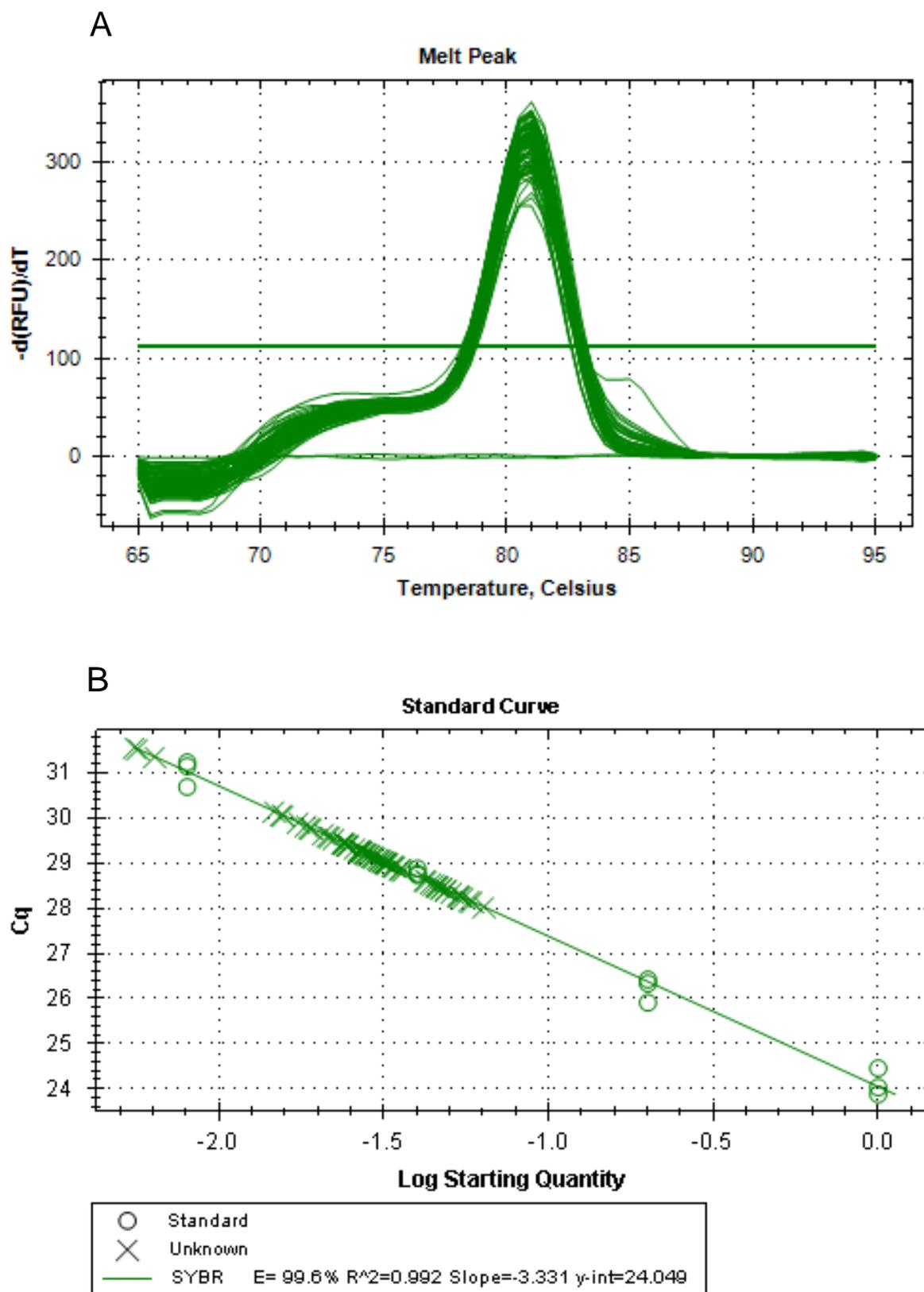
**Supplementary Figure S3.3.** Aphid cage: siRNA injection into wheat. A photograph illustrating the cage used to contain aphids over the site of siRNA injection on a wheat leaf. A 15 ml polypropylene tube was cut into 45 mm sections. Adult aphids were placed on the leaf after which it was threaded through the modified polypropylene tube which was then held in place by cotton wool inserted at the top and bottom of the leaf at each end of the tube. The cotton wool was adjusted to allow aphids to move around freely within a  $\pm 25 \text{ mm}^2$  area centered around the site of injection.



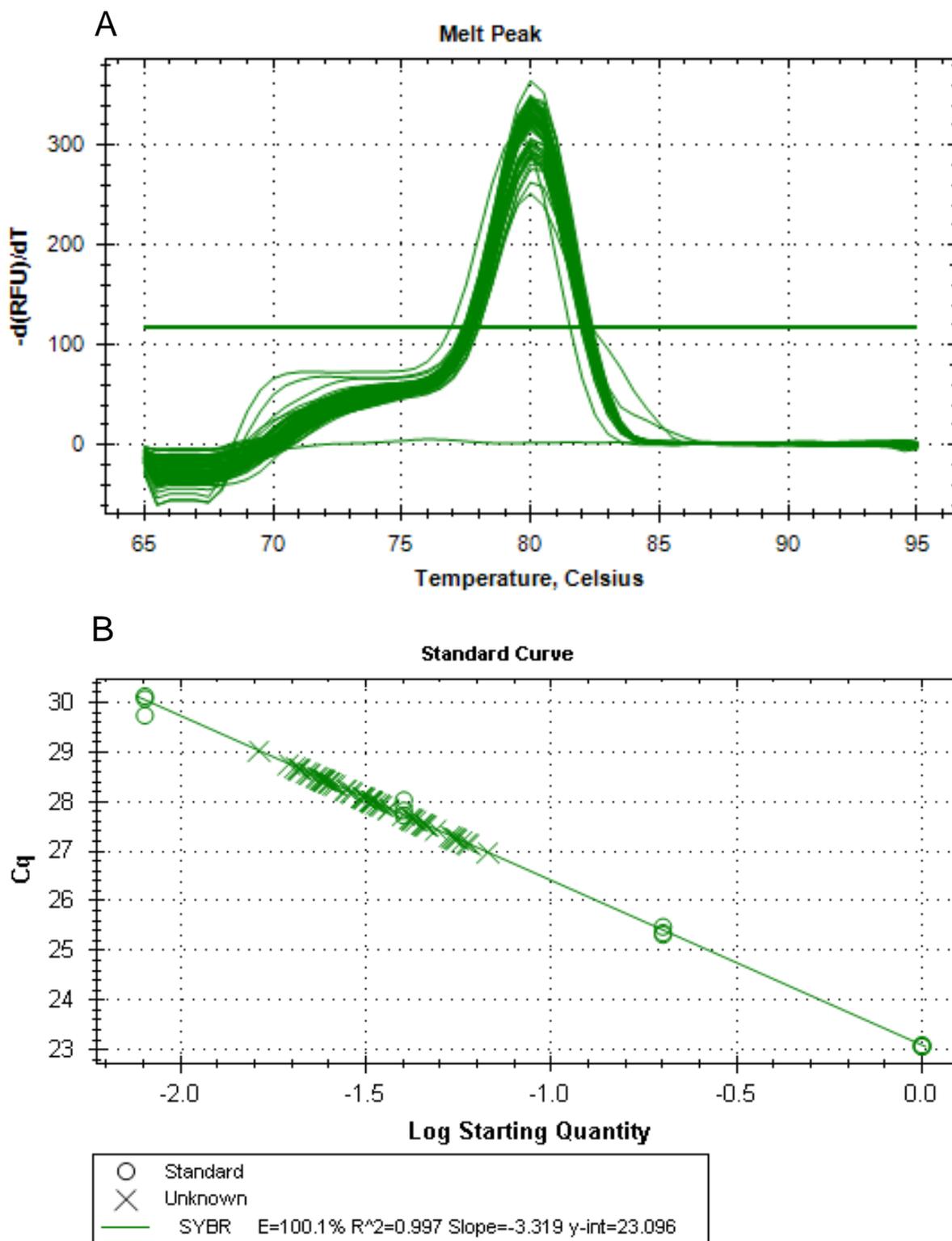
**Supplementary Figure S3.4.** Melt curve (A) and standard curve (B) for *Dnc002*. The melt curve indicates a single product.



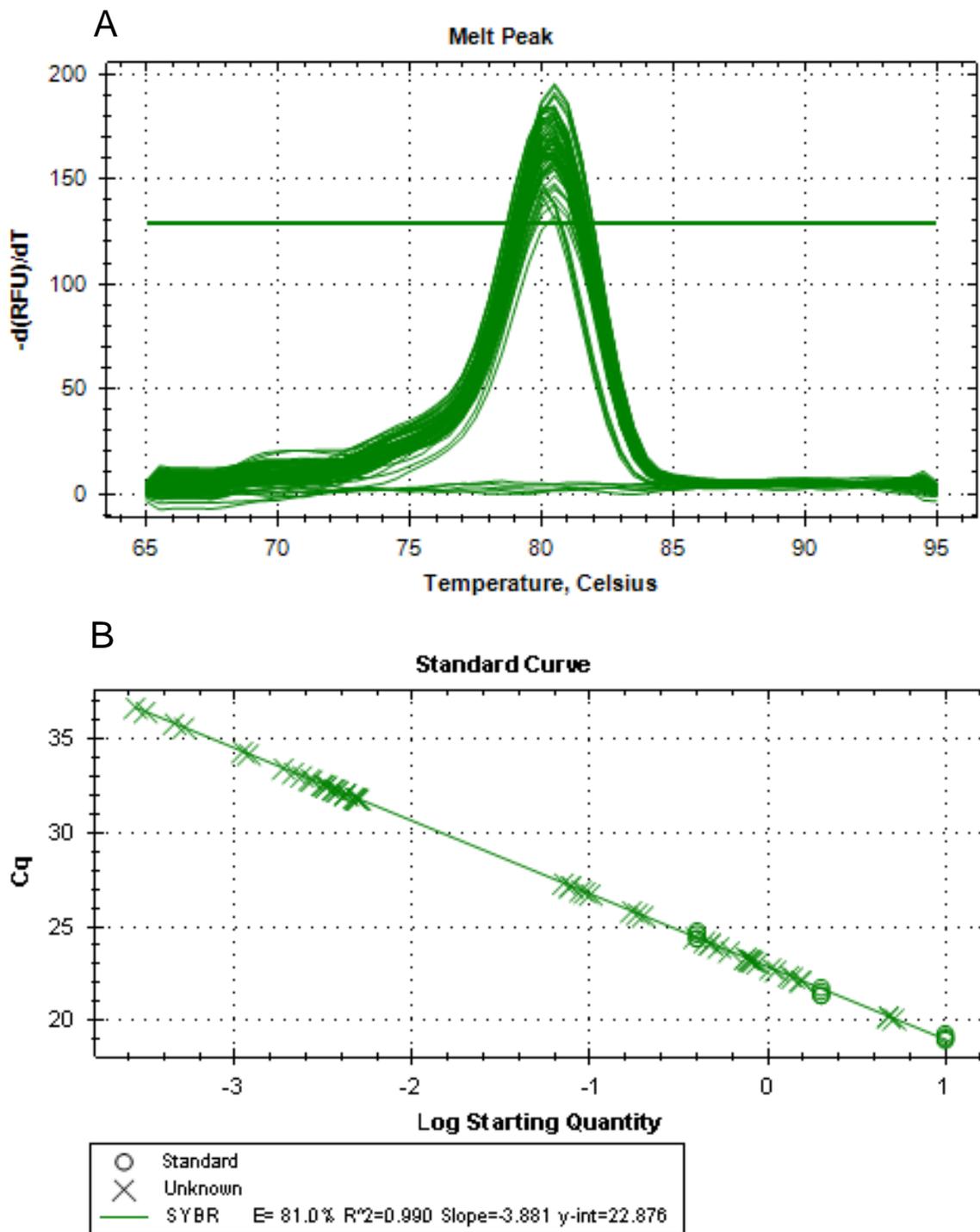
**Supplementary Figure S3.5.** Melt curve (**A**) and standard curve (**B**) for *Dncpr1-8*. The melt curve indicates a single product.



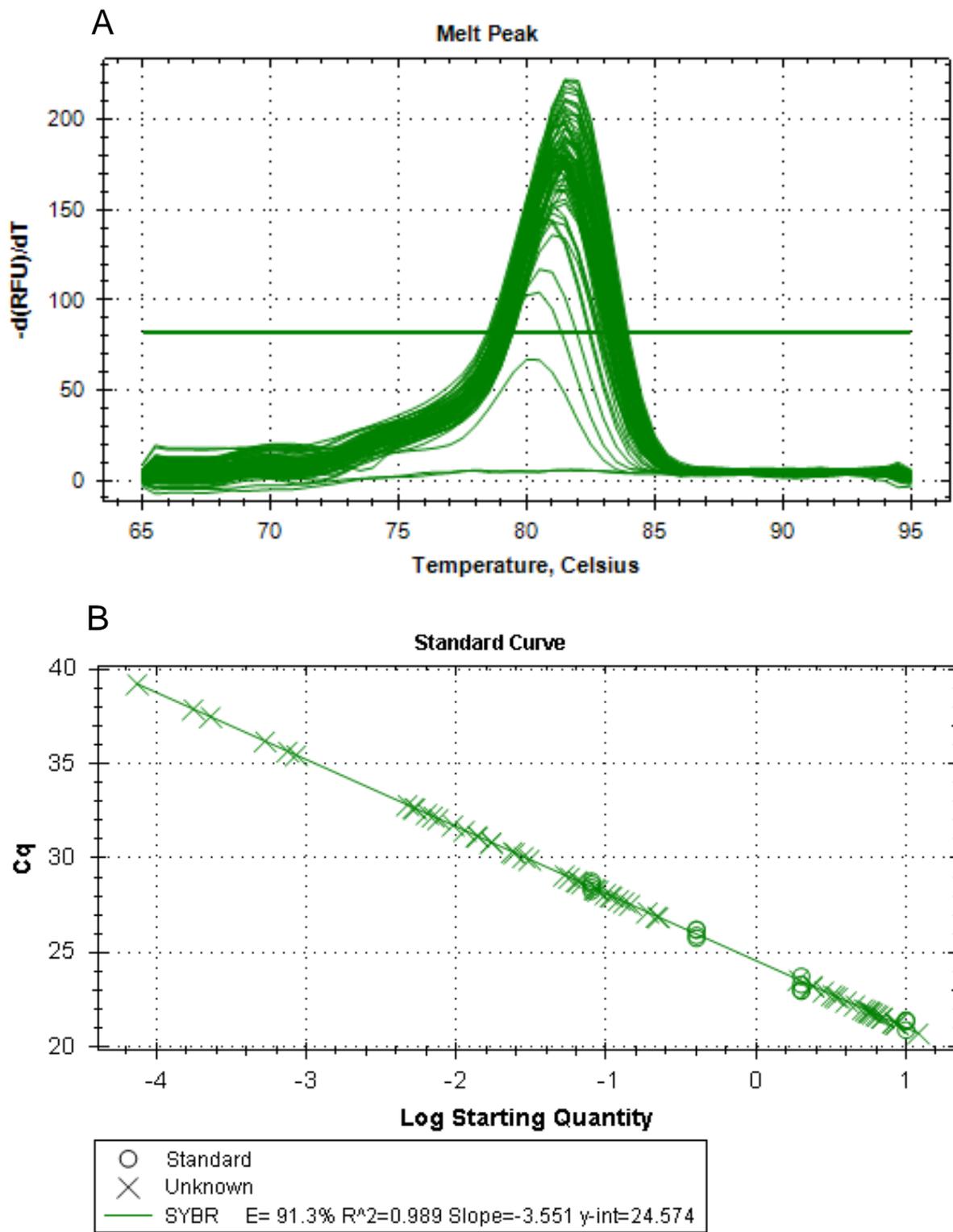
**Supplementary Figure S3.6.** Melt curve (A) and standard curve (B) for *L27*. The melt curve indicates a single product.



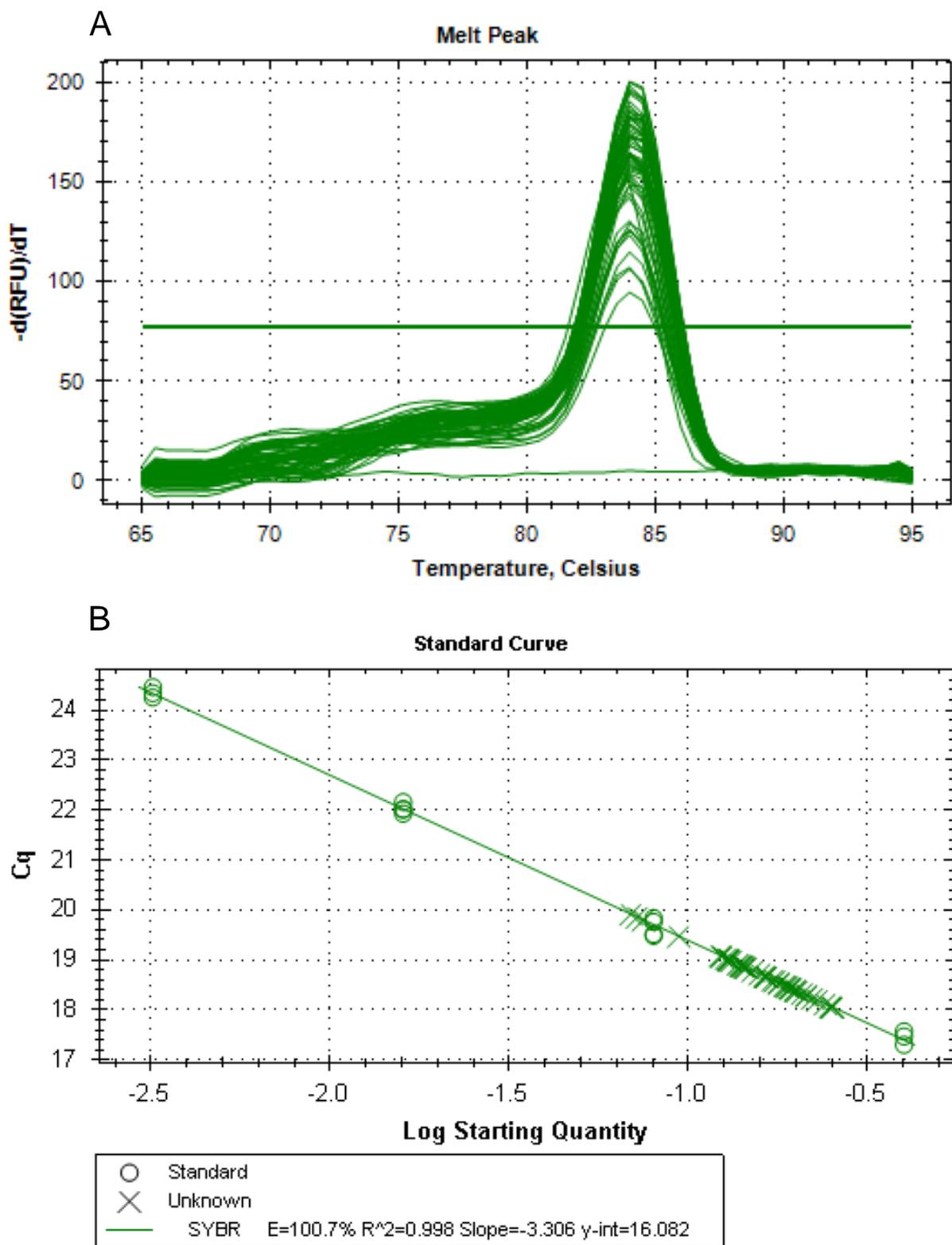
**Supplementary Figure S3.7.** Melt curve (A) and standard curve (B) for *L32*. The melt curve indicates a single product.



