A targeted investigation of *Diuraphis noxia* (Hemiptera: Aphididae) methylation

By

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Declaration

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Preface

The findings obtained and presented in this thesis are the outcomes of a study conducted between January 2014 and December 2016 under the supervision of Professor Anna-Maria Botha, in the Department of Genetics at Stellenbosch University.

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Abstract

Diuraphis noxia (Kurdjumov, Hemiptera: Aphididae – or Russian wheat aphid, RWA) is an economically important phloem-feeding pest of wheat and barley. The most effective method for controlling RWA infestation of wheat is the deployment of resistant cultivars. However, new biotypes – aphid populations expressing virulence towards these cultivars – continue to develop. Consequently, a dire need exists to understand the molecular mechanism underlying increases in aphid virulence. The epigenetic modification of methylation has been proposed as one such mechanism, yet its effect on virulence remains largely unexplored. The aim of the study was thus to determine if methylation plays a role in biotypification and the associated increase in aphid virulence. To this end, two methods, namely methylation-sensitive amplification polymorphism (MSAP or MS-AFLP) and restriction site-specific fluorescent labelling (RSSFL), were tested for their ability to detect and quantify RWA methylation. The former was successful on both counts, specifically in the CG and CC dinucleotide contexts. Use of this methodology also revealed 22 polymorphic loci between the least and most virulent South African biotypes, SA1 and SAM, with 18 resulting from an increase in methylation during SAM's biotypification from SA1. Restriction site-specific fluorescent labelling is a novel technique that makes use of a fluorescently labelled adaptor, which binds to the sticky ends produced after the restriction of DNA using the isoschizomers HpaII and MspI. Although unable to detect or quantify methylation, RSSFL was able to detect trends in methylation. Various aspects of the DNA methyltransferases (DNMTs), which catalyse methylation, were also investigated. A homology search identified four putative RWA *DNMT* genes, namely *DNMT1*, *DNMT2*, *DNMT3A* and *DNMT3B*. Sequencing of these genes detected only one single nucleotide polymorphism between biotypes SA1 and SAM. Baseline *DNMT* expression, quantified using RT-qPCR, revealed significant differences in *DNMT3A* expression, which could be explained by the virulence of the respective biotypes. An antibody specific to 5-methylcytosine (5mC) was used to quantify both the DNMT protein activity (by detecting the relative number of methyl groups transferred by the DNMTs to a universal substrate)

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and the global 5mC levels, both of which did not differ significantly between the biotypes. The 5mC levels ranged from 0.1% to 0.16% and were in line with levels reported for numerous insects. Global hydroxymethylation levels were quantified using an antibody specific to 5-hydroxymethylcytosine (5hmC, a demethylation intermediate). Biotype SAM's 5hmC level was significantly higher than that of biotypes SA1, SA2 and SA3. Based on the results obtained, it is recommended that future studies of RWA methylation first perform RSSFL, followed by either MSAP or antibody-mediated methylation quantification (or both), depending on the needs of the specific study. The results also made clear the fact that methylation, and the removal thereof is related to differences in RWA virulence. Although many aspects of methylation were similar between the biotypes, local increases in methylation proved beneficial to the development of the highly virulent biotype SAM. During biotypification SAM also attained an increased ability to demethylate its genome, which affords this biotype greater flexibility to adapt to changing environments, by means of alterations in gene regulation. An increased demethylation capacity might therefore be a key contributory factor to increases in aphid virulence and hence biotypification.