**Supplementary Figure S3.8.** Melt curve (A) and standard curve (B) for the primers *Dncpr1-8*-siRNA F and universal stem-loop R. The melt curve indicates a single product.



**Supplementary Figure S3.9.** Melt curve (A) and standard curve (B) for the primers *Dnc002*-siRNA F and universal stem-loop R. The melt curve indicates a single product.



**Supplementary Figure S3.10.** Melt curve (A) and standard curve (B) for wheat 18S. The melt curve indicates a single product.

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SA1_Sanger_DNA      -----GGTATACGTCCAAAAAATCACCATGAACA
SAM_NGS             CATCCCAACATACGGCAATTTTCTGATCACGGTATACGTCCAAAAAATCACCATGAACA
                      *****

SA1_Sanger_DNA      CTTTGGTGAGTTAAATAATATTTCTTTAAATCTTCTTAAAAAAGACATAGCAATATTATC
SAM_NGS             CTTTGGTGAGTTAAATAATATTTCTTTAAATCTTCTTAAAAAAGACATAGCAATATTATC
                      *****

SA1_Sanger_DNA      ATTTTATTTTTTAATTTAGTTATACAACGCATTGTATAATAGTTTCAATTTATAAACAGT
SAM_NGS             ATTTTATTTTTTAATTTAGTTATACAACGCATTGTATAATAGTTTCAATTTATAAACAGT
                      *****

SA1_Sanger_DNA      TTAATTTAAAGAAAAAATAGTATARTTACTGAAATTATTTATTATTTTCGTACTTTATCA
SAM_NGS             TTA-----
                      ****

SA1_Sanger_DNA      TACACTTATGAATTTTTAGTATTTTTGGTATACGAGAATATCTTATTATTTTATAAATAT
SAM_NGS             -----

SA1_Sanger_DNA      CTTATAAAATAAATGCTCATATTATGTTATACTTATTTTTTAAATTAATGAAACTACGAA
SAM_NGS             -----

SA1_Sanger_DNA      AAAATTAATTTTAACTCAAATTTTCAAATTTTTTAAGTTCAAATAAGTACCTCAATTTAT
SAM_NGS             -----

SA1_Sanger_DNA      ATTATGAACAGTGTAAGTATAATATTTACGTTTACTGCAACCATTATTATATTAGAAT
SAM_NGS             -----

SA1_Sanger_DNA      CAGTCTTATTATTTTTGTACTTCATAAAAAAATGCCTGAAAATTAAAATTTAAAAGATAT
SAM_NGS             -----

SA1_Sanger_DNA      CTCTACAATTTAACGCATAGGTAATCTTATTAATCGTAATCATATTTTAACCAAATCCAT
SAM_NGS             -----TTAACGCATAGGTAATCTTATTAATCGTAATCATATTTTAACCAAATCCAT
                      *****

SA1_Sanger_DNA      ATTTTTAGTTTCAATTTAAAATTGACATTACACCTGTAAAAGTTTTTCACAGTATATC---
SAM_NGS             ATTTTTAGTTTCAATTTAAAATTGACATTACACCTGTAAAAGTTTTTCACAGTATATCTTC
                      *****

SA1_Sanger_DNA      -----TTCACAGCAAAAAATATGCATA
SAM_NGS             ACAGCAAAAAATATGCATAAAATTATTTCTTDCATGATTTTCACAGCAAAAAATATGCATA
                      *****

SA1_Sanger_DNA      AAATTATTTCTTTCCTCAGCACTTTTATACAATTTTCGTGCCTTCGTTTTTAGGTAGTGTT
SAM_NGS             AAATTATTTCTTTCCTCAGCACTTTTATACAATTTTCGTGCCTTCGTTTTTAGGTAGTGTT
                      *****

SA1_Sanger_DNA      AGTAGCTGTCATCGCAGCGGTGTCTGCTGCGGCCCCACCTCAGGAAGCTGCCAAAGCTTT
SAM_NGS             AGTAGCTGTCATCGCAGCGGTGTCTGCTGCGGCCCCACCTCAGGAAGCTGCCAAAGCTTT
                      *****

SA1_Sanger_DNA      TACTTTCAGTGGATTCCCATCCAACCAAGCCTACTACCCAGGCCAACAAAGGGTACTACCC
SAM_NGS             TACTTTCAGTGGATTCCCATCCAACCAAGCCTACTACCCAGGCCAACAAAGGGTACTACCC
                      *****

SA1_Sanger_DNA      AGGATACATTGGTTACCAGGGTTATCAAGGTTACAGCGGATTCCGTAATGGATACTACCC
SAM_NGS             AGGATACATTGGTTACCAGGGTTATCAAGGTTACAGCGGATTCCGTAATGGATACTACCC
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SA1_Sanger_DNA      GGGACAACAAGGATACTACCCAGGATACCAAGGTTACCAGGGATACTACCCAGGATACCA
SAM_NGS              GGGACAACAAGGATACTACCCAGGATACCAAGGTTACCAGGGATACTACCCAGGATACCA
*****

SA1_Sanger_DNA      AGGTTACCAGGGATACTACCCAGGATATCAAGGTTTAATTTTCGTTAATTATACGTCTAAA
SAM_NGS              AGGTTACCAGGGATACTACCCAGGATATCAAGGTTTAATTTTCGTTAATTATACGTCTAAA
*****

SA1_Sanger_DNA      ACAC TGCAGAGTCACATGATGTGTTATAAGTTTCTTATAATTTACTATTTTCACATAGGT
SAM_NGS              ACAC TGCAGAGTCACATGATGTGTTATAAGTTTCTTATAATTTACTATTTTCACATAGGT
*****

SA1_Sanger_DNA      TACAACCGCGGTTACTACCCAGGTGCCCCAGCCGTCTACCCACCGTCACCCCGCCCCA
SAM_NGS              TACAACCGCGGTTACTACCCAGGTGCCCCAGCCGTCTACCCACCGTCACCCCGCCCCA
*****

SA1_Sanger_DNA      ATCATCGCACCAGTGCCAGTCGCGCCCAAGGCTGTTTCTCCAGTGTACAAACCCGTAGAC
SAM_NGS              ATCATCGCACCAGTGCCAGTCGCGCCCAAGGCTGTTTCTCCAGTGTACAAACCCGTAGAC
*****

SA1_Sanger_DNA      AACAAAGTGCCAGCTATCCTTAAACAATCGCAAGAAGCTGACTTGAACGGATTCAAATAC
SAM_NGS              AACAAAGTGCCAGCTATCCTTAAACAATCGCAAGAAGCTGACTTGAACGGATTCAAATAC
*****

SA1_Sanger_DNA      GGGTTAGTATTATAAATTGGTACATTATTATTTCGATCGGTTTTCTGCATCACAGCGAATGG
SAM_NGS              GGGTTAGTATTATAAATTGGTACATTATTATTTCGATCGGTTTTCTGCATCACAGCGAATGG
*****

SA1_Sanger_DNA      YGGATTAAAATTGAGAAATAGAGACCCGCGCCAAATGGCATGTCCACAAAAAATACATTG
SAM_NGS              TGGATTAAAATTGAGAAATAGAGACCCGCGCCAAATGGCATGTCCACAAAAAATACATTG
*****

SA1_Sanger_DNA      TTTGTAAATAACGGATATATTTGTGTGCATTTTCAGATACGAAACCGAAAACGGCATCGT
SAM_NGS              TTTGTAAATAACGGATATATTTGTGTGCATTTTCAGATACGAAACCGAAAACGGCATCGT
*****

SA1_Sanger_DNA      CGCCCAGGCTGCTGGATACGTTAAGAACGCCGGTTCCGAAAACGCCGTCCAAGTGATCGA
SAM_NGS              CGCCCAGGCTGCTGGATACGTTAAGAACGCCGGTTCCGAAAACGCCGTCCAAGTGATCGA
*****

SA1_Sanger_DNA      AGGCTCGTATGCCTACATCGGTGACGATGGTGCTCCAGTCGAAGTCAAGTACTACGCTGA
SAM_NGS              AGGCTCGTATGCCTACATCGGTGACGATGGTGCTCCAGTCGAAGTCAAGTACTACGCTGA
*****

SA1_Sanger_DNA      CGAGACCGGTTACCACGCAGCCGAAACGTCGTCCCAGCACTCCCCAGAGATCGCCAA
SAM_NGS              CGAGACCGGTTACCACGCAGCCGAAACGTCGTCCCAGCACTCCCCAGAGATCGCCAA
*****

SA1_Sanger_DNA      GTCTTTGGAATTAATCGCCTCCCAACCACAGAAACCAGAAGACTCCAAAAAGAA-----
SAM_NGS              GTCTTTGGAATTAATCGCCTCCCAACCACAGAAACCAGAAGACTCCAAAAAGAAGTAAAC
*****

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**Supplementary Figure S3.11.** SA1 *Dncpr1-8* (DNA) alignment. Sanger sequence of SA1 *Dncpr1-8* (DNA) aligned to *Dncpr1-8* obtained from the SAM genome sequence (Burger and Botha 2017). Alignment performed using MUSCLE (3.8) (Edgar 2004).

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SAM_Sanger_DNA -----CGGTATACGTCCAAAAAATCACCATGAACA
SAM_NGS CATCCCAACATACGGCAATTTTCTGATCACGGTATACGTCCAAAAAATCACCATGAACA
*****

SAM_Sanger_DNA CTTTGGTGAGTTAAATAATATTTCTTTAAATCTTCTTAAAAAAGACATAGCAATATTATC
SAM_NGS CTTTGGTGAGTTAAATAATATTTCTTTAAATCTTCTTAAAAAAGACATAGCAATATTATC
*****

SAM_Sanger_DNA ATTTTATTTTTTAATTTAGTTATACAACGCATTGTATAATAGTTTCAATTTATAAACAGT
SAM_NGS ATTTTATTTTTTAATTTAGTTATACAACGCATTGTATAATAGTTTCAATTTATAAACAGT
*****

SAM_Sanger_DNA TTAATTTAAAGAAAAAATAGTATARTTACTGAAATTATTTATTATTTTCGTACTTTATCA
SAM_NGS TTA-----
****

SAM_Sanger_DNA TACACTTATGAATTTTTAGTATTTTTGGTATACGAGAATATCTTATTATTTTATAAATAT
SAM_NGS -----

SAM_Sanger_DNA CTTATAAAATAAATGCTCATATTATGTTATACTTATTTTTTAAATTAATGAAACTACGAA
SAM_NGS -----

SAM_Sanger_DNA AAAATTAATTTTAACTCAAATTTTCAAATTTTTTAAGTTCAAATAAGTACCTCAATTTAT
SAM_NGS -----

SAM_Sanger_DNA ATTATGAACAGTGTAAGTATAATATTTACGTTTACTGCAACCATTATTATATTAGAAT
SAM_NGS -----

SAM_Sanger_DNA CAGTCTTATTATTTTTGTACTTCATAAAAAAATGCCTGAAAATTTAAAATTTAAAAGATAT
SAM_NGS -----

SAM_Sanger_DNA CTCTACAATTTAACGCATAGGTAATCTTATTAATCGTAATCATATTTTAAACCAAATCCAT
SAM_NGS -----TTAACGCATAGGTAATCTTATTAATCGTAATCATATTTTAAACCAAATCCAT
*****

SAM_Sanger_DNA ATTTTTAGTTTCAATTTAAAATTGACATTACACCTGTAAAAGTTTTTCACAGTATATC---
SAM_NGS ATTTTTAGTTTCAATTTAAAATTGACATTACACCTGTAAAAGTTTTTCACAGTATATCTTC
*****

SAM_Sanger_DNA -----TTCACAGCAAAAAATATGCATA
SAM_NGS ACAGCAAAAAATATGCATAAAATTATTTCTTDCATGATTTTCACAGCAAAAAATATGCATA
*****

SAM_Sanger_DNA AAATTATTTCTTTCCTCAGCACTTTTATACAATTTTCGTGCCTTCGTTTTTAGGTAGTGTT
SAM_NGS AAATTATTTCTTTCCTCAGCACTTTTATACAATTTTCGTGCCTTCGTTTTTAGGTAGTGTT
*****

SAM_Sanger_DNA AGTAGCTGTCATCGCAGCGGTGTCTGCTGCGGCCCCACCTCAGGAAGCTGCCAAAGCTTT
SAM_NGS AGTAGCTGTCATCGCAGCGGTGTCTGCTGCGGCCCCACCTCAGGAAGCTGCCAAAGCTTT
*****

SAM_Sanger_DNA TACTTTCAGTGGATTCCCATCCAACCAAGCCTACTACCCAGGCCAACAAAGGGTACTACCC
SAM_NGS TACTTTCAGTGGATTCCCATCCAACCAAGCCTACTACCCAGGCCAACAAAGGGTACTACCC
*****

SAM_Sanger_DNA AGGATACATTGGTTACCAGKGTATCAAGGTTACAGCGGATTCCGTAATGGATACTACCC
SAM_NGS AGGATACATTGGTTACCAGGGTTATCAAGGTTACAGCGGATTCCGTAATGGATACTACCC
*****

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SAM_Sanger_DNA      GGGACAACAAGGATACTACCCAGGATACCAAGGTTACCAGGGATACTACCCAGGATACCA
SAM_NGS              GGGACAACAAGGATACTACCCAGGATACCAAGGTTACCAGGGATACTACCCAGGATACCA
*****

SAM_Sanger_DNA      AGGTTACCAGGGATACTACCCAGGATATCAAGGTTTAATTTTCGTTAATTATACGTCTAAA
SAM_NGS              AGGTTACCAGGGATACTACCCAGGATATCAAGGTTTAATTTTCGTTAATTATACGTCTAAA
*****

SAM_Sanger_DNA      ACACTGCAGAGTCACATGATGTGTTATAAGTTTCTTATAATTTACTATTTTCACATAGGT
SAM_NGS              ACACTGCAGAGTCACATGATGTGTTATAAGTTTCTTATAATTTACTATTTTCACATAGGT
*****

SAM_Sanger_DNA      TACAACCGCGTTACTACCCAGGTGCCCCAGCCGTCTACCCCACCGTCACCCCGCCCCA
SAM_NGS              TACAACCGCGTTACTACCCAGGTGCCCCAGCCGTCTACCCCACCGTCACCCCGCCCCA
*****

SAM_Sanger_DNA      ATCATCGCACCAGTGCCAGTCGCGCCCAAGGCTGTTTCTCCAGTGTACAAACCCGTAGAC
SAM_NGS              ATCATCGCACCAGTGCCAGTCGCGCCCAAGGCTGTTTCTCCAGTGTACAAACCCGTAGAC
*****

SAM_Sanger_DNA      AACAAAGTGCCAGCTATCCTTAAACAATCGCAAGAAGCTGACTTGAACGGATTCAAATAC
SAM_NGS              AACAAAGTGCCAGCTATCCTTAAACAATCGCAAGAAGCTGACTTGAACGGATTCAAATAC
*****

SAM_Sanger_DNA      GGGTTAGTATTATAATTGGTACATTATTATTTCGATCGGTTTTCTGCATCACAGCGAATGG
SAM_NGS              GGGTTAGTATTATAATTGGTACATTATTATTTCGATCGGTTTTCTGCATCACAGCGAATGG
*****

SAM_Sanger_DNA      YGGATTAATAATTGAGAAATAGAGACCCGCGCCAAATGGCATGTCCACAAAAAATACATTG
SAM_NGS              TGGATTAATAATTGAGAAATAGAGACCCGCGCCAAATGGCATGTCCACAAAAAATACATTG
*****

SAM_Sanger_DNA      TTTGTAAATAACGGATATATTTGTGTGCATTTTCAGATACGAAACCGAAAACGGCATCGT
SAM_NGS              TTTGTAAATAACGGATATATTTGTGTGCATTTTCAGATACGAAACCGAAAACGGCATCGT
*****

SAM_Sanger_DNA      CGCCCAGGCTGCTGGATACGTTAAGAACGCCGGTTCCGAAAACGCCGTCCAAGTGATCGA
SAM_NGS              CGCCCAGGCTGCTGGATACGTTAAGAACGCCGGTTCCGAAAACGCCGTCCAAGTGATCGA
*****

SAM_Sanger_DNA      AGGCTCGTATGCCTACATCGGTGACGATGGTGCTCCAGTCGAAGTCAAGTACTACGCTGA
SAM_NGS              AGGCTCGTATGCCTACATCGGTGACGATGGTGCTCCAGTCGAAGTCAAGTACTACGCTGA
*****

SAM_Sanger_DNA      CGAGACCGGTTACCACGCAGCCGAAACGTCGTCGTCGACCACTCCCCAGAGATCGCCAA
SAM_NGS              CGAGACCGGTTACCACGCAGCCGAAACGTCGTCGTCGACCACTCCCCAGAGATCGCCAA
*****

SAM_Sanger_DNA      GTCTTTGGAATTAATCGCCTCCCAACACAGAAACGAAAGACTCCAAAAAGAA-----
SAM_NGS              GTCTTTGGAATTAATCGCCTCCCAACACAGAAACGAAAGACTCCAAAAAGAAAGTAAAC
*****

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**Supplementary Figure S3.12.** SAM *Dncpr1-8* (DNA) alignment. Sanger sequence of SAM *Dncpr1-8* (DNA) aligned to *Dncpr1-8* obtained from the SAM genome sequence (Burger and Botha 2017). Alignment performed using MUSCLE (3.8) (Edgar 2004).



**Supplementary Figure S3.13.** Protein alignment and secondary structure of the CPRR1-8 G- and T-alleles. The T-allele was found in SAM clone #2, while the other SA1 and SAM clones result in the same amino acid sequence as the G-allele. The length of beta-strands was different and an additional coil was observed on the original G-allele when the secondary structure of the two alleles were compared. The secondary structure was annotated using the EMBOSS 6.5.7 tool garnier (Garnier *et al.* 1978). Graphic was generated in Geneious 9.1.8 (Biomatters). ■, alpha helix; →, beta strand; U, turn; ~, coil.

```

SA1_Sanger_cDNA      -----TCCAAAAAAAAATCACCATGAACA
SAM_NGS              CATCCCAACATACGGCAATTTTCTGATCACGGTATACGTCCAAAAAAAAATCACCATGAACA
                      *****

SA1_Sanger_cDNA      CTTTGGTAGTGTTAGTAGCTGTCATCGCAGCGGTGTCTGCTGCGGCCCCACCTCAGGAAG
SAM_NGS              CTTTGGTAGTGTTAGTAGCTGTCATCGCAGCGGTGTCTGCTGCGGCCCCACCTCAGGAAG
                      *****

SA1_Sanger_cDNA      CTGCCAAAGCTTTTACTTTTCAGTGGATTCCCATCCAACCAAGCCTACTACCCAGGCCAAC
SAM_NGS              CTGCCAAAGCTTTTACTTTTCAGTGGATTCCCATCCAACCAAGCCTACTACCCAGGCCAAC
                      *****

SA1_Sanger_cDNA      AAGGGTACTACCCAGGATACATTGGTTACCAGGGTTAYCAAGGTTACAGCGGATTCCGTA
SAM_NGS              AAGGGTACTACCCAGGATACATTGGTTACCAGGGTTATCAAGGTTACAGCGGATTCCGTA
                      *****

SA1_Sanger_cDNA      ATGGATACTACCCGGGACAACAAGGATACTACCCAGGATACCAAGGTTACCAGGGATACT
SAM_NGS              ATGGATACTACCCGGGACAACAAGGATACTACCCAGGATACCAAGGTTACCAGGGATACT
                      *****

SA1_Sanger_cDNA      ACCCAGGATACCAAGGTTACCAGGGATACTACCCAGGATATCAAGGTTACAACCGCGGTT
SAM_NGS              ACCCAGGATACCAAGGTTACCAGGGATACTACCCAGGATATCAAGGTTACAACCGCGGTT
                      *****

SA1_Sanger_cDNA      ACTACCCAGGTGCCCCAGCCGTCTACCCACCCTCACCCCGCCCCAATCATCGCACCAG
SAM_NGS              ACTACCCAGGTGCCCCAGCCGTCTACCCACCCTCACCCCGCCCCAATCATCGCACCAG
                      *****

SA1_Sanger_cDNA      TGCCAGTCGCGCCCCAAGGCTGTTTCTCCAGTGTACAAACCCGTAGACAACAAAGTGCCAG
SAM_NGS              TGCCAGTCGCGCCCCAAGGCTGTTTCTCCAGTGTACAAACCCGTAGACAACAAAGTGCCAG
                      *****

SA1_Sanger_cDNA      CTATCCTTAAACAATCGCAAGAAGCTGACTTGAACGGATTCAAATACGGATACGAAACCG
SAM_NGS              CTATCCTTAAACAATCGCAAGAAGCTGACTTGAACGGATTCAAATACGGATACGAAACCG
                      *****

SA1_Sanger_cDNA      AAAACGGCATCGTCGCCCAGGCTGCTGGATACGTTAAGAACGCCGGTCCGAAAACGCCG
SAM_NGS              AAAACGGCATCGTCGCCCAGGCTGCTGGATACGTTAAGAACGCCGGTCCGAAAACGCCG
                      *****

SA1_Sanger_cDNA      TCCAAGTGATCGAAGGCTCGTATGCCTACATCGGTGACGATGGTGTCCAGTCGAAGTCA
SAM_NGS              TCCAAGTGATCGAAGGCTCGTATGCCTACATCGGTGACGATGGTGTCCAGTCGAAGTCA
                      *****

SA1_Sanger_cDNA      AGTACTACGCTGACGAGACCGGTTACCACGCAGCCGAAACGTCGTCCCGACCACTCCCC
SAM_NGS              AGTACTACGCTGACGAGACCGGTTACCACGCAGCCGAAACGTCGTCCCGACCACTCCCC
                      *****

SA1_Sanger_cDNA      CAGAGATCGCCAAGTCTTTGGAATTAATCGCCTCCC-----
SAM_NGS              CAGAGATCGCCAAGTCTTTGGAATTAATCGCCTCCCAACCACAGAAACCAGAAGACTCCA
                      *****

SA1_Sanger_cDNA      -----
SAM_NGS              AAAAGAAATCACTCTAATATTATTTATGAGTTATGTAAAATACATG

```

**Supplementary Figure S3.14.** SA1 *Dncpr1-8* transcript (cDNA) alignment. Sanger sequence of the biotype SA1 *Dncpr1-8* transcript (cDNA) aligned to the *in silico* predicted SAM *Dncpr1-8* transcript from the SAM genome sequence (Burger and Botha 2017). Alignment performed using MUSCLE (3.8) (Edgar 2004).

```

SAM_Sanger_cDNA -----TCCAAAAAAAAATCACCATGAACA
SAM_NGS CATCCCAACATACGGCAATTTTCTGATCACGGTATACGTCCAAAAAAAAATCACCATGAACA
*****

SAM_Sanger_cDNA CTTTGGTAGTGTTAGTAGCTGTCATCGCAGCGGTGTCTGCTGCGGCCCCACCTCAGGAAG
SAM_NGS CTTTGGTAGTGTTAGTAGCTGTCATCGCAGCGGTGTCTGCTGCGGCCCCACCTCAGGAAG
*****

SAM_Sanger_cDNA CTGCCAAAGCTTTTACTTTTCAGTGGATTCCCATCCAACCAAGCCTACTACCCAGGCCAAC
SAM_NGS CTGCCAAAGCTTTTACTTTTCAGTGGATTCCCATCCAACCAAGCCTACTACCCAGGCCAAC
*****

SAM_Sanger_cDNA AAGGGTACTACCCAGGATACATTGGTTACCAGGGTTAYCAAGGTTACAGCGGATTCCGTA
SAM_NGS AAGGGTACTACCCAGGATACATTGGTTACCAGGGTTATCAAGGTTACAGCGGATTCCGTA
*****

SAM_Sanger_cDNA ATGGATACTACCCGGGACAACAAGGATACTACCCAGGATACCAAGGTTACCAGGGATACT
SAM_NGS ATGGATACTACCCGGGACAACAAGGATACTACCCAGGATACCAAGGTTACCAGGGATACT
*****

SAM_Sanger_cDNA ACCCAGGATACCAAGGTTACCAGGGATACTACCCAGGATATCAAGGTTACAACCGCGGTT
SAM_NGS ACCCAGGATACCAAGGTTACCAGGGATACTACCCAGGATATCAAGGTTACAACCGCGGTT
*****

SAM_Sanger_cDNA ACTACCCAGGTGCCCCAGCCGTCTACCCACCCTCACCCCGCCCCAATCATCGCACCAG
SAM_NGS ACTACCCAGGTGCCCCAGCCGTCTACCCACCCTCACCCCGCCCCAATCATCGCACCAG
*****

SAM_Sanger_cDNA TGCCAGTCGCGCCCCAAGGCTGTTTCTCCAGTGTACAAACCCGTAGACAACAAAGTGCCAG
SAM_NGS TGCCAGTCGCGCCCCAAGGCTGTTTCTCCAGTGTACAAACCCGTAGACAACAAAGTGCCAG
*****

SAM_Sanger_cDNA CTATCCTTAAACAATCGCAAGAAGCTGACTTGAACGGATTCAAATACGGATACGAAACCG
SAM_NGS CTATCCTTAAACAATCGCAAGAAGCTGACTTGAACGGATTCAAATACGGATACGAAACCG
*****

SAM_Sanger_cDNA AAAACGGCATCGTCGCCCAGGCTGCTGGATACGTTAAGAACGCCGGTCCGAAAACGCCG
SAM_NGS AAAACGGCATCGTCGCCCAGGCTGCTGGATACGTTAAGAACGCCGGTCCGAAAACGCCG
*****

SAM_Sanger_cDNA TCCAAGTGATCGAAGGCTCGTATGCCTACATCGGTGACGATGGTGTCCAGTCGAAGTCA
SAM_NGS TCCAAGTGATCGAAGGCTCGTATGCCTACATCGGTGACGATGGTGTCCAGTCGAAGTCA
*****

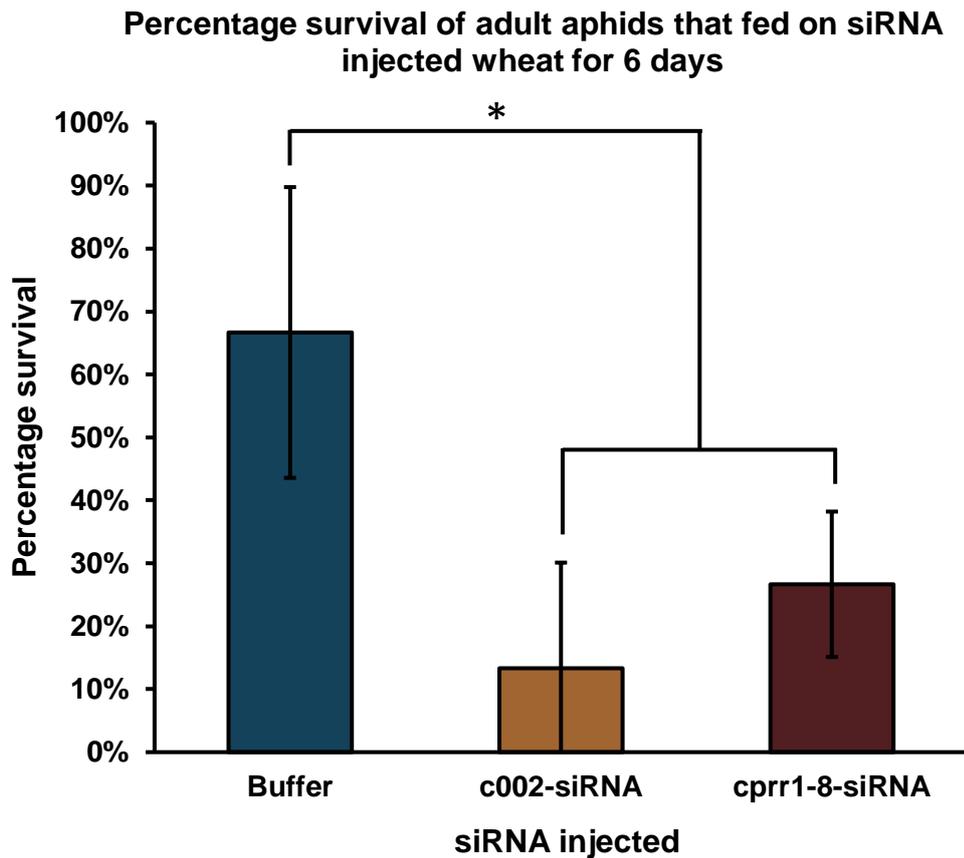
SAM_Sanger_cDNA AGTACTACGCTGACGAGACCGGTTACCACGCAGCCGAAACGTCGTCCCGACCACTCCCC
SAM_NGS AGTACTACGCTGACGAGACCGGTTACCACGCAGCCGAAACGTCGTCCCGACCACTCCCC
*****

SAM_Sanger_cDNA CAGAGATCGCCAAGTCTTTGGAATTAATCGCCTCCCAACCACAGAAAC-----
SAM_NGS CAGAGATCGCCAAGTCTTTGGAATTAATCGCCTCCCAACCACAGAAACCAGAAGACTCCA
*****

SAM_Sanger_cDNA -----
SAM_NGS AAAAGAAATCACTCTAATATTATTTATGAGTTATGTAAAATACATG

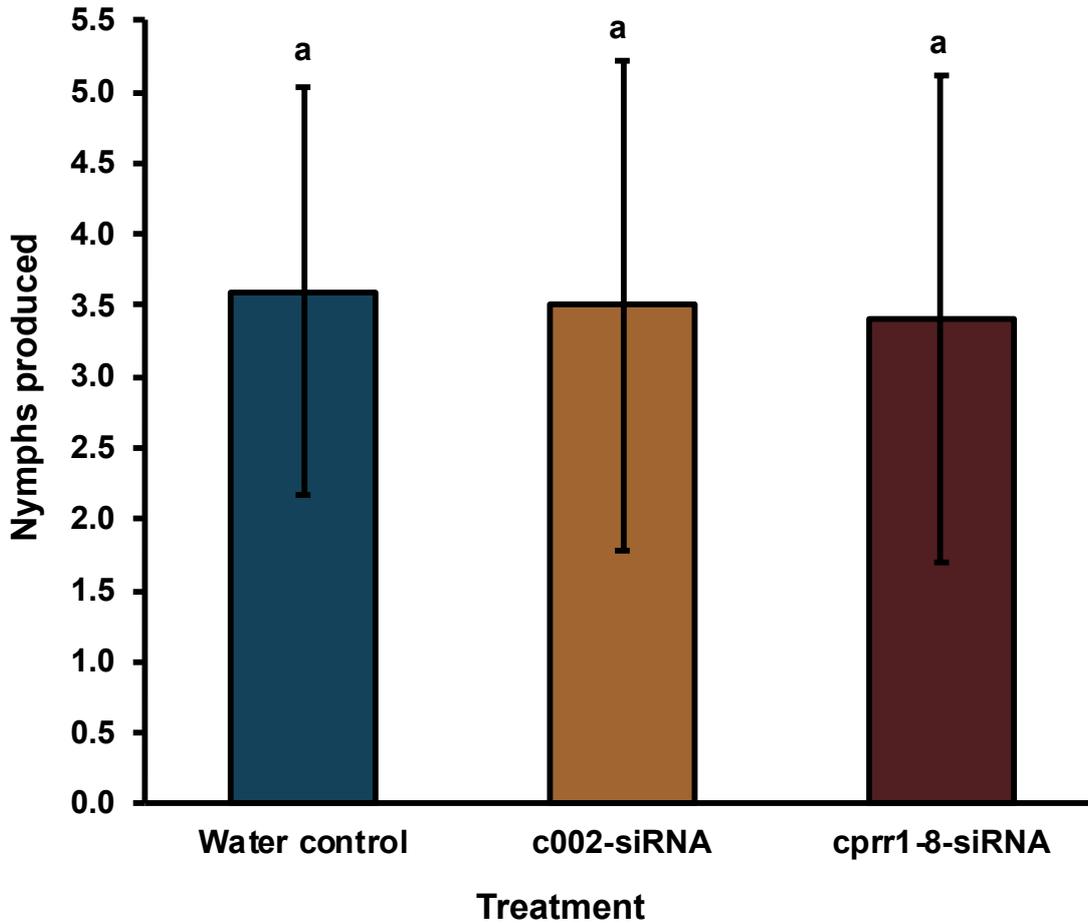
```

**Supplementary Figure S3.15.** SAM *Dncpr1-8* transcript (cDNA) alignment. Sanger sequence of SAM *Dncpr1-8* transcript (cDNA) aligned to the *in silico* predicted SAM *Dncpr1-8* transcript from the SAM genome sequence (Burger and Botha 2017). Alignment performed using MUSCLE (3.8) (Edgar 2004).

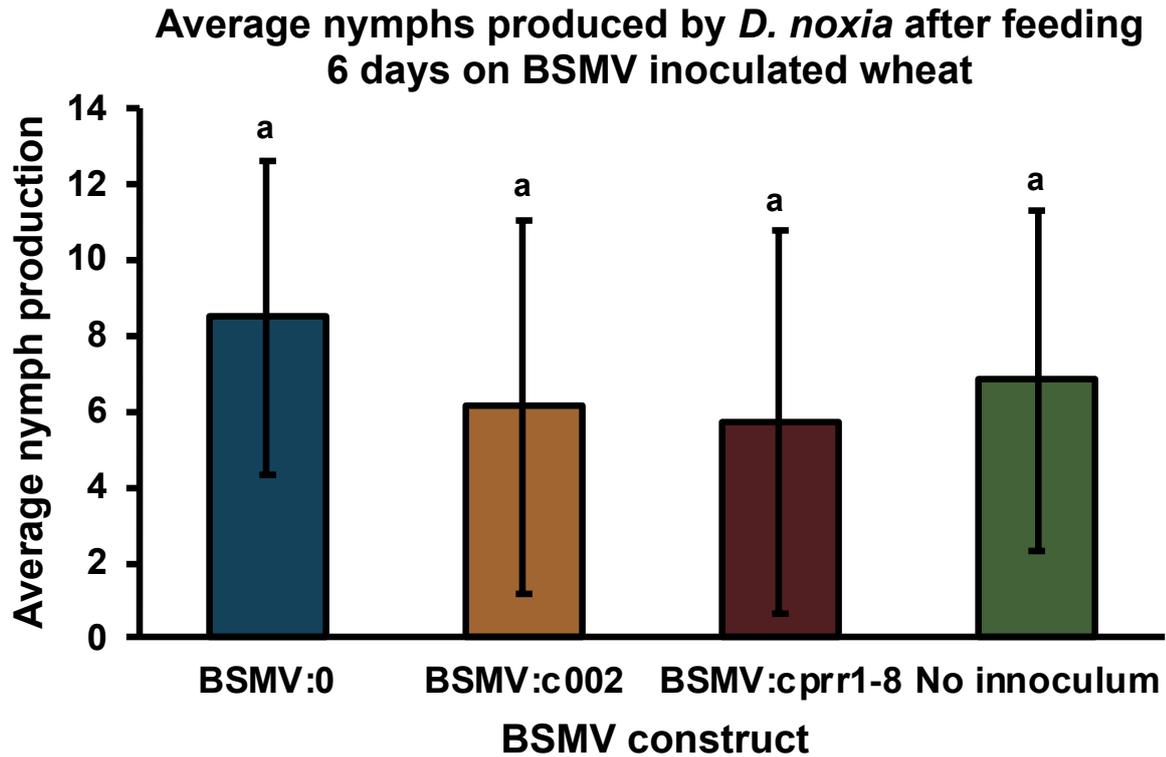


**Supplementary Figure S3.16.** Percentage survival of *Diuraphis noxia* biotype SAM feeding on wheat leaves injected with siRNA that targets the *D. noxia* genes *c002* or *cpr1-8* for a period of 6 days. Buffer, 10 mM Tris (pH 7); *c002*-siRNA, siRNA targeting *c002* dissolved in 10mM Tris (pH 7); *cpr1-8*-siRNA, siRNA targeting *cpr1-8* dissolved in 10 mM Tris (pH 7); \*, significantly different at  $P \leq 0.05$ . Significant differences determined using a two-sided Dunnett test. Error bars represent SD.

**Average nymphs produced by adult aphids after 4 days of feeding on siRNA supplemented artificial media**



**Supplementary Figure S3.17.** Average nymph production of *Diuraphis noxia* biotype SAM after feeding on siRNA supplemented artificial feeding medium (composition described in Materials and Methods). 1  $\mu\text{g}/\mu\text{l}$  *Dnc002*- or *Dncprr1-8*-siRNA was added to the medium. Equal alphabets indicate no statistically significant difference (Tukey's HSD test,  $P \leq 0.05$ ).



**Supplementary Figure S3.18.** Average nymph production of *Diuraphis noxia* SAM after feeding on wheat (Gamtoos-R) inoculated modified *barley stripe mosaic virus* (BSMV) for a period of 6 days. BSMV:0, unmodified BSMV virus-induced gene silencing vector; BSMV:c002, BSMV containing *Dnc002* transcript sequence; BSMV:cpr1-8, BSMV containing *Dncpr1-8* transcript sequence. Equal alphabets indicate no statistically significant difference (Tukey's HSD test,  $P \leq 0.05$ ).

**Supplementary Table S3.1.** Additional primer sequences. Primers were used for the construction of the virus-induced gene silencing vector, gene expression analysis or determination of siRNA concentration.