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Uittreksel

Diuraphis noxia (Kurdjumov, Hemiptera: Aphididae – of Russiese koringluis, RKL) is ’n ekonomies belangrike floeëmvoedende plaag van koring en gars. Die doeltreffendste beheermaatreël vir RKL-infestering in koring is die gebruik van weerstandige kultivars. Nuwe biotipes – koringluispopulasies wat virulensie teenoor hierdie kultivars toon – ontwikkel egter voortdurend. Gevolglik bestaan ’n dringende behoefte om te verstaan watter molekulêre meganisme onderliggend aan toenemende koringluisvirulensie is. Die epigenetiese-modifikasie deur metilering is voorgestel as so ’n meganisme, maar die effek daarvan op virulensie is nog nie goed ondersoek nie. Die doel van hierdie studie is dus om te bepaal of metilering ’n rol in die vorming van biotipes en die gevolglike toename in koringluisvirulensie speel. Twee metodes, naamlik metilering-sensitiewe amplifikasiepolimorfisme (MSAP of MS-AFLP) en beperkingsarea-spesifieke fluoresserende etikettering (RSSFL), is getoets vir hul vermoë om RKL-metilering uit te wys en te kwantifiseer. Eersgenoemde metode het beide gedoen, spesifiek in die konteks van die CG en CC dinukleotiedpare. Die gebruik van hierdie metode het ook 22 polimorfiese lokusse tussen die minste en mees virulente Suid-Afrikaanse biotipes, SA1 en SAM, uitgewys. Verder is bevind dat 18 lokusse die resultaat van ’n toename in metilering tydens SAM se biotipevorming vanuit SA1 is. Beperkingsarea-spesifieke fluoresserende etikettering is ’n nuwe tegniek wat gebruik maak van ’n fluoresserend-gemerkde verbinder wat bind aan die beperkingsensiemoorhange wat ontstaan na beperkingsnyding van DNS deur isoskisomere HpaII en MspI te gebruik. Hoewel RSSFL nie metilering kon kwantifiseer nie, kon dit wel tendense in metilering uitwys. Verskeie aspekte van die DNS-metieltransferases (DNMTs) wat metilering kataliseer, is ook ondersoek. ’n Homologiesoektog het vier vermeende *DNMT*-gene in die RKL-genoom geïdentifiseer, naamlik *DNMT1*, *DNMT2*, *DNMT3A* en *DNMT3B*. Volgordebepaling van hierdie gene het slegs een enkelnukleotied-polimorfisme tussen biotipes SA1 en SAM uitgewys. Basislyn-*DNMT*-uitdrukking, wat deur middel van RT-qPCR gekwantifiseer is, het betekenisvolle verskille in die uitdrukking van *DNMT3A* uitgewys, wat deur die virulensie van die onderskeie biotipes verklaar kan word. ’n

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Teenliggaam, spesifiek aan 5-metielsitosien (5mC), is gebruik om sowel die DNMT-proteïenaktiwiteit (deur vasstelling van die relatiewe aantal metielgroepe wat deur die DNMTs na ’n universele substraat oorgedra is) as die globale 5mC-vlakke te kwantifiseer; beide het nie betekenisvolle verskille tussen die biotipes getoon nie. Die 5mC-vlakke het tussen 0.1% tot 0.16% gewissel en was in lyn met vlakke wat in verskeie ander insekte gemeet is. Globale vlakke van hidroksiemetilering is met ’n teenliggaam wat spesifiek teen 5-hidroksiemetielsitosien (5hmC, ’n demetileringstussenganger) is, gekwantifiseer. Biotipe SAM se 5hmC-vlak was betekenisvol hoër as dié van biotipes SA1, SA2 en SA3. Op grond van hierdie resultate word voorgestel dat toekomstige ondersoeke na RKL-metilering eerstens RSSFL uitvoer, gevolg deur óf MSAP óf teenliggaam-bemiddelde metileringskwantifisering (of beide), afhangende van die behoeftes van die betrokke ondersoek. Die resultate maak dit ook duidelik dat metilering en die verwydering daarvan verband hou met verskille in RKL-virulensie. Hoewel baie aspekte van metilering tussen die betrokke biotipes ooreenstem, het plaaslike toenames in metilering voordelig geblyk vir die ontwikkeling van die hoogs virulente biotipe SAM. Gedurende biotipevorming het SAM ook ’n verhoogde vermoë om sy genoom te demetileer verkry, wat hierdie biotipe van groter buigsaamheid voorsien om by veranderende omgewings aan te pas. Laasgenoemde is waarskynlik deur middel van wysigings in geenregulering. ’n Verhoogde vermoë om te demetileer is derhalwe moontlik ’n sleutelfaktor wat tot toenames in plantluisvirulensie en gevolglik biotipevorming, bydra.

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List of abbreviations

3'	Downstream
5'	Upstream or fifth carbon of pyrimidine ring of cytosine
5caC	5-carboxylcytosine
5fC	5-formylcytosine
5gmC	β-glucosyl-5-hydroxymethylcytosine
5hmC	5-hydroxymethylcytosine or hydroxymethylcytosine
5hmU	5-hydroxymethyluracil or hydroxymethyluracil
5mC	5-methylcytosine or methylcytosine
A	Adenine
Aba-Seq	AbaSI sequencing
AFLP	Amplified fragment length polymorphism
AID/APOBEC	Activation-induced cytidine deaminase/apolipoprotein B mRNA editing enzyme, catalytic polypeptide
ANOVA	Analysis of variance
Asp	Aspartate
Avr	Avirulence
BER	Base excision repair
βGT	β-glucosyltransferase
BLAST(n/p)	(Nucleotide/Protein) Basic local alignment search tool
bp	Base pair(s)
BS-Seq	Bisulfite sequencing
C	Cytosine
CAF	Central Analytical Facility
cDNA	Complementary DNA
CDS	Coding domain sequence
CG/CpG	Cytosine followed by guanine in 5' to 3' direction
-chip	Followed by microarray
CMS	Cytosine-5-methylenesulfonate
CTCF	CCCTC-binding factor
DAMPs	Damage-associated molecular patterns
dH_2O	Distilled water
Dn	*Diuraphis noxia*
DNA	Deoxyribonucleic acid