Name of primer	Sequences of primers (5'-3')	Template	Amplicon length (bp)	Annealing temp. (°C)	Reference
<i>c002_VIGS_Pacl</i> F	TATATATTAATTAAGAGCAGGAAGAAGTGTCCGA	cDNA	441	56	This study
<i>c002_VIGS_Nofl</i> R	AAATATGCGGCCGCGACGTTATTGGTCCGAGT				
<i>cpr1-8_VIGS_Pacl</i> F	TATATATTAATTAACCCGTATGAGAAGCCGACTG	cDNA	434	56	This study
<i>cpr1-8_VIGS_Nofl</i> R	AAATATGCGGCCGCCCATCTTGGTGGGAGCTCTG				
<i>L32</i> F	CGTCTTCGGACTCTGTTGTCAA	cDNA	74	56.3	Shakesby <i>et al.</i> 2009
<i>L32</i> R	CAAAGTGATCGTTATGACAAACTCAA				
<i>L27</i> F	ACCAGCACGATTTTACCAGATTTTC	cDNA	90	56.3	Sinha and Smith 2014
<i>L27</i> R	CGTAGCCTGCCCTCGTGTA				
<i>18S</i> F	TGCCCTATCAACTTTTCGATGG	cDNA	111	54	Botha <i>et al.</i> 2010
<i>18S</i> R	TGGATGTGGTAGCCGTTTCTC				
SP6	TATTTAGGTGACACTATAG	Plasmid DNA	1219	50	Knoche and Kephart 1999
T7	TAATACGACTCACTATAGGG				
<i>Dncpr1-8-siRNA</i> RT stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTTAAA	RNA	65	N/A	Varkonyi-Gasic <i>et al.</i> 2007
<i>Dnc002-siRNA</i> RT stem-loop	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCTCCG	RNA	65	N/A	Varkonyi-Gasic <i>et al.</i> 2007
<i>Dncpr1-8-siRNA</i> F	AGCTTCTTGCGATTG	cDNA	41	60	This study
<i>Dnc002-siRNA</i> F	AUUUCAGAGAGACAU	cDNA	41	60	This study
Universal stem-loop R	GTGCAGGGTCCGAGGT	cDNA	41	60	Varkonyi-Gasic <i>et al.</i> 2007

## Chapter 4

### Ingestion of siRNA by *Myzus persicae*

## 4.1 Introduction

*Myzus persicae*, also known as the green peach aphid, is a global aphid pest of crop plants. It not only causes direct damage by feeding on over 40 families of plants, but also serves as vector for over 100 plant viruses (Kennedy *et al.* 1962; Centre for Agriculture and Biosciences International 2018). Controlling this pest is hindered by its resistance to multiple insecticide classes by using more than one mode of resistance in some strains (Bass *et al.* 2011). It also has a very high reproduction rate as is common to many aphid species (Davis *et al.* 2006), necessitating quick reaction to an infestation.

Comparison of the salivary proteome of Russian wheat aphid (*Diuraphis noxia*) biotypes SA1 and virulent biotype SAM genome sequence and salivary proteins revealed differences believed to be related to the formation of virulence (Cloete 2015). When one of these virulence proteins, an RR-1 cuticle protein (*cpr1-8*), was silenced in Russian wheat aphid, a decreased survival rate of these aphids was observed while feeding on resistant wheat (Chapter 3). A cuticle protein family also containing a Rebers and Riddiford (1988) consensus, the RR-2 gene family, was shown to be upregulated when *M. persicae* fed on *Nicotiana benthamiana* compared to *Brassica rapa* (Chinese cabbage) (Mathers *et al.* 2017). While Brassicaceae is known for producing glucosinolates, *Nicotiana* sp. produce nicotine among other chemicals to defend against insects and other herbivores (Hopkins *et al.* 2009; Todd *et al.* 2010). Although two different approaches were used to identify *cpr1-8* and the RR-2 gene family, these cuticle proteins seem likely to be involved in protection against plant defense compounds. Another protein identified from the SA1 and SAM comparison, *tpa-1*, is known to be involved in innate immunity of *Caenorhabditis elegans* (Van der Hoeven *et al.* 2012). In *C. elegans* it is involved in the activation of the p38 mitogen-activated protein kinase cascade in reaction to a pathogen derived stimulus and also in the regulation of reactive oxygen species in the cells lining the intestinal lumen. To determine whether the function of these proteins is conserved amongst different aphid species, siRNA targeting the same gene was fed to a generalist aphid species, *Myzus persicae* with the aim to knockdown gene expression.

## 4.2 Materials and Methods

### 4.2.1 Biological material

*Arabidopsis thaliana* Col-0 was grown for 30 days at a constant temperature of 23°C, humidity of 60% and a 16h photoperiod (200  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ). Thereafter the photoperiod was

reduced to 6h to slow further growth. *M. persicae* US clone G006 was maintained on potato. Clone G006 was obtained from the James Hutton Institute, Dundee, UK. To increase the reproduction rate of the colony it was kept at 25°C with an increase to 28°C for 2h during the day (Davis *et al.* 2006). Only apterous adult aphids were used in experiments.

#### 4.2.2 siRNA design

**Table 4.1.** *Mpcpr1-8*-, *Mptpa*- and *Dnc002*-siRNA sequences. siRNAs were designed to contain a 19 bp duplex region and a 2 nt 3'-overhang on either side.

Sequence target	Duplex siRNA sequence
<i>Mpcpr1-8</i>	5' -GAAACCAGAAGACUCCAAAAA-3'       3' -GUCUUUGGUCUUCUGAGGUUU-5'
<i>Mptpa</i>	5' -GUUCAAAACAAGGUCUCAAUG-3'       3' -AACAAGUUUGUUCAGAGUUU-5'
<i>Mpc002</i>	5' -CCUUCGAGAGUCUGAUAAAAC-3'       3' -UCGGAAGCUCUCAGACUAUUU-5'

BLASTn was used to search the *M. persicae* genome for homologs to *D. noxia cpr1-8*, *tpa-1* and *c002* (Altschul *et al.* 1990; Mathers *et al.* 2016). The Custom RNAi Design Tool was used to design the *Mpcpr1-8*-, *Mptpa-1*- and *Mpc002*-siRNAs (Rozen and Skaletsky 1999) (Table 4.1). The parameters were changed to select siRNAs with a 19 nucleotide (nt) duplex region and a 2 nt 3'-overhang. siRNA sequences returned were screened to verify its theoretical efficiency at incorporating into the RISC and guiding RISC to the target mRNA. It was specifically ensured that siRNAs cohered to the 'asymmetric thermodynamic rule' which is based on the fact that the siRNA strand that is bound less stably at the 5'-end is preferentially incorporated into RISC (Khvorova *et al.* 2003). It also contained a G/C content of 30-52%, as well as the absence of internal repeats (Horn and Boutros 2013). The synthesized siRNA was obtained from IDT (<https://www.idtdna.com/>).

### 4.2.3 Clip cage construction



**Figure 4.1:** Clip cage used to contain *Myzus persicae* on *Arabidopsis thaliana* leaves.

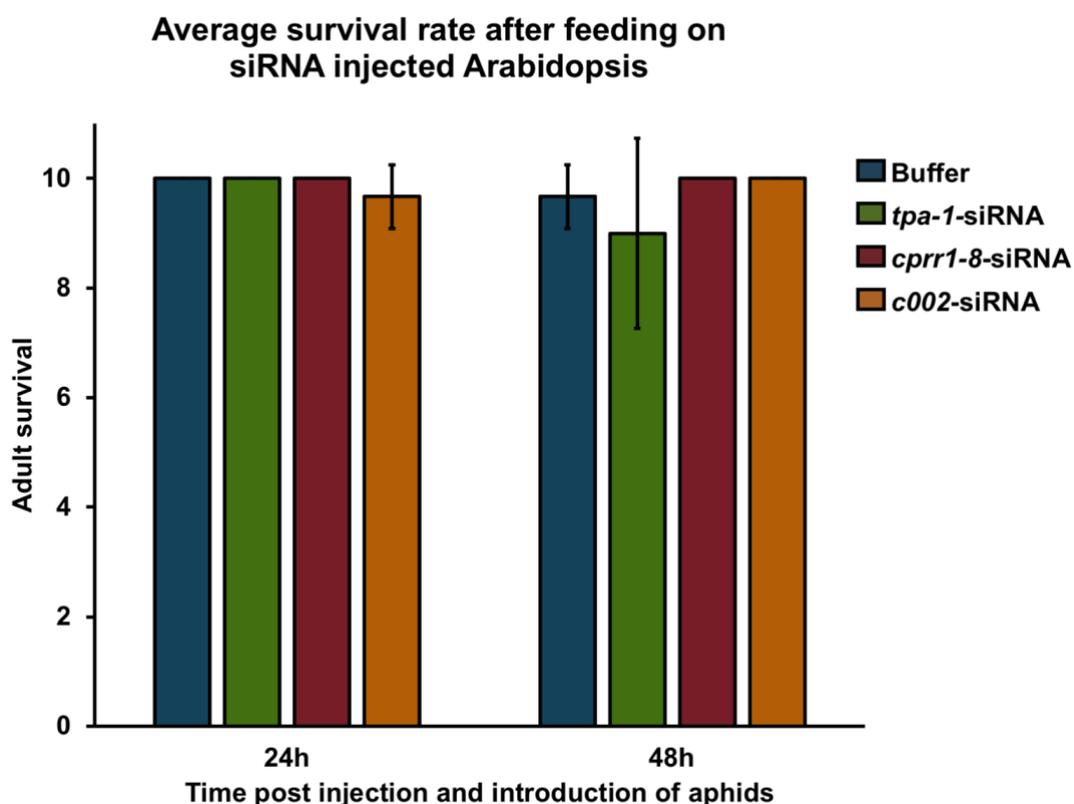
In order to contain *M. persicae* on *A. thaliana* leaves for the siRNA experiment described below, clip cages were required. It was built from 10 mm and 5 mm sections of 15 ml polypropylene tubes (Greiner Bio-One, Austria) for the top and bottom parts, respectively. To one side of each of the top and bottom parts, cylindrical sponges (5 mm high, outer diameter of 25 mm and inner diameter of 10 mm) were glued using Super Contact (Bostik, USA) This allows the pressure from the tubes to be spread over a larger area of the leaf and to close spaces from which the aphids could escape. To the other sides, fine nylon mesh was glued to using Blits Stick Super Glue (Bostik) for air flow within the clip cage. With the foam-sides facing one another, a top and bottom half was attached to a hair clip by pushing heated hair clip prongs into the polypropylene cylinders. The top and bottom halves were fixed to the hair clip using Gorilla Epoxy (Gorilla Glue, USA). An example of a clip cage in use can be seen in Figure 4.1.

### 4.2.4 siRNA ingestion by *M. persicae* through injection into *A. thaliana* leaves

Using a 10  $\mu$ l, model 1701 Hamilton syringe with a 25.4 mm needle of 34 gauge, and 45° tip, 1  $\mu$ l of 2  $\mu$ g/ $\mu$ l siRNA dissolved in 10 mM 4-morpholinepropanesulfonic acid (MOPS) (Sigma-Aldrich, USA) at pH 7.0 was injected into the midrib one of the rosette leaves. Ten mM MOPS (Sigma-Aldrich) at pH 7.0 was injected as a control. Only one leaf per plant was

injected. Injections were performed by inserting the needle perpendicular to the midrib. A clip cage was used to contain 10 adult *Myzus persicae* on the injected leaf, over the injected sites (Figure 4.1). *M. persicae* was allowed to feed on the injected leaf for a period of 24h or 48h. After feeding for the set period of time, phenotypic data was collected, including survival of the adult aphids and nymphs produced by the adult aphids. As many nymphs were produced that tended to become mobile when disturbed, photographs were taken immediately after a clip cage was opened to ensure accurate counting. For the two time points, an ANOVA with Tukey's HSD test was used to determine if the siRNA used had a significant effect on the phenotypic parameters measured (XLstat, Addinsoft).

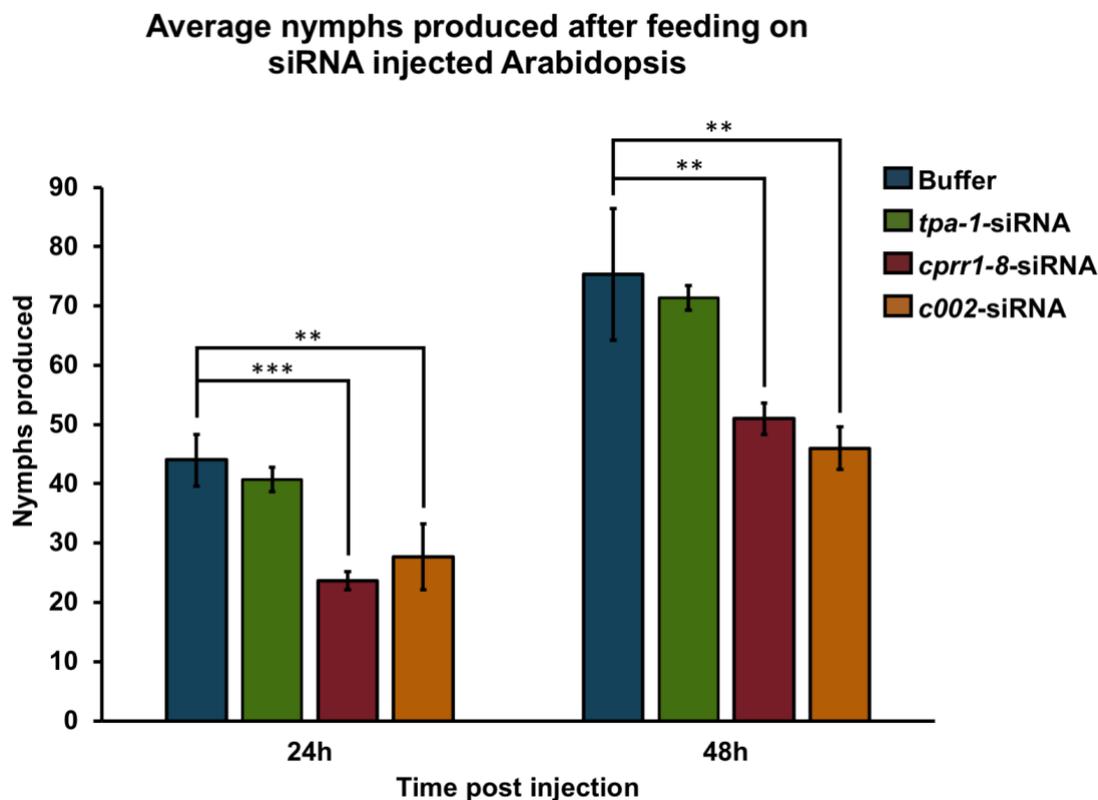
### 4.3 Results



**Figure 4.2.** The effect of siRNA ingestion by *Myzus persicae* on its survival. Ingestion occurred by feeding on *Arabidopsis thaliana* injected with 2  $\mu$ g of the respective siRNAs. Bars represent the mean of three biological repeats (a single biological repeat consisted of 10 adult aphids). The timepoints represent independent experiments. Buffer, 10 mM MOPS (pH 7.0); *tpa-1*-siRNA, siRNA targeting *Mptpa-1* dissolved in 10 mM MOPS (pH 7.0); *cpr1-8*-siRNA, siRNA targeting *Mpcpr1-8* dissolved in 10 mM MOPS (pH 7.0); *c002*-siRNA, siRNA targeting *Mpc002* dissolved in 10 mM MOPS (pH 7.0). An ANOVA with Tukey's HSD test was used to determine if statistically significant differences exist between means.

Ingestion of *Mpcpr1-8*- or *Mpc002*-siRNA did not influence the survival of *M. persicae* (Figure 4.2) but did result in significantly lower nymph production which was observed at 24

hpi and 48 hpi (Figure 4.3). The average nymph production was about half that of the control injection when fed on *Mpcpr1-8*- or *Mpc002*-siRNA for 24h. After 48h, the difference in nymph production between *Mpcpr1-8*- and *Mpc002*-siRNA fed, and buffer fed was less than before. After ingestion of *tpa-1*-siRNA, *M. persicae* did produce slightly less nymphs at 24 and 48h post introduction, but these differences were not statistically significant.



**Figure 4.3.** The effect of siRNA ingestion by *Myzus persicae* on its reproduction rate. Ingestion occurred by feeding on *Arabidopsis thaliana* injected with 2 µg of the respective siRNAs. Bars represent the mean nymph production of three biological repeats (a single biological repeat consisted of 10 adult aphids). The timepoints represent independent experiments. Error bars represent standard deviation. Buffer, 10 mM MOPS (pH 7.0); *tpa-1*-siRNA, siRNA targeting *Mptpa-1* dissolved in 10 mM MOPS (pH 7.0); *cpr1-8*-siRNA, siRNA targeting *Mpcpr1-8* dissolved in 10 mM MOPS (pH 7.0); *c002*-siRNA, siRNA targeting *Mpc002* dissolved in 10 mM MOPS (pH 7.0); \*\*  $P < 0.01$ ; \*\*\*  $P < 0.001$  (ANOVA with Tukey's HSD test).

#### 4.4 Discussion

After ingestion of the control siRNA, *c002*-siRNA, the reduction in fecundity observed, whilst having no effect on survival of *M. persicae* is supported by previous studies performed by Pitino *et al.* (2011). In this study, dsRNA was delivered to *M. persicae* through permanent plant transformation. Based on the comparable phenotype that was observed here, the method of siRNA delivery through injection into the host plant is thus as effective as feeding on dsRNA expressing plants.

Ingestion of *cpr1-8*-siRNA by *M. persicae* resulted in a similar phenotype as when *c002*-siRNA was ingested (Figures 4.2 and 4.3). Assuming that this phenotype is a result of gene silencing, it clearly indicates that reduced expression of these genes has a pronounced effect on the fecundity of *M. persicae*. While around double the number of nymphs were produced from aphids feeding on *Mpc002*- and *Mpcpr1-8* siRNA at 48h compared to 24h, at 48h aphids feeding on buffer injected leaves produced 10% less than double the number produced at 24h (Figure 4.3). A possible explanation for this occurrence could be that the higher population density seen on the buffer injected leaves could stimulate the aphids to slow down reproduction and conserve resources to use for other purposes.

That fact that there was no phenotypic response by *M. persicae* when it fed on *Mptpa-1*-siRNA could be the result of different causes. Firstly, in the aphid-plant combination tested, *tpa-1* may not play as important a role as it is believed to play in the *D. noxia*-wheat interaction. Therefore, reduced expression may not be as detrimental to *M. persicae* when feeding on *A. thaliana*. It may also be that *Mptpa-1* was not in fact silenced in *M. persicae* and thus it performed similar to the control injection. The experiments described in this chapter were performed at the University of Leeds, UK. As there was limited time available at this location, gene expression analysis could not be performed. In the future, in order to determine if *Mptpa-1* was silenced while *M. persicae* fed on *Mptpa-1*-siRNA, RT-qPCR will need to be performed on *M. persicae*.

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## **Chapter 5**

### **Summary**

Breeding of *D. noxia* resistant wheat cultivars has been relatively successful based on the number of resistance genes documented (Li *et al.* 2018). However, many of these resistance genes have been overcome by *D. noxia* through the development of new aphid biotypes. Of the available seventeen resistance genes against *D. noxia*, only *Dn7* remains effective against the biotypes found in the USA and South Africa (not all genes have been screened against South African biotypes). As the process of breeding for pest resistance in crops is a time consuming and costly process, the mechanism of virulence formation in *D. noxia* requires investigation. This would allow more informed breeding and effective management of the available resistance genes.

*Diuraphis noxia* biotype SAM is virulent to all the resistance genes against which it has been screened including *Dn1*, *Dn2*, *Dn4*, *Dn5*, *Dn7*, *Dn8*, and *Dn9* while SA1, from which SAM is derived, is only virulent against *dn3* (Jankielsohn 2016; Botha *et al.* 2014; Burger *et al.* 2017). This makes SAM an excellent model to study virulence. In the past, peptides in the salivary glands were compared between SA1 and SAM using tandem mass spectrometry (Cloete 2015). After the comparative analysis between the salivary proteome of SA1 and SAM, several differences were found including the cuticle protein *Dncpr1-8*, which was found to be present in the salivary proteome of SAM but not in that of SA1. Whole genome sequence analysis was also used to compare the two biotypes (Burger and Botha 2017) and again, SNPs were detected in *Dncpr1-8* between SA1 and SAM.

In this study, the objective was set to compare siRNA delivery methods, and then by using the most efficient delivery system, shed some light on the putative function of cuticle protein *Dncpr1-8* in *D. noxia*. This putative function observed in *D. noxia* was then compared to another aphid species, *M. persicae*. Four different siRNA delivery methods were compared of which two represents delivery without the use of a plant as feeding substrate, namely through artificial media feeding and injection into the hemolymph. siRNA delivery through direct injection into the aphid hemolymph was quickly determined not to be a viable method with the equipment available. The injection resulted in death regardless of the solution injected. This was likely due to the small aphid size (1.4-2.6 mm long, Centre for Agriculture and Biosciences International 2018) compared to the needle used. siRNA delivery into the hemolymph could therefore still be possible by using a smaller glass needle. Feeding on artificial media resulted in no interference as shown by *Dncpr1-8* expression and the survival and nymph production observed. This method is also laborious and required the largest amount of siRNA. As it is hypothesized that the salivary proteome improves the palatability (ingestion) of phloem constituents, it can be argued that such delivery system

may not fully represent the interaction between the salivary proteome and the feeding environment *in vivo*. It has previously been shown that the salivary proteome harvested from feeding on artificial media differ from that obtain after feeding on plants, making artificial feeding less suitable for studies to elucidate function of salivary proteins (Habibi *et al.* 2001).

Two *in planta* delivery systems were then compared, namely plant virus mediated delivery and a new method of delivery, i.e. direct injection of siRNA into the leaf. Even though both systems resulted in interference as defined by aphid response, direct injection *in planta* resulted in a larger phenotypic effect, was less laborious and cheaper to perform. The plant virus mediated delivery also gave inconsistent results, possibly as a result of variable virus infection, while with direct delivery it was easier to administer the appropriate concentration (dose) of siRNA as the aphids can be placed over the injection site. siRNA delivery through injection *in planta* also resulted in the first report of gene silencing through RNAi in *D. noxia*. Gene silencing was not only observed in adult aphids at 48 hours post introduction, but also in the newborn nymphs at 96 hours post introduction of the foundress adults. Collectively, the data showed that the method of direct siRNA injection *in planta* resulted in a much larger phenotypic effect than the other siRNA/dsRNA delivery methods tested.

Injection of siRNA into the host plant did not only result in an apparent phenotype in the *D. noxia*-wheat combination, but also when *M. persicae* fed on *Mpcpr1-8*- and *Mpc002*-siRNA injected into *A. thaliana*. This method of siRNA delivery is thus effective in more than one aphid-plant system and could therefore possibly be applied to other plant feeding insects. When the goal is rapid phenotypic characterization through gene silencing, this is an effective method to use. As is common to the other methods of transient siRNA delivery, the phenotypic effect could potentially revert back to normal after a period of time, depending on the gene targeted. In the timeframes used in this study, this reversion was not observed. Should prolonged periods of gene silencing be desired, this novel delivery method could be followed by the more laborious method of plant transformation once it is known that a phenotypic effect is observed during silencing a specific target gene. This course of action could be followed in the future for the genes investigated in this study. It would be especially interesting to observe if *M. persicae* would also have a reduced survival rate as seen in *D. noxia* if allowed to feed on *Mpcpr1-8*-dsRNA for a prolonged period of time.

The ingestion of *Dncpr1-8*-siRNA by *D. noxia* resulted in a significant reduction in survival and nymph production while feeding on resistant wheat. Before this, elevated catalase and peroxidase levels indicated stress in the aphids. A reduction in peroxidase activity in wheat indicated that the *Dncpr1-8*-siRNA fed *D. noxia* was not perceived as a threat to wheat.

When *M. persicae* fed on *Mpcpr1*-siRNA injected *A. thaliana* a different, but also apparent phenotype was observed in the form of reduced nymph production. The fact that ingestion of *cprr1-8*-siRNA not only affect the fitness of *D. noxia* feeding on wheat but also *M. persicae* feeding on *A. thaliana*, means that *cprr1-8* is not only important in the *D. noxia*-wheat combination, but also other insect-plant interactions. The exact function of this protein in the context of the aphid-plant interaction, however still requires further investigation. As expression of this protein is increased in the virulent biotype SAM and gene silencing results in reduced fitness, it can be deduced that *cprr1-8* offers some kind of protection against resistant wheat. Being a cuticle protein, *cprr1-8* could be located in the cuticle of the aphid but may also be found in the stylet or slightly further down in the intestinal tract (Uzest *et al.* 2007). In the future, the exact anatomical location of this protein should be determined. This could be done using Immunolabeling combined with confocal microscopy. Once the location is known, the effect of *cprr1-8* silencing on the organ in question can be examined.

Burger and Botha (2017) found SNPs between the SA1 and SAM *Dncpr1-8*. In this study, that finding could not be replicated, however one novel SNP was found in SAM clone #2 that did result in an amino acid change and also had an effect on the tertiary structure of the protein. This clone was only found in 1 out of 11 clones and thus it is unlikely that this allele alone results in higher virulence in SAM.

In order to confirm why *cprr1-8* was present in the SAM salivary gland, but absent from the SA1 salivary gland (Cloete 2015), gene expression analysis was performed in this study which confirmed that *Dncpr1-8* is expressed at a higher level in SAM than in SA1. It is also reported here that a difference in DNA methylation patterns between the two biotypes exist. Not only was methylation present at a higher frequency, but a higher number of sites were also methylated in biotype SAM. This could explain the differential gene regulation of *Dncpr1-8* observed. Although DNA methylation has been associated with gene regulation (Walsh *et al.* 2010), the exact mechanism is not fully understood in insects. It is thus difficult to determine with certainty if and how gene regulation will be affected based on different DNA methylation patterns as observed between SA1 and SAM.

Considering the asexual reproduction of *D. noxia* and short evolutionary history between SA1 and SAM, a large genetic change between the biotypes cannot be expected. A mechanism like aforementioned DNA methylation that is independent of a large change in DNA sequence is thus more likely to cause the increased virulence in SAM. There are however alternative means to achieve a large change in gene expression with minimal DNA sequence alteration, such as the modification of one or two key transcription factors which

could alter regulation of many genes. A transcriptome analysis of the two biotypes could identify if certain groups of genes are differentially regulated which could in turn be associated with a specific transcription factor.

The use of RNAi for crop protection against insect pests is a promising concept. This involves the genetic engineering of a crop plant to produce dsRNA identical in sequence to a transcript of a vitally important gene for the target pest. As RNAi is very specific, there should be no effects on non-target organisms. Targeting a large area of a transcript using long dsRNA will also hinder the development of resistance in the pest. As a siRNA is about 23-19 nt in length, a mutation about every 19-23 bp is required in the transcript target area for the pest to be resistant. The use of *cprr1-8* as a target for RNAi-mediated pest control shows promise, especially considering the reduction in survival rate of *D. noxia* during *Dncprr1-8* silencing. The effect of *Mpcprr1-8* silencing in *M. persicae* manifested in the form of reduced nymph production. This might not influence the aphid population enough for a pest control application, but as mentioned before, prolonged exposure might have a different outcome. RR chitin binding domains, as found in *cprr1-8*, are unique to insects (Victor *et al.* 2018). This reduces the chance of having unintentional off-target effects in other organisms. The RR-1 and RR-2 domains are also conserved among insects, potentially allowing one to target multiple insects with the same dsRNA construct. There is thus a strong basis to explore plant-mediated *cprr1-8* RNAi as a means of insect pest control in future studies.

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# **Appendix 1**

## **Patent**

**REPUBLIC OF SOUTH AFRICA****PATENTS ACT, 1978****PROVISIONAL SPECIFICATION**  
(Section 30(1) – Regulation 27)

OFFICIAL APPLICATION NO		LODGING DATE		AGENT'S REFERENCE
21	01	22		P3482ZA00/SC/pf
<b>FULL NAME(S) OF APPLICANT(S)</b>				
71	STELLENBOSCH UNIVERSITY			
<b>FULL NAME(S) OF INVENTOR(S)</b>				
72	BOTHA-OBERHOLSTER, Anna-Maria SWIEGERS, Hendrik Willem BURGER, Nicolaas Francois Visser			
<b>TITLE OF INVENTION</b>				
54	siRNA for Controlling Pest Infestations and Method of Use			

## **siRNA FOR CONTROLLING PEST INFESTATIONS AND METHOD OF USE**

### **FIELD OF THE INVENTION**

The present invention relates to methods for controlling pest infestation using a siRNA molecule. The invention provides methods for making transgenic plants that express the siRNA molecule, as well as pesticidal agents and commodity products produced by the plants. The invention also provides transgenic plants that are resistant to insect pest infestation.

### **BACKGROUND TO THE INVENTION**

Insect pests are one of the largest causes of crop losses in the agricultural sector. The emergence of host plant resistance is a natural way in which crop losses due to insect pests are sometimes limited, but unfortunately this is often counteracted by the rapid emergence of new insect biotypes that are virulent to the now previously resistant cultivar.

The Russian wheat aphid (RWA) (*Diuraphis noxia*, Kurdjomov) is one such example. This aphid has a narrow host range, consisting mainly of wheat, barley and other grasses (Morrison and Peairs, 1998) and is found in all the major wheat producing countries. Of the 14 resistance genes in wheat (Xu et al. 2015), only *Dn7* and *Dn2401* remain effective to existing *D. noxia* biotypes in the USA (Puterka et al. 2015). The same is true in South Africa, where only *Dn7* confers effective resistance against the four biotypes present (*Dn2401* is yet to be screened against South African biotypes) (Jankielsohn 2011, 2016).

Chemical insecticides can also be used to control some insect pests, but this is not always desirable, as the insecticides may be harmful to the environment.

There is therefore a need to develop agricultural crops which have durable resistance to insect pests.

## SUMMARY OF THE INVENTION

According to a first embodiment of the invention, there is provided a nucleic acid molecule comprising annealed complementary strands, wherein:

a first strand of the nucleic acid molecule comprises a polyribonucleotide having the nucleotide sequence 5' CUUAAACAAUCGCAAGAAGCU 3' (SEQ ID NO: 24), and

a second strand of the nucleic acid molecule comprises a polyribonucleotide having the nucleotide sequence 5' UCAGCUUUCUUGCGAUUGUUUA 3' (SEQ ID No: 25), or

the first and second strands comprise polyribonucleotides having at least 80% sequence identity to SEQ ID NO: 24 or SEQ ID NO: 25, wherein ingestion of the polyribonucleotides by a pest inhibits the biological activity of the pest.

The nucleic acid molecule may be for use in inhibiting the biological activity of a pest, such as by decreasing survival of the pest, reducing fertility of the pest, or reducing the fitness of the pest in any other manner.

According to a further embodiment of the invention, there is provided an isolated polynucleotide or set of polynucleotides encoding the nucleic acid molecule described above.

According to a further embodiment of the invention, there is provided a composition for inhibiting the biological activity of a pest, the composition comprising the nucleic acid molecule described above.

According to a further embodiment of the invention, there is provided a composition for inhibiting the biological activity of a pest, the composition comprising a polynucleotide or set of polynucleotides encoding the nucleic acid molecule described above.

The composition may be a pesticide.

According to a further embodiment of the invention, there is provided a cell transformed with a polynucleotide or set of polynucleotides encoding the nucleic acid molecule described above.

The cell may be a plant cell.

According to a further embodiment of the invention, there is provided a plant transformed with a polynucleotide or set of polynucleotides encoding the nucleic acid molecule described above.

According to a further embodiment of the invention, there is provided a method for controlling pest infestation, the method comprising providing a pest with plant material comprising the nucleic acid molecule described above.

According to a further embodiment of the invention, there is provided a method for controlling pest infestation, the method comprising:

- (a) introducing the nucleic acid molecule described above into a plant; and
- (b) providing the plant, or portion thereof, to the pest.

According to a further embodiment of the invention, there is provided a method for controlling pest infestation, the method comprising:

- (a) introducing a polynucleotide or set of polynucleotides into a plant; and
- (b) providing the plant, or portion thereof, to the pest,

wherein the polynucleotide or set of polynucleotides encodes the nucleic acid molecule described above.

According to a further embodiment of the invention, there is provided a method for improving crop yield, the method comprising:

- a) introducing a polynucleotide or set of polynucleotides into a plant; and
- b) cultivating the plant to allow polynucleotide expression, wherein the expression inhibits feeding by a pest and loss of yield due to pest infestation,

wherein the polynucleotide or set of polynucleotides encodes the nucleic acid molecule described above.

The nucleic acid molecule may suppress a target gene in a pest that has ingested a portion of the crop plant.

According to a further embodiment of the invention, there is provided a method for producing a commodity product, the method comprising:

- a) introducing a polynucleotide or set of polynucleotides into a plant cell;
- b) growing the plant cell under conditions suitable for generating a plant; and
- c) producing a commodity product from the plant or part thereof,

wherein the polynucleotide or set of polynucleotides encodes the nucleic acid molecule described above.

The plant may be selected from the group consisting of wheat, barley, sugarcane, maize, rice, rye, sorghum, soya, potato, cassava, sugar beet, banana, citrus, apple, watermelon, mango, cucumber, tomato, brassica species, other vegetables, cotton and ornamental plants such as roses.

The pest may be an insect, such as an aphid (e.g. *Diuraphis noxia*, *Myzus persicae*, *Aphis fabae*, *Aphis glycines*, *Brevicoryne brassicae*, *Aphis gossypii*, *Macrosiphum euphorbiae*, *Acyrtosiphon pisum*, *Dysaphis plantaginea*, *Aphis craccivora*, *Schizaphis graminum*, *Rhopalosiphum padi*, *Rhopalosiphum maidis*, *Sitobion avenae*), Hemipteran (e.g. whitefly) or other insects (e.g. *Thrips*, Lepidoptera larva, Diptera larva, Coleoptera larva, Tetranychidae, Gryllidae and Caelifera).

## BRIEF DESCRIPTION OF THE FIGURES

**Figure 1** Graphic view of the *Diuraphis noxia* *cpr1-8* coding domain sequence, including annotations.

**Figure 2a and b** *D. noxia* larval cuticle protein *cpr1-8* (SEQ ID NO: 1).

**Figure 3** Mature mRNA *D. noxia* larval cuticle protein *cpr1-8* (SEQ ID NO: 2).

**Figure 4** CDS *Diuraphis noxia* larval cuticle protein *cpr1-8* (SEQ ID NO: 3).

**Figure 5** Next generation sequence: *D. noxia* larval cuticle protein *cpr1-8* (SEQ ID NO: 4)

**Figure 6** Relative qPCR expression of *Dnc002* (A) and *Dncpr1-8* (B) in *D. noxia* biotype SA1 and SAM after feeding on susceptible wheat cultivar 'Gamtoos-S' (GamS) or resistant 'Gamtoos-R' (GamR) for at least 10 days.

**Figure 7** Percentage survival of *D. noxia* biotype SAM after feeding on wheat leaves injected with siRNA (19 nt duplex region and a 2 nt 3'-overhang) that targets the genes *c002*

or *cprr1-8*. siRNA was dissolved in 10 mM Tris (pH 7.0) before injection. GamS, susceptible wheat cultivar 'Gamtoos-S'; GamR, resistant wheat cultivar 'Gamtoos-R'; No injection, wheat leaves without injection; Buffer, 10 mM Tris (pH 7); *c002*-siRNA, siRNA targeting *c002* dissolved in 10 mM Tris (pH 7); *cprr1-8*-siRNA, siRNA targeting *cprr1-8* dissolved in 10 mM Tris (pH 7).

**Figure 8** Percentage survival of *D. noxia* biotype SAM after feeding on wheat leaves injected with siRNA that targets the *D. noxia* genes *c002* or *cprr1-8*. GamS, *D. noxia* susceptible wheat cultivar 'Gamtoos-S'; GamR, *D. noxia* resistant wheat cultivar 'Gamtoos-R'; Buffer, 10mM Tris (pH 7); *c002*-siRNA, siRNA targeting *c002* dissolved in 10mM Tris (pH 7); *cprr1-8*-siRNA, siRNA targeting *cprr1-8* dissolved in 10mM Tris (pH 7); \*\*, significantly different at  $P \leq 0.01$ ; \*\*\*, significantly different at  $P \leq 0.001$ . Error bars represent SEM.

**Figure 9** *D. noxia* biotype SAM was allowed to feed on wheat cultivar Gamtoos-R injected with *Dnc002*-siRNA, *Dncprr1-8*-siRNA or buffer (control, no siRNA). *D. noxia* biotype SAM was also allowed to feed on wheat cultivar Gamtoos-R and Gamtoos-S that was not injected (**control**). Relative expression of *D. noxia* biotype SAM *c002* (**A**) *cprr1* (**B**) was determined with qPCR while SAM fed on wheat injected with siRNA. , Gamtoos-S, No injection; , Gamtoos-R, No injection; , Gamtoos-R, Buffer injection; , Gamtoos-R, *c002*-siRNA injection; , Gamtoos-R, *cprr1-8*-siRNA injection. qPCR was also used to determine the *c002*-siRNA (**C**) or *cprr1-8*-siRNA (**D**) concentration in the injected wheat leaf on which *D. noxia* biotype SAM fed. Error bars represent SEM.

**Figure 10** Relative *cprr1-8* expression after feeding on *cprr1-8*-siRNA injected wheat. Nymphs of *D. noxia* feeding on 'Gamtoos R' injected with *cprr1-8* were used for gene expression analysis of *cprr1-8* via RT-qPCR.

**Figure 11** (**A**) Catalase and (**B**) peroxidase activity of *D. noxia* biotype SAM after feeding on wheat leaves injected with *cprr1-8*- or *c002*-siRNA respectively. siRNA was dissolved in 10 mM Tris (pH 7.0) before injection.  GamS, *D. noxia* susceptible wheat cultivar 'Gamtoos-S';  GamR, *D. noxia* resistant wheat cultivar 'Gamtoos-R';  No injection, wheat leaves without injection; Buffer, 10 mM Tris (pH 7);  *c002*-siRNA, siRNA targeting *c002* dissolved in 10 mM Tris (pH 7);  *cprr1-8*-siRNA, siRNA targeting *cprr1-8* dissolved in 10 mM Tris (pH 7).

## DETAILED DESCRIPTION OF THE INVENTION

A method for controlling pest infestation is described herein. A siRNA molecule is provided for this purpose, the siRNA molecule comprising a first polyribonucleotide strand comprising the nucleotide sequence 5' CUUAAACAAUCGCAAGAAGCU 3' (SEQ ID NO: 24), and a second polyribonucleotide strand comprising a nucleotide sequence 5' UCAGCUUCUUGCGAUUGUUUA 3' (SEQ ID No: 25), or the first and second strands comprising polyribonucleotides having at least 80% sequence identity to SEQ ID NO: 24 and SEQ ID NO: 25, wherein ingestion of the siRNA molecule by a pest inhibits the biological activity of the pest. The use of the siRNA molecule is a natural and environmentally friendly method for controlling insect pests.