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DNase	Deoxyribonuclease
DNMT	DNA methyltransferase
dNTPs	Deoxynucleotide triphosphates
dsRNA	Double-stranded RNA
E	Efficiency
EDTA	Ethylenediaminetetraacetic acid
e.g.	*exempli gratia* (for example)
ELISA	Enzyme-linked immunosorbent assay
et al.	*et alia* (and others)
F	Forward
G	Guanosine
g	Gram or g-force
g/l	Gram per litre
Gly	Glycine
GST	Glutathione-S-transferase
GTP	Guanosine triphosphate
H	A, C or T
H_2O_2	Hydrogen peroxide
HMeDIP	Hydroxymethyl-DNA immunoprecipitation
HMeDIP-Seq	Hydroxymethyl-DNA immunoprecipitation sequencing
HPLC/MS/MS	High performance liquid chromatography tandem mass spectrometry
HPR	Host plant resistance
IDT	Integrated DNA Technologies
i.e.	*id est* (that is)
Inc.	Incorporation
IPM	Integrated pest management
kg/ha	Kilograms per hectare
$KRuO_4$	Potassium perruthenate
l	Litre
LB	Luria Broth
LSD	Least significant difference
Ltd	Limited
M	Molar or molecular marker
Mb	Megabases
MBD(2/4)	Methyl-CpG-binding domain protein (2 or 4)

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MBD-Fc	Recombinant fusion protein that binds double-stranded DNA at methylated CpG sites
MCIp	Methyl-CpG immunoprecipitation
Mcr	Modified cytosine restriction
MeCP2	Methyl-CpG-binding protein 2
MeDIP	Methylated DNA immunoprecipitation
MeDIP-Seq	Methylated DNA immunoprecipitation sequencing
mg/ml	Milligram per millilitre
mg protein/ml	Milligram protein per millilitre
Min	Minutes
MIRA	Methylated CpG island recovery assay
ml	Millilitre
mm	Millimetre
mM	Millimolar
mRNA	Messenger RNA
MS-AFLP	Methylation-sensitive amplified fragment length polymorphism
MSAP	Methylation-sensitive amplification polymorphism
MS-RFLP	Methylation-sensitive restriction fragment length polymorphism
m/v	Mass per volume
NCBI	National Center for Biotechnology Information
ng	Nanogram
ng/µl	Nanogram per microlitre
nm	Nanometre
OD	Optical density
OD/h/µg	Optical density per hour per microgram
OxBS-Seq	Oxidative bisulfite sequencing
P	Amount of positive control in nanograms
PAMPs	Pathogen-associated molecular patterns
PCR	Polymerase chain reaction
pH	Power/potential of Hydrogen
pmole	Picomole
Pty	Proprietary
***p* value**	Probability value
Pvu-Seq	PvuRts1I sequencing
qPCR	Quantitative PCR

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Q score	Quality score
R	Resistance or relative expression or reverse
R^2	Coefficient of determination
RefSeq	Reference sequence
RFU	Relative fluorescence units
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
ROS	Reactive oxygen species
RPM	Revolutions per minute
RSSFL	Restriction site-specific fluorescent labelling
RT-qPCR	Reverse transcription qPCR
RWA	Russian wheat aphid
S	Amount of sample in nanograms
SA1	South African RWA biotype 1
SA2	South African RWA biotype 2
SA3	South African RWA biotype 3
SA4	South African RWA biotype 4
SAM	South African Mutant biotype
Sec	Seconds
siRNA	Small-interfering RNA
SNP	Single nucleotide polymorphism
T	Thymine
T_a	Annealing temperature
TAB-Seq	Tet-assisted bisulfite sequencing
TAE	Tris/Acetic acid/EDTA
Taq	*Thermus aquaticus*
TBE	Tris/Boric acid/EDTA
TDG	Thymine DNA glycosylase
TE	Tris-EDTA
TET	Ten-eleven translocation enzyme
T_m	Melting temperature
tRNA	Transfer RNA
U	Uracil or units
UDP-Glc	Uridine diphosphate glucose

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µg	Microgram
µg/ml	Microgram per millilitre
µl	Microlitre
US(A)	United States (of America)
US$	United States Dollar
US1	United States RWA biotype 1
US2	United States RWA biotype 2
V	Volts
Val	Valine
VOC	Volatile organic compounds
v/v	Volume per volume
x	Times
°C	Degrees Celsius
%	Percent
%ID	Percent identical sites
%QC	Percent query coverage
λ	Wavelength
(+n)	Number of selective nucleotides
™	Trademark
®	Registered trademark
*****	Perfect alignment of a base between all sequences
#	Number of
[]	Concentration