Methods are known in the art for administering the siRNA to pests. For example, the siRNA molecule can be injected into or ingested by the pest. Ingestion can occur by feeding the pest with an artificial food containing the siRNA, by causing the plant to transiently or stably express the siRNA, or by injecting a plant with the siRNA. In a preferred embodiment of the invention, plants or plant cells are transformed with a polynucleotide or set of polynucleotides encoding the siRNA, so as to produce a transgenic plant which expresses the siRNA.

Plants and plant cells transformed with a polynucleotide or set of polynucleotides encoding the siRNA are also provided.

Compositions comprising the siRNA, comprising a polynucleotide or set of polynucleotides encoding the siRNA, or comprising a plant or plant part expressing the siRNA are further provided. In some instances, these compositions can be a pesticide, e.g. for inhibiting the biological activity of a pest.

The siRNA molecule can be used to control pest infestation, improve crop yield or produce a commodity product.

When the siRNA is ingested by a pest which feeds on the crop, or when the siRNA is otherwise administered to the pest, the biological activity of the pest may be altered, and in particular the fitness or virulence of the pest may be reduced. For example, the survival rate of the pest may be decreased or its reproduction abilities may be reduced. This may be as a result of a target gene in the pest being suppressed. For example, the target gene can be the *cpr1-8* gene.

Although the examples which follow describe the effect of the siRNA molecule on aphids from the species *Diuraphis noxia*, the inventors have also found that an orthologous siRNA molecule reduces the fitness of another aphid species, *Myzus persicae*. A person skilled in the art will understand that the siRNA molecule could also be used to control other crop pests, such as other aphid species (e.g. *Aphis fabae*, *Aphis glycines*, *Brevicoryne brassicae*, *Aphis gossypii*, *Macrosiphum euphorbiae*, *Acyrtosiphon pisum*, *Dysaphis plantaginea*, *Aphis craccivora*, *Schizaphis graminum*, *Rhopalosiphum padi*, *Rhopalosiphum maidis*, *Sitobion avenae*), Hemipteran (e.g. whitefly) or other insects (e.g. *Thrips*, Lepidoptera larva, Diptera larva, Coleoptera larva, Tetranychidae, Gryllidae and Caelifera).

Similarly, a person skilled in the art will understand that the invention is not intended to be limited to the introduction of the siRNA molecule into what plants, and that other plants could also be transformed with the siRNA. For example, other suitable plants include crop plant such as wheat, barley, sugarcane, maize, rice, rye, sorghum, soya, potato, cassava, sugar beet, banana, citrus, apple, watermelon, mango, cucumber, tomato, brassica species, other vegetables, cotton and ornamental plants such as roses.

In the context of the present invention, the term “virulence” refers to the ability of an insect to feed and proliferate on a particular plant.

The most virulent South African biotype of *D. noxia* is SAM (South African Mutant) (Botha et al. 2014). This biotype was developed from biotype SA1 (the least virulent SA biotype, only virulent to *dn3*) (Jankielsohn, 2011, 2016) by feeding it aphid-resistant wheat cultivars, thus placing it under continuous selection pressure. As a result, SAM has been shown to express virulence against all described *Dn* genes found in wheat (Burger et al. 2017, Burger and Botha, 2017). This characteristic makes biotype SAM a useful model in studies to elucidate the mechanism of virulence against resistance genes.

The RR1 protein, which can be isolated from the salivary gland, is unique to the biotype SAM. It is encoded by the *cpr1-8* gene. The function of RR1 during aphid feeding was investigated to determine whether this protein is associated with the virulence of SAM. This was done using RNA interference (RNAi)-mediated gene silencing or knockdown.

The RNAi process relies on double-stranded RNA (dsRNA) precursors, specifically lowering transcript abundance of a target gene when introduced into cells (Fire et al., 1998). The process involves the cleavage of the dsRNA precursors into siRNA (~21–23 nucleotides in size) by the enzyme Dicer-2 (Dcr-2) (Meister and Tuschl, 2004). The resulting siRNAs are

then incorporated into an RNA-induced silencing complex (RISC). Argonaute-2 (Ago-2), the catalytic component of RISC, uses one of the siRNA strands as a template to recognize and degrade the complementary mRNA (Meister and Tuschl, 2004).

Three siRNA delivery techniques were attempted and compared: direct injection of dsRNA or siRNA into aphid haemolymph (Mutti et al., 2006, 2008; Jaubert-Possamai et al., 2007); feeding of dsRNA from an artificial diet (Shakesby et al., 2009; Whyard et al., 2009); plant-mediated RNAi to initiate down-regulation of gene targets (Bhatia et al., 2012; Guo et al., 2014; Coleman et al., 2015; Pitino et al., 2011; Pitino and Hogenhout, 2013). However, because aphids are so small (>3 mm in size, <http://www.cabi.org/isc/datasheet/9887>), microinjection requires specialized equipment. Wheat also has a large genome (17 000 MB, Gill et al., 2004) and unlike *Arabidopsis*, proved cumbersome to transform (Le Roux et al., 2015). Thus, alternative strategies to achieve RNAi-mediated gene silencing in *D. noxia* were required and a novel siRNA delivery method was developed.

The invention is described in more detail below in the following non-limiting examples.

## Materials and Methods

### *Aphid populations*

Colonies of parthenogenetic (apterous) female aphids of South African *D. noxia* biotypes SA1 and SAM, expressing different levels of virulence, were separately established in BugDorm cages (MegaView Science Education Services Co. Ltd., Taiwan) in an insectary with the following conditions:  $22.5 \pm 2.5^{\circ}\text{C}$ , 40% relative humidity, and continuous artificial lighting from high pressure sodium lamps.

The aphid colonies were maintained on near isogenic wheat lines. SA1 was maintained on Tugela (*D. noxia* susceptible, biotype SA1, Hewitt et al., 1984), while SAM was maintained on Tugela*Dn1*, a wheat cultivar containing the *Dn1* resistance gene (Swanevelder et al., 2010). All cultivars were planted in sand-filled pots and watered daily with a fertilizer that consisted of 2 g Microplex (Ocean Agriculture (Pty) Ltd, South Africa), 164 g Sol-u-fert (Kynoch Fertilizers (Pty) Ltd, South Africa) and 77 ml potassium nitrate per 100 liters of water.

As several studies have been conducted on *c002* (Mutti et al., 2006, 2008; Coleman et al., 2015; Visser 2017), this gene from *D. noxia* was included as reference in the present study.

### *Plant material and growth conditions*

Near isogenic wheat lines (NILs), GamtoosR (GamR, resistant) and GamtoosS (GamS, susceptible) were grown under greenhouse conditions using natural lighting and kept at a temperature of  $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ . The resistance in GamR was obtained after a 1RS/1BL translocation from rye (*Secale cereale* L.) (Marais et al., 1994), and is denoted *Dn7*. GamR is known to express antixenosis and antibiosis against aphids during feeding (Zaayman et al., 2009; Lapitan et al., 2007a,b; Botha et al., 2010). Antibiosis is observed when the plant reduces the reproductive fitness of aphids feeding on it, while antixenosis is the non-preference of a cultivar as host (Painter, 1951, 1958). Seeds were planted in pots filled with crusher dust and watered twice daily using drip irrigation. These plants were used for siRNA injection experiments. Plants used to maintain aphid colonies were grown under King Plus 800W LED lights for a 12h photoperiod and at a temperature of  $20^{\circ}\text{C} \pm 1^{\circ}\text{C}$ .

### *Sample preparation and RNA extraction*

For RNA extraction of both aphid and plant material, samples were immediately flash-frozen in liquid nitrogen and grounded in 1.5 ml Eppendorf tubes using a micropestle. RNA from aphids was extracted using an RNeasy Mini Kit (Qiagen) and on-column DNase I treatment (Qiagen) following the manufacturer's protocol. RNA from wheat was extracted by adding 600  $\mu\text{l}$  TRI Reagent® (Zymo Research) to the ground material, after which the Direct-zol™ RNA MiniPrep Plus kit (Zymo Research) was used by following the manufacturer's protocol and stored at  $-80^{\circ}\text{C}$  until further use.

Complementary DNA (cDNA) synthesis was conducted using an iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocol. The quantity of the cDNA was assessed through Qubit analysis (Thermo Fisher Scientific; Central Analytical Services, CAF, Stellenbosch University), whereafter the cDNA was stored at  $-80^{\circ}\text{C}$  until further use.

### *Sequence characterization of gene *Dncpr1-8* that encodes the protein RR1 from *Diuraphis noxia**

Primers were designed using the available sequenced SAM genome, SAM v1.0 (GenBank accession: ID GCA\_001465515.1 and BioProject PRJNA29716; Burger and Botha, 2017) (Table 1). Using the primers and synthesized cDNA as template, the *Dncpr1-8* and *Dnc002* genes were Sanger sequenced at CAF, Stellenbosch University, and the obtained sequences were submitted to the basic local alignment search tool (BLASTn and BLASTx, Altschul et al., 1990, 1997) at the National Centre for Biotechnology Information (NCBI) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to confirm the identity of genes *Dncpr1-8* (Figures 1-

5) and *Dnc002*. The protein coding region was analyzed for amino acid content through the use of the Geneious (v7.1.5) platform (Kearse et al., 2012).

**Table 1.** Sequence of primers used for sequence verification and gene expression analysis of *cprr1-8* and *c002*

Name of primer	Sequences of primers (5'-3')	Amplicon length (bp)	Annealing temp. (°C)
<i>c002</i> CDS F	GAGCAGGAAGAAGTGTCCGA (SEQ ID NO: 5)	441	59
<i>c002</i> CDS R	AGCACGTTATTGGTCCGAGT (SEQ ID NO: 6)		
<i>c002</i> qPCR F	CCCGTATGAGAAGCCGACTG (SEQ ID NO: 7)	123	60
<i>c002</i> qPCR R	CCATCTTGGTGGGAGCTCTG (SEQ ID NO: 8)		
<i>cprr1-8</i> CDS F	TTACTACCCAGGTGCCCCA (SEQ ID NO: 9)	434	59
<i>cprr1-8</i> CDS R	CTGTGGTTGGGAGGCGATTA (SEQ ID NO: 10)		
<i>cprr1-8</i> qPCR F	CCCATCCAACCAAGCCTA (SEQ ID NO: 11)	123	56
<i>cprr1-8</i> qPCR R	TAGTATCCTTGTTGTCCCGG (SEQ ID NO: 12)		

#### Design of siRNA

Once the sequence identities of the respective genes were confirmed, the Custom RNAi Design Tool (Owczarzy et al. 2008) was used to design siRNAs targeting the *Dncprr1-8* and *Dnc002*. The standard parameters were changed to select siRNAs with a 19 nt duplex region and a 2 nt 3'-overhang. siRNA sequences returned were screened to verify their theoretical efficiency (Table 2). According to Khvorova et al. (2003), siRNA has to adhere to the 'asymmetric thermodynamic rule' which is based on the principle that the siRNA strand that is bound less stably at the 5'-end is preferentially incorporated into RISC. The siRNAs were also designed to contain an ideal GC content of 30-52%, as well as the absence of internal repeats (Horn and Boutros 2013). The synthesized siRNA was obtained from IDT (<https://www.idtdna.com/>).

**Table 2.** Sequences of siRNAs

Name of siRNA	Sequence of sense (5'-3')	Sequence of antisense (5'-3')
<i>c002</i> -siRNA	ATTTCAGAGAGACATCGGAGG (SEQ ID NO: 13)	TCCGATGTCTCTCTGAAATTG (SEQ ID NO: 14)
<i>cpr1-8</i> -siRNA	TAAACAATCGCAAGAAGCTGA (SEQ ID NO: 15)	AGCTTCTTGCGATTGTTTAAG (SEQ ID NO: 16)

*Aphid feeding on siRNA-containing artificial media*

An artificial feeding media developed specifically for *D. noxia* was used for aphid feeding (Bahlmann (2005)). It was modified to contain the following: 0.10 g L-methionine (Merck), 0.20 g L-leucine (Sigma–Aldrich), 0.10 g L-tryptophan (Merck), 20.00 g sucrose (Merck), 0.20 g MgCl<sub>2</sub>.6H<sub>2</sub>O (Merck), and 0.25 g K<sub>2</sub>HPO<sub>4</sub> (Sigma–Aldrich). The pH was adjusted to 7.0 using KOH (Merck) and ddH<sub>2</sub>O was added to a final volume of 100 ml. The media was then filter sterilized (0.2 µm pore size) and stored at 4°C.

Adult *D. noxia* of between 350 µm and 500 µm in size were placed individually inside a glass test tube with a 14 mm outside diameter. Parafilm M (Bemis, Oshkosh, WI, USA) was stretched close to its maximum capacity and placed over the opening. One microliter of siRNA (25 µg/µl) dissolved in RNase-free water (Ambion), or water for the control, was added to 24 µl artificial feeding media and placed on the stretched Parafilm M. Another layer of Parafilm M was then placed over the artificial media, spreading the media between the two layers. The test tubes were placed vertically in a stand with the open end at the bottom. Yellow tape was placed below the test tubes to encourage the aphids to feed (Kieckhefer et al. 1976). The experiment was repeated ten times (n=10) for each siRNA (i.e., *Dnc002* and *Dncpr1-8*) and control (only media). The survival rate and the number of nymphs produced by each foundress was determined daily for four days.

*Aphid feeding on siRNA-injected wheat*

The leaves of 30-day-old wheat plants were injected with 1 µl of 1 µg/µl siRNA dissolved in 10 mM Tris (Sigma–Aldrich) pH 7.0, at two locations in the midvein ±5 mm apart, resulting in a total of 2 µg siRNA injected into each leaf. 10 mM Tris (Sigma–Aldrich) at pH 7.0 was injected as a control. A 10 µl, model 1701 Hamilton syringe with a 25.4 mm needle of 34 gauge, and 45° tip (Hamilton) was used for the injections. To contain aphids at the injection site, 15 ml polypropylene tubes (Greiner) were cut 45 mm from the opening, after which the closed end of the bottom tube was also removed to produce two tubes of ±45 mm in length. After 15 adult aphids were placed on the leaf between the injection sites, the leaf was threaded through the

modified polypropylene tube, which was then held in place by cotton wool inserted at the top and bottom of the leaf at each end of the tube. The cotton wool was adjusted to allow aphids to move around freely within a  $\pm 25 \text{ mm}^2$  area centered around the injection sites. The cages were supported by wire wrapped around the tubes, and anchored to a wooden rod. The foundress aphids were then allowed to feed for a period of 6h or 48h before the survivors were counted and sampled for further analysis. Leaf samples were taken at the same time points (6h and 48h), as well as directly after injection (0h). The experiment was performed in triplicate for every time point, and repeated twice over time ( $n = 18$ ).

#### *Foundress survival and nymph reproduction*

Aphid reproductive measurements were taken following the protocol as previously described (Van Eck et al., 2010) with small modifications. Directly after injection of siRNA, the aphids ( $n=15$ ) (biotype: SAM) were caged on the emerged third leaf of each plant, with each plant considered a biological repeat, with three biological repeats per treatment ( $n=3$ ). As the siRNA titer only lasts for a limited time period, the mothers were considered the foundress, and her nymph production recorded from the second day of settlement (24 h). Aphid nymph numbers were measured daily and the mean total number of nymphs was calculated as a measure of fertility ( $n=15$ ).

#### *Gene expression analysis in aphids using qPCR*

Silencing/knockdown of candidate genes was confirmed via qPCR. Primers were all designed to be 20 bp in length, to amplify a product of 123 bp in size and bind to the coding domain sequences of the *Dnc002* and *Dncprr1-8* genes. cDNA was synthesized as described, of which 0.5 ng was subsequently used for qPCR analysis. The qPCR setup comprised 5  $\mu\text{l}$  SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad) and one of the following primer sets: 0.5  $\mu\text{M}$  of both the forward and reverse sets specific to *Dnc002* or *Dncprr1-8*, 0.4  $\mu\text{M}$  forward and 0.6  $\mu\text{M}$  reverse specific to *L32* or 0.6  $\mu\text{M}$  of both the forward and reverse specific to *L27* in 10  $\mu\text{l}$  total volume reactions. The PCR cycling profile consisted of two initial steps of 50 °C for 1 min and 95 °C for 5 min, followed by 40 cycles at 95°C for 10 s, 20 s at annealing temp specified in Table 1 and 72°C for 20 s. A melt curve was also performed at 0.5°C increments every 5 s from 65°C to 95°C. Relative expression was calculated by means of the mathematical model by Pfaffl (2001) using untreated aphids sampled at day 0 as the calibrator and normalized to ribosomal proteins *L27* (Sinha and Smith 2014) and *L32* (Shakesby et al. 2009; De Jager et al. 2014).

*siRNA concentration in wheat*

A section of leaf material 10 mm in length, which included the two injection sites in the middle, was used for RNA extraction as described above. Stemloop primers specific to the synthetic siRNAs were designed as described by Varkonyi-Gasic et al. (2007). Each 20 µl cDNA synthesis reaction contained 3 mM MgCl<sub>2</sub>, 0.5 mM of each dNTP, 30 µM random hexamer primers, 0.5 µM specific stemloop primer, 150 ng RNA template, 1 µl ImProm-II™ Reverse Transcriptase (Promega) and 4 µl ImProm-II™ 5X Reaction Buffer (Promega). 5 ng of the cDNA was used in each 10 µl qPCR reaction as well as 5 µl SsoAdvanced universal SYBR® Green supermix (Bio-Rad), 1 µM universal stemloop reverse primer (Varkonyi-Gasic et al. 2007) and 1 µM specific forward primer (DnC002-siRNA F or RR1-siRNA F, Table 3). The thermal cycling protocol described by Varkonyi-Gasic et al. (2007) was followed to perform the reactions. 18S expression levels for each sample were determined in a 10 µl qPCR reaction consisting of 0.2 ng cDNA, 5 µl SsoAdvanced Universal SYBR® Green Supermix and 0.4 µM of both the forward and reverse primers. After an initial 3 min step at 95 °C, 40 cycles of 95 °C for 10 s, 54 °C for 30 s and 72 °C for 30 s were followed to amplify the product. The concentration of the siRNAs was calculated relative to 18S expression using the mathematical model by Pfaffl (2001).

**Table 3.** Sequences of primers used for reverse transcription and qPCR of siRNAs

Name of primer	Sequences of primers (5'-3')
<i>c002</i> siRNA SL RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCTCCG (SEQ ID NO: 17)
<i>c002</i> siRNA F	GCCACCATTTTCAGAGAGACAT (SEQ ID NO: 18)
<i>cprr1-8</i> siRNA SL RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTTAAA (SEQ ID NO: 19)
<i>cprr1-8</i> siRNA F	GCACAGCTTCTTGCGATTG (SEQ ID NO: 20)
Universal stemloop R	GTGCAGGGTCCGAGGT (SEQ ID NO: 21)
18S RNA forward	TGCCTATCAACTTTCGATGG (SEQ ID NO: 22)
18S RNA reverse	TGGATGTGGTAGCCGTTTCTC (SEQ ID NO: 23)

*Aphid protein assays*

Aphids were ground in ice-cold 50 mM phosphate buffer (pH of 7.5), 1 mM phenylmethylsulfonyl fluoride and 1 mM dithiothreitol. The extract was centrifuged at 4 °C for 15 min at 17 200 rpm. The supernatant was removed and kept on ice until protein assays were performed.

Catalase activity was determined colometrically by adding an aliquot of protein extract to 0.2 M phosphate buffer (pH 6.5) and 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> as previously described (Johansson and Borg 1988) and the degradation of H<sub>2</sub>O<sub>2</sub> was observed at 260 nm. Enzyme activity was expressed as  $\mu$ mol H<sub>2</sub>O<sub>2</sub>.mg protein<sup>-1</sup>.min<sup>-1</sup>.

Peroxidase activity was measured by adding an aliquot of protein extract to 0.2 M phosphate buffer (pH 5.0), 100 mM H<sub>2</sub>O<sub>2</sub> and 30 mM guaiacol (Zieslin and Benzaken 1991). The formation of tetraguaiacol was a linear function of enzyme concentration and peroxidase activity was expressed as mmol tetraguaiacol min<sup>-1</sup>. mg<sup>-1</sup> protein.

#### *Wheat protein assays*

Liquid N<sub>2</sub> was used to freeze wheat leaf material while it was ground. To this, 100 mM potassium phosphate (pH 7.5), 1 mM ethylenediaminetetraacetic acid and 1% polyvinylpyrrolidone was added to further homogenize the leaf material using a micropestle. The supernatant was collected after centrifugation at 4 °C for 15 min at 17 200 rpm (Rao et al. 1997).

Peroxidase activity was determined as previously described (Hildebrand et al. 1986) and horse radish peroxidase (BioLabs, Inqaba, Pretoria, ZA) was used as a standard. Hydrogen peroxide (0.06%) was added into a mixture containing 2  $\mu$ g of leaf extract, 6 mM guaiacol, 25 mM potassium phosphate buffer (pH 6.0) and 24% distilled water. The formation of tetraguaiacol was a linear function of enzyme concentration and peroxidase activity was expressed as mmol tetraguaiacol min<sup>-1</sup>. mg<sup>-1</sup> protein.

#### *Aphid and plant protein concentrations*

All protein concentrations were determined following a method described by Bradford (1976) using bovine serum albumin (Bio-Rad, USA) as standard. The Glomax spectrophotometer (Promega, USA) was used for this purpose as described by Rybutt and Parish (1982).

#### *Data analysis*

All sequence analysis was conducted utilizing the BLASTn and BLASTx tools (Altschul et al. 1990; Altschul et al. 1997) and SWISSPROT (O'Donovan et al. 2002) with *E*-values lower than 1e-10 considered as significant. CutProtFam-Pred: Detection and classification of putative structural cuticular proteins from sequence alone, based on profile Hidden Markov Models was applied to confirm the RR1 protein identity (<http://bioinformatics.biol.uoa.gr/CutProtFam->

Pred) (Ioannidou et al., 2014) All statistical analyses were conducted by using GraphPad PRISM 7 Software Tools ([https://www.graphpad.com/guides/prism/7/statistics/index.htm?usingstatistical\\_analyses\\_step\\_by\\_s.htm](https://www.graphpad.com/guides/prism/7/statistics/index.htm?usingstatistical_analyses_step_by_s.htm)) with significance set at  $\alpha = 0.05$ . Aphid fertility measurements were calculated as described by Van Eck et al. (2010), using the mean total number of nymphs born per day.

## Results and Discussion

Arthropod cuticle is a composite, bipartite system, made of chitin filaments embedded in a proteinaceous matrix, which serves as a protective barrier, and provide structural and mechanical support (Neville 1993). The physical properties of cuticle are determined by the structure and the interactions of its two major components, which are cuticular proteins (CPs) and chitin (Neville 1993). The proteinaceous matrix consists mainly of structural CPs, while the majority of these belonging to the CPR family, containing a conserved R&R region (Rebers and Riddiford Consensus) (Willis 1999; Rebers and Willis 2001). Two major subfamilies of the CPR family (i.e., RR-1 and RR-2) (Karouzou et al., 2007) have been identified from conservation at sequence level and some correlation with the cuticle type (Ioannidou et al., 2014). Some proteins containing the RR-1 motif were found in soft (flexible) cuticles, while the proteins containing the RR-2 motif were found in hard (rigid) cuticles, but this distinction is not firmly established (Andersen 2000).

### *Sequence characterization of D. noxia Dncpr1-8*

After obtaining the *in silico* predicted sequence for cuticle protein (RR-1) from the available SAM genome sequence (g3915.t2), it was Sanger sequenced. Analysis of the available sequences revealed that the *Dncpr1-8* gene were 3,873 bp long, containing 5 exons, a non-cytoplasmic domain and includes a 35-36 amino acid motif known as the chitin-binding Rebers and Riddiford (R&R) consensus (Willis 1999; Rebers and Willis 2001) (Figure 1).

In the present study, the function of *Dncpr1-8* was investigated as it has been shown to be expressed in the more virulent biotype SAM, but not in SA1 when feeding on RWA resistant wheat plants. In the study of Burger and Botha (2017), the authors found two single nucleotide polymorphisms between the biotypes, resulting in substitutions and codon changes in the gene (i.e., GTC>AGC resulting in V > S (valine, a hydrophobic charged amino acid, became serine, an uncharged amino acid); TCGC>AAAA resulting in IA > KK (isoleucine and alanine, hydrophobic charged amino acids, became lysine, an amino acid with positively charged side chains)).

### *Assessing the relative expression of Dnc002 and Dncpr1-8 in D. noxia biotypes SA1 and SAM*

In order to assess whether there is any difference in the inherent expression of *Dncpr1-8* and *Dnc002* in biotypes SA1 and SAM when feeding on resistant (GamR) and susceptible (GamS) NiLs, the biotypes were fed on these lines for 10 days. After feeding for 10 days, qPCR expression analyses were conducted for *Dncpr1-8* and *Dnc002* and relative expression was calculated (Figure 6). The obtained results revealed that even though the level of *Dncpr1-8* expression was higher in SAM relative to its parent SA1, it was not statistically significant (Figure 6B). While the expression of *Dnc002* didn't differ between the aphid biotypes when feeding on GamS, although higher in SAM when feeding on GamR, this also wasn't statistically significant (Figure 6A).

### *Optimizing siRNA of Dnc002 and Dncpr1-8*

After determining that the relative expression of these genes didn't differ significantly in biotype SAM irrespective of its host, different siRNA delivery systems were compared. Firstly, siRNA was delivered through direct injection in the insect haemolymph (Mutti et al., 2006, 2008; Jaubert-Possamai et al., 2007). This technique proved impossible due to the size of the aphids. All the aphids died shortly after injection, irrespective of being injected with no fluid, buffer or siRNA (data not shown).

Delivery of the siRNA was then conducted using feeding on artificial media (Shakesby et al., 2009; Whyard et al., 2009) or *in planta* (feeding on siRNA injected plants (Lapitan et al. 2007)), whereafter reproduction and foundress survival (Figures 7 and 8) were assessed. The aphids were allowed to feed for 48h before counting the number of nymphs produced to ensure settling of the foundresses. In the artificial feeding experiment, all foundresses survived irrespective of the feeding medium (Figure 8). In contrast, 48 hours post injection (hpi) with *Dncpr1-8*-siRNA and *Dnc002*-siRNA, significantly more foundresses died after feeding on these plants than on any other treatment.

In the artificial feeding experiment, although not statistically significant, more nymphs were produced by foundresses feeding on artificial media containing *Dncpr1-8*-siRNA, than on just artificial medium or medium containing *Dnc002*-siRNA (Figure 8). *In planta* knockdown with both *Dncpr1-8*-siRNA and *Dnc002*-siRNA resulted in significantly lower nymph production by foundresses feeding on these plants, when compared to foundresses feeding on GamS and buffer injected plants.

*siRNA of Dnc002 and Dncpr1-8*

To investigate the functions of *Dnc002*-siRNA and *Dncpr1-8* in the salivary glands of virulent biotype SAM while feeding on one of the most RWA resistant wheat varieties, GamR (containing *Dn7*), biotype SAM was allowed to feed on uninjected, buffer injected and plants injected with either 2 µg *Dnc002*-siRNA (Figure 9A) or 2 µg *Dncpr1-8*-siRNA (Figure 9C) and relative gene expression measured. When biotype SAM fed on *Dnc002*-siRNA for 48h, overexpression was observed relative to untreated and buffer injected leaves at 6h and 48h after introduction of aphids. Upregulation of *Dnc002* was also observed when SAM fed on GamS.

At 6h after aphid introduction, *Dncpr1-8* expression measured in SAM differs according to the plants they fed on and was in the following order (from highest): uninjected GamR > buffer injected GamR > *Dncpr1-8*-siRNA > GamS. However, the *Dncpr1-8* expression differed only statistically between aphids that fed on GamS, GamR and buffer injected plants ( $P \leq 0.05$ ). At 48 hpi the expression of *Dncpr1-8* in aphids feeding on GamS was significantly lower than that measured after 6 hpi, and also lower than in aphids feeding on *Dncpr1-8*-siRNA injected GamR plants. In fact, the levels of *Dncpr1-8* of the latter aphids were comparable to those feeding on GamS and much lower than those feeding on uninjected GamR plants.

At 48 hpi the expression of *Dncpr1-8* was the lowest in aphids feeding on *Dncpr1-8*-siRNA followed by GamS, buffer injected GamR and the highest expression was observed in aphids feeding on GamR. Although not significant, *Dncpr1-8* expression was lower in both aphids that fed on *Dncpr1-8*-siRNA injected GamR and GamS at 48 hpi compared to 6 hpi. Between the same time points *Dncpr1-8* expression of aphids that fed on buffer injected GamR also decreased, but not to the same extent as *Dncpr1-8*-siRNA injected plants and GamS. When feeding on GamR, *Dncpr1-8* expression stayed roughly the same.

To validate that the response measured in the feeding aphids can be directly correlated to siRNA present in the plants, the levels of *Dncpr1-8*-siRNA and *Dnc002*-siRNA were quantified using stemloop primers and qPCR analyses to reveal the siRNA concentration relative to wheat 18S expression (Figures 9B and D). These results confirmed that *Dnc002*-siRNA and *Dncpr1-8*-siRNA were present in the siRNA injected leaves and absent from the untreated leaves. Six hours after injection the siRNA was still present at levels equivalent to levels measured directly after injection. After 48h a decrease in siRNA was observed, although it was still present in significant quantities indicating relative stability within the plant. The

measured levels of siRNA were significantly higher ( $P \leq 0.05$ ) in the *Dncpr1-8*-siRNA and *Dnc002*-siRNA injected plants, when compared to all other plants.

#### *Transgenerational effect of siRNA*

To validate whether the interference also affects the unborn embryos of the feeding foundresses, newly born nymphs were sampled on 0, 96 and 144 hpi and assayed for the expression of *Dncpr1-8* (Figure 10). Interestingly, the effect of knockdown was most severe in newly born nymphs produced 96 hpi, and differed significantly from that in nymphs produced from foundresses feeding on uninjected GamR plants. This finding is in accordance with earlier findings in *Myzus persicae* (Coleman et al., 2014), where the *Mpc002* was down-regulated in nymphs born from mothers exposed to *c002*-dsRNA-producing transgenic Arabidopsis plants. In that study, Coleman followed the impact of RNAi over three generations of aphids and revealed that aphids reared on *c002*-dsRNA producing transgenic plants experienced a 60% decline in aphid reproduction levels compared with a 40% decline of aphids reared on Rack1-dsRNA- and MpPIntO2-dsRNA producing plants.

#### *D. noxia*-host interaction

In higher organisms, reactive oxygen species (ROS) are regularly generated by mitochondrial electron transport, when partially reduced and highly reactive metabolites of  $O_2$  such as superoxide anion ( $O_2^{\cdot-}$ ) and hydrogen peroxide ( $H_2O_2$ ) are formed during cellular respiration. Excessive release of ROS damages lipids, proteins, and DNA, which leads to oxidative stress, loss of cell function, and programmed cell death (Freeman and Crapo 1982; Starkov 2008). ROS are also actively released by hosts, in response to cellular invasion by pathogens as first line of defense, and occur in all eukaryotic cells. To regulate oxidative stress, the eukaryotic cell produces different ROS-scavenging enzymes, such as superoxide dismutase (which reduces  $O_2^{\cdot-}$  to  $H_2O_2$ ), glutathione peroxidase and catalase (which reduces  $H_2O_2$  to  $H_2O$ ) (Thannickal and Franburg 2000).

In the context of this study, it is deemed to happen in both the host plant (Fahnenstich et al., 2008) and insect species (Miller et al., 2000; Molina-Cruz et al., 2008). An increase in peroxidase activity also occurs in wheat after *D. noxia* infestation, which is indicative of the activation of systemic acquired resistance (SAR) (Van der Westhuizen et al., 1998; Smith et al., 2005, 2010; Botha et al., 2010, Botha 2013), albeit the induction is delayed in susceptible varieties (Van der Westhuizen et al., 1998). However, virulent *D. noxia* biotype SAM avoids detection by its host plant during feeding and a limited increased in peroxidase activity and

SAR is measured (Botha et al., 2014), making this aphid an ideal model to study plant-aphid interactions.

To elucidate the functions of *Dnc002* and *Dncpr1-8* in the salivary glands of virulent biotype SAM during feeding on GamR (a wheat expressing antibiosis and antixenosis (Painter 1951, 1958)), biotype SAM was allowed to feed on uninjected, buffer injected and plants injected with either 2 µg *Dnc002*-siRNA or 2 µg *Dncpr1-8*-siRNA, whereafter the activities of reactive oxygen species (ROS) were assayed in the feeding aphids and host (Figure 11).

As peroxidase (POX) is a ROS enzyme and a marker of the oxidative burst during the host defense throughout the interaction of wheat and *Diuraphis noxia* (Van der Westhuizen et al., 1998), it was assayed at 0, 6 and 48 hpi (Figure 11A). When comparing the POX activity between uninjected, infested GamS and GamR plants, higher POX activity was measured in the plants after infestation, with the highest POX activity assayed in the GamR 48 hpi ( $P \geq 0.05$ ), which is indicative of the induction of the host defense response (Van der Westhuizen et al., 1998; Botha et al., 2010, Botha 2013). However, even though POX activity increased slightly in the *Dnc002*-siRNA and *Dncpr1-8*-siRNA injected plants after 6 hpi, it decreased after 48 hpi to that at 0h. This observation suggests that unlike aphids feeding on uninjected GamR plants, the aphids feeding on the *Dnc002*-siRNA and *Dncpr1-8*-siRNA injected plants were not perceived as invasive. Hence the decrease in the transcription of host defense proteins like POX, as these are expected to increase as part of the systemic acquired resistance pathway in the resistant GamR plants and remained elevated to provide prolonged basal resistance (Van der Westhuizen et al., 1998; Smith et al., 2005, 2010; Lapitan et al., 2007b; Botha et al., 2010, Botha 2013).

Although aphid survival rate was still unaffected 6h after feeding on siRNA (Figures 7 and 8), POX and catalase (CAT) activity increased in aphids feeding on uninjected GamS and GamR, buffer injected and *Dnc002*-siRNA or *Dncpr1-8*-siRNA injected plants over the 48h period (Figure 11A). However, POX activity was only significantly higher in aphids feeding on *Dnc002*-siRNA or *Dncpr1-8*-siRNA injected plants ( $P \geq 0.05$ ) 6 hpi (Figure 7B). CAT activity was also elevated at 6 hpi in aphids that fed on *Dncpr1-8*- and *Dnc002*-siRNA injected plants, with CAT activity in *Dnc002*-siRNA fed aphids being significantly higher than in aphids that fed on buffer injected plants ( $P \geq 0.05$ ). ROS metabolism influences critical parameters of insect physiology, including fecundity (DeJong et al., 2007; Diaz-Albiter et al., 2011) and immune response (Ha et al., 2005a, 2005b). As POX and CAT activity is indicative of cellular stress experienced in response to the aphids' feeding environment, the results suggest that both genes afford the aphids some level of "protection" while feeding on the antixenotic and

antibiotic GamR, as partial knockdown of these genes decreased foundress survival by approximately 50% and affected nymph production significantly during *in planta* feeding experiments. In a field setting, a reduction of the aphid reproduction by 40-60% would dramatically decrease aphid population growth, contributing to a substantial reduction in agricultural losses (Coleman et al. 2014).

## Conclusion

Plant-mediated RNA interference (RNAi) has been successfully used as a tool to study gene function in aphids, and in this study it was applied to demonstrate the importance of *Dncpr1-8* in *D. noxia* fecundity. As demonstrated, partial knockdown of these genes decreased foundress survival by approximately 50% and affected nymph production significantly during *in planta* feeding experiments. A novel method to achieve gene silencing, not only in *D. noxia* but also in other aphid species (data not shown), is also described. This method allows observation of the interaction of plant and aphid during a gene silencing experiment, while this is not the case for siRNA/dsRNA delivery through artificial media, a popular method for aphid-gene silencing studies. It is also not invasive like direct injection of siRNA or dsRNA into the aphid hemolymph and less laborious than plant transformation if elucidating gene function is the immediate goal. The present study also demonstrated transgenerational knockdown, in that expression of the *Dncpr1-8* gene in the newly born embryos was decreased, making this method highly useful and feasible in aphid-plant interaction studies.

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Dated this 5<sup>th</sup> day of March 2018



VON SEIDELS Intellectual Property Attorneys

for the applicant

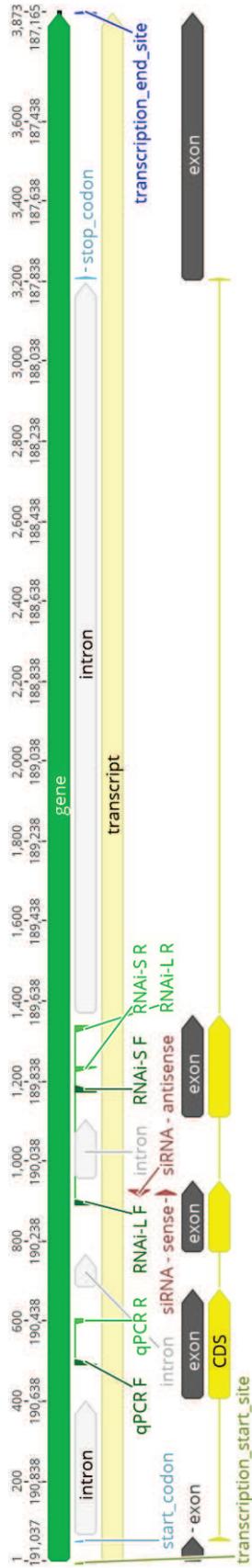


Figure 1

*A. Coold*

VON SEIDELS  
FOR THE APPLICANT

CATCCCAACATACGGCAATTTTCTGATCACGGTATACGTCCAAAAAATCACCATGAACACTTTTGTGAGT  
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Figure 2a

*A. Coold*

VON SEIDELS  
FOR THE APPLICANT

**Annotations:**

Transcription start site

Exon, Exon

Start codon

Coding domain sequence, Coding domain sequence

Intron

Stop codon

Transcription end site

**Primer binding site sequence:**qPCR F – **CCCATCCAACCAAGCCTA**qPCR R – **CCGGGACAACAAGGATACTA** (Primer 5'-3': TAGTATCCTTGTGTCCCGG)RNAi-L F – **GTAGACAACAAAGTGCCAGC**RNAi-L R – **AATTAATCGCCTCCAACCA** (Primer 5'-3': TGGTTGGGAGGCGATTAATT)RNAi-S F – **AAAACGCCGTCCAAGTGATC**RNAi-S R – **GGTGCTCCAGTCGAAGTCAA** (Primer 5'-3': TTGACTTCGACTGGAGCACC)**Figure 2b**

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VON SEIDELS  
FOR THE APPLICANT

STELLENBOSCH UNIVERSITY  
PROVISIONAL SPECIFICATION

10 Sheets/ Sheet 4

CATCCCAACATACGGCAATTTTCTGATCACGGTATACGTCCAAAAAATCACC**ATGAACACTTTGGTAGTG**  
**TTAGTAGCTGTCATCGCAGCGGTGTCTGCTGCGGCCCCACCTCAGGAAGCTGCCAAAGCTTTTACTTTTCAG**  
**TGGATTCCCATCCAACCAAGCCTACTACCCAGGCCAACAAAGGGTACTACCCAGGATACATTGGTTACCAGG**  
**GTTATCAAGGTTACAGCGGATTCCGTAATGGATACTACCCGGGACAAACAAGGATACTACCCAGGATACCAA**  
**GGTTACCAGGGATACTACCCAGGATACCAAGGTTACCAGGGATACTACCCAGGATATCAAGGTTACAACCG**  
**CGGTTACTACCCAGGTGCCCCAGCCGTCTACCCACCGTACCCCCGCCCCAATCATCGCACCAGTGCCAG**  
**TCGCGCCCAAGGCTGTTTCTCCAGTGTACAAACCCGTAGACAACAAAGTGCCAGCTATCCTTAAACAATCG**  
**CAAGAAGCTGACTTGAACGGATTCAAATACGGATACGAAACCGAAAAACGGCATCGTCGCCCAGGCTGCTGG**  
**ATACGTTAAGAACGCCGGTTCGAAAAACGCCGTCCAAGTGATCGAAGGCTCGTATGCCCTACATCGGTGACG**  
**ATGGTGCTCCAGTCGAAGTCAAGTACTACGCTGACGAGACCCGGTTACCACGCAGCCGGAAACGTCGTCCCG**  
**ACCACTCCCCCAGAGATCGCCAAGTCTTTGGAAATTAATCGCCTCCCAACCAACAGAAACCAGAAGACTCCAA**  
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 TTKTRATTTTTANNAATATAATTGTATTTATTTATAC

**Annotations:**

**Coding domain sequence, Coding domain sequence**

**Primer binding site sequence:**qPCR F – **CCCATCCAACCAAGCCTA**qPCR R – **CCGGGACAACAAGGATACTA** (Primer 5'-3': TAGTATCCTTGTGTCCCGG)RNAi-L F – **GTAGACAACAAAGTGCCAGC**RNAi-L R – **AATTAATCGCCTCCCAACCA** (Primer 5'-3': TGGTTGGGAGGCGATTAATT)RNAi-S F – **AAAACGCCGTCCAAGTGATC**RNAi-S R – **GGTGCTCCAGTCGAAGTCAA** (Primer 5'-3': TTGACTTCGACTGGAGCACC)**Figure 3**



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 VON SEIDELS  
FOR THE APPLICANT

ATGAACACTTTGGTAGTGTTAGTAGCTGTCATCGCAGCGGTGTCTGCTGCGGCCCCACCTCAGGA  
 AGCTGCCAAAGCTTTTACTTTTCAGTGGATTCCCATCCAACCAAGCCTACTACCCAGGCCAACAAG  
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 CTTGAACGGATTCAAATACGGATACGAAACCGAAAACGGCATCGTCGCCCAGGCTGCTGGATACG  
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 CGTCCCACCCTCCCCAGAGATCGCCAAGTCTTTGGAATTAATCGCCTCCCAACCACAGAAAC  
 CAGAAGACTCCAAAAAGAAATCACTCTAA

**Primer binding site sequence:**qPCR F – CCCATCCAACCAAGCCTAqPCR R – CCGGGACAACAAGGATACTA (Primer 5'-3':  
TAGTATCCTTGTTGTCCCGG)RNAi-L F – GTAGACAACAAAGTGCCAGCRNAi-L R – AATTAATCGCCTCCCAACCA (Primer 5'-3':  
TGGTTGGGAGGCGATTAATT)RNAi-S F – AAAACGCCGTCCAAGTGATCRNAi-S R – GGTGCTCCAGTCGAAGTCAA (Primer 5'-3':  
TTGACTTCGACTGGAGCACC)**Figure 4**

GCCGTCTACCCACCGTCACCCCGCCCCAATCATCGCACCAGTGCCAGTCGCGCCCAAGGCTGT  
 TTCTCCAGTGTACAAACCCGTAGACAACAAAGTGCCAGCTATCCTTAAACAATCGCAAGAAGCTG  
 ACTTGAACGGATTCAAATACGGATACGAAACCGAAAACGGCATCGTCGCCCAGGCTGCTGGATAC  
 GTTAAGAACGCCGGTTCCGAAAACGCCGTCCAAGTGATCGAAGGCTCGTATGCCTACATCGGTGA  
 CGATGGTGCTCCAGTCGAAGTCAAGTACTACGCTGACGAGACCGGTTACCACGCAGCCGGAAACG  
 TCGTCCCACCCTCCCCAGAGATCGCCAAGTCTTTGGAAT

**Figure 5**



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 VON SEIDELS  
 FOR THE APPLICANT

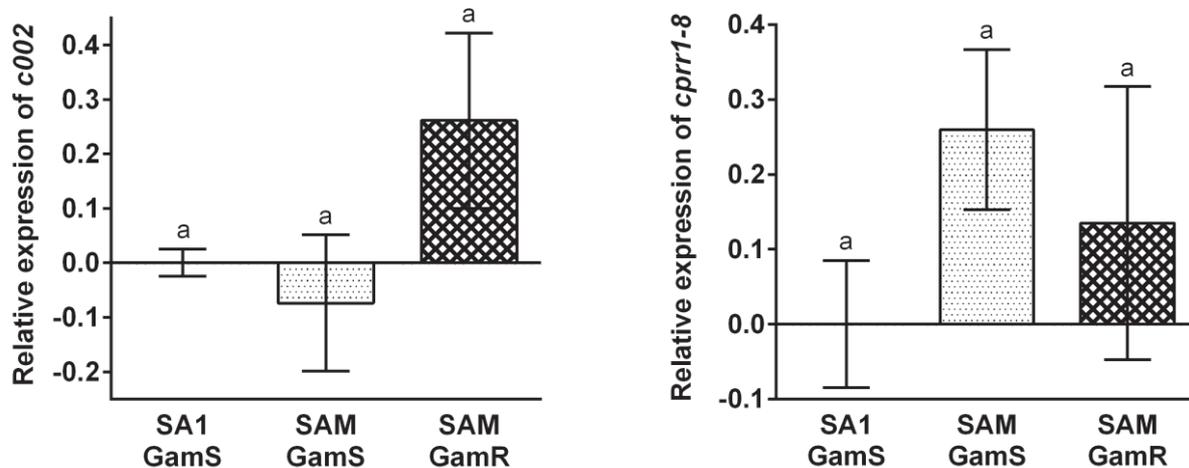


Figure 6

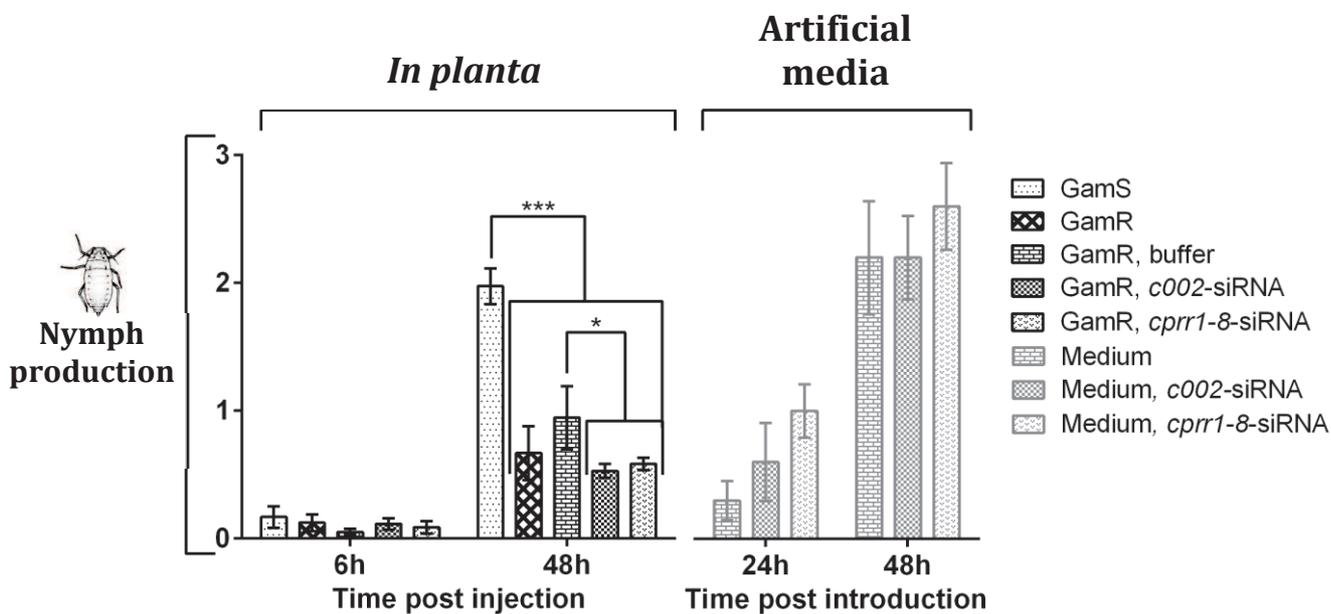


Figure 7

*A. Coold*

VON SEIDELS  
FOR THE APPLICANT

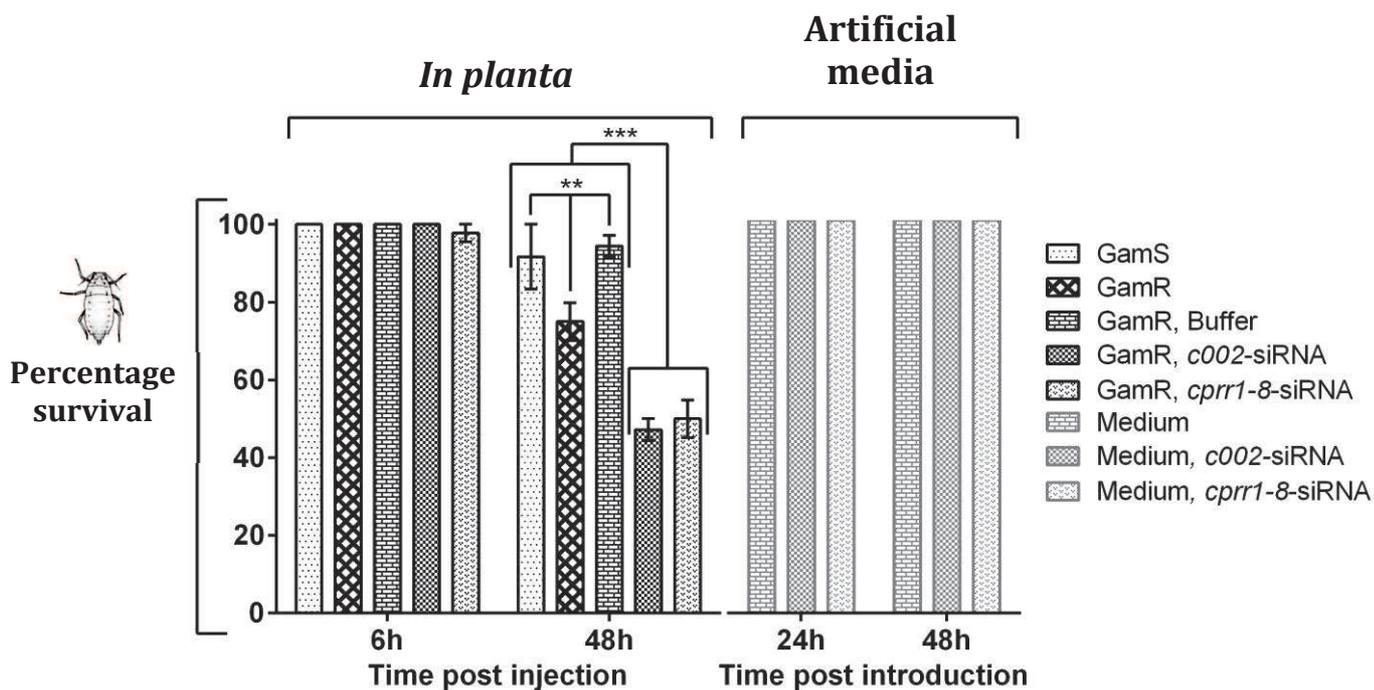


Figure 8

*A. Coold*

VON SEIDELS  
FOR THE APPLICANT

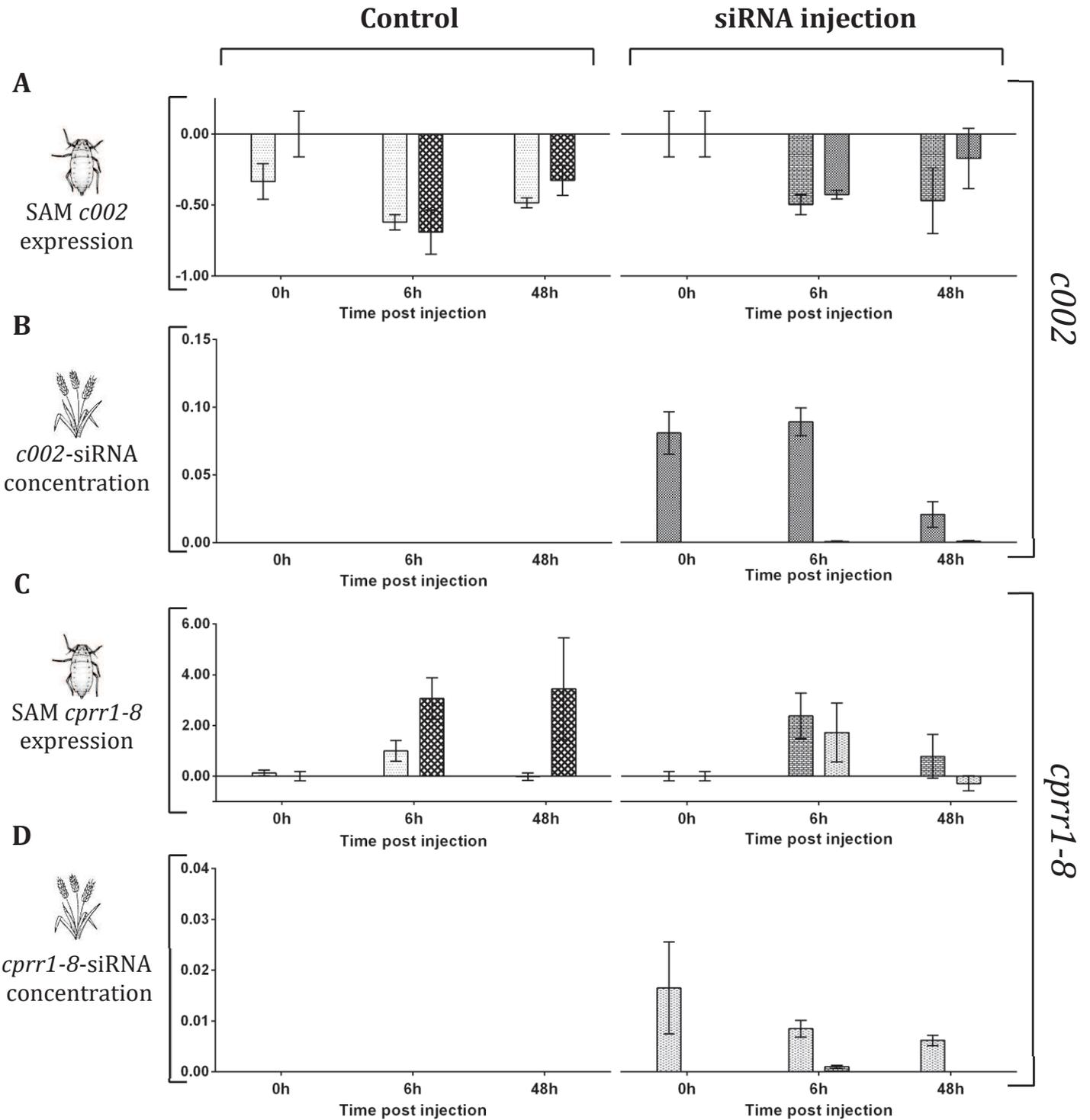
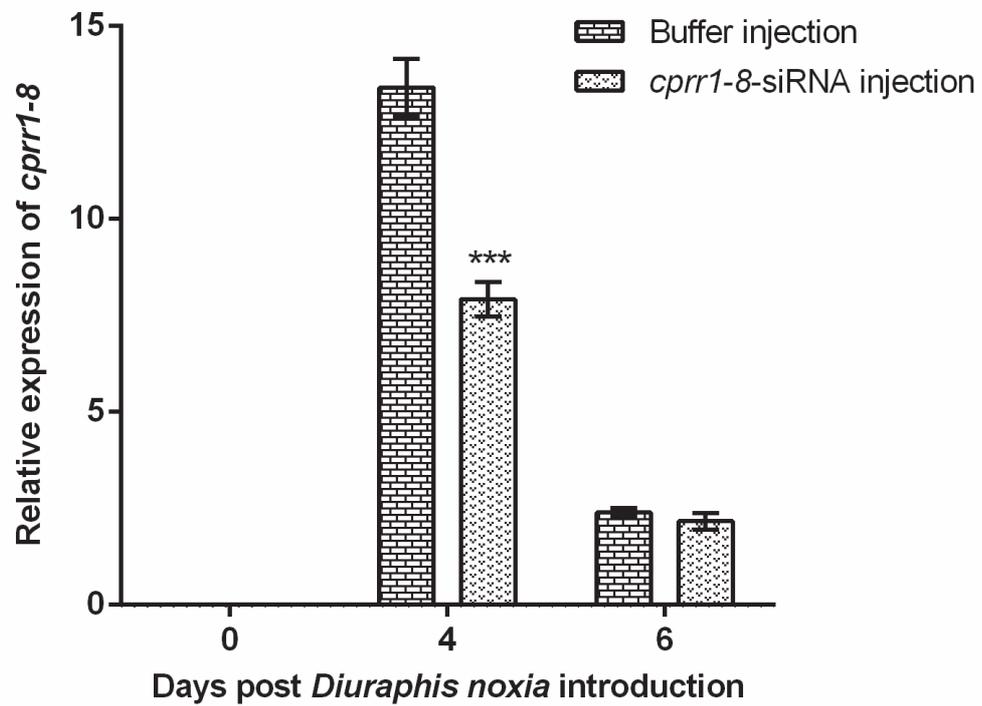


Figure 9

*A. Coold*

VON SEIDELS  
FOR THE APPLICANT

**Figure 10**

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VON SEIDELS  
FOR THE APPLICANT