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Chapter 1

Introduction

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1.1 Introduction

Wheat (*Triticum aestivum* (L.)) is a staple grain in many countries including South Africa (Altman *et al*. 2009; McFall and Fowler 2009). Wheat is also, however, one of the two main hosts, the other being barley (*Hordeum vulgare* (L.)), of the cereal pest *Diuraphis noxia* (Kurdjumov, Hemiptera: Aphididae – or Russian wheat aphid, RWA) (Botha *et al*. 2005; Porter *et al*. 2009; Botha 2013). Russian wheat aphid infestation causes significant yield penalties to wheat production, with South Africa and the United States of America (USA) being the worst afflicted countries (Basky 2003; Botha *et al*. 2005; Porter *et al*. 2009). Host plant resistance (HPR), the introduction of genes that confer resistance against *D. noxia* (designated as *Dn* genes) into wheat cultivars, has proved the most effective strategy for managing RWA infestation and dispersal in both South Africa and the USA (Porter *et al*. 2009). The efficacy of HPR is, however, threatened by the emergence of new biotypes, morphologically similar aphid populations which render previously resistant cultivars susceptible, and thus display increasingly higher levels of virulence (Botha *et al*. 2005, 2010; Tagu *et al*. 2008; Sinha and Smith 2014). The molecular mechanism driving biotypification is currently unknown (Shufran and Payton 2009; Botha *et al*. 2014a), but urgently needs to be elucidated to enable the breeding and deployment of wheat cultivars with more durable resistance (Sinha and Smith 2014).

In South Africa, there are four naturally occurring RWA biotypes, as well as one highly virulent mutant biotype, SAM, which is laboratory-contained (Van Zyl and Botha 2008; Swanevelder *et al*. 2010; Jankielsohn 2014, 2016). The naturally occurring biotypes, named in order of emergence and increasing virulence, are SA1 < SA2 < SA3 < SA4 (Jankielsohn 2014, 2016). Biotype SA1, the original population of aphids in South Africa, is the most avirulent and only damages wheat cultivars containing the recessive *dn3* gene (Jankielsohn 2011). In stark contrast to this, biotype SAM which developed from SA1, is able to overcome the resistance of all introduced and/or documented *Dn* genes (Van Zyl and Botha 2008; Swanevelder *et al*. 2010; Botha 2013; Botha *et al*. 2014a).

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Despite the successful introduction/breeding of *Dn* genes into wheat cultivars, none of these genes, nor the genes encoding aphid effector proteins with which resistance proteins interact, have been cloned (Botha *et al*. 2005, 2014b; Smith and Clement 2012). Consequently, some scientists have begun researching other factors which could increase aphid virulence, such as differences in energy production between aphid biotypes (De Jager 2014) or biotypic differences in the genome of *Buchnera aphidicola*, the sole RWA endosymbiont (Swanevelder *et al*. 2010). In 2012, Gong *et al*. identified differences in the methylation levels of four RWA salivary-gland transcribed genes (i.e., putative effector genes) between two US biotypes, which by definition display different virulence levels. This provided the first evidence that methylation, and alterations thereof, may be a contributing factor to increases in RWA virulence during biotypification.

Methylation is known to be involved in a number of aphid processes, including insecticide resistance (*Myzus persicae*), as well as growth rate, morph distribution and pigmentation (*Acyrthosiphon pisum*) (Field *et al*. 1989, 2004; Dombrovsky *et al*. 2009). Wing polyphenism, a type of phenotypic plasticity displayed by aphids, is strongly believed to be under epigenetic regulation, and may therefore also be mediated by methylation (Tagu *et al*. 2008; Srinivasan and Brisson 2012). Cues for changes in, or alterations to the methylation of an organism can be either environmental or intrinsic (Bonasio *et al*. 2010; Feil and Fraga 2012; Foret *et al*. 2012; Yan *et al*. 2015). In the case of the RWA, the introduction of resistance genes into wheat cultivars constitutes an environmental change/stimulus. Much like aphid wing polyphenism (Srinivasan and Brisson 2012), increases in aphid virulence could be as a result of environmental cues sensed by the aphid foundress that are translated into heritable changes in the offspring. However, as no in-depth studies of RWA methylation have been performed, the role of methylation in biotypification remains hypothetical.

The aim of the current study was thus to determine if methylation plays a role in biotype development and the related increase in virulence. To accomplish this, different aspects of methylation of the RWA biotypes SA1, SA2, SA3 and SAM were investigated. The availability of

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biotype SAM proved especially useful in resolving the questions related to biotypification. Biotype SAM and its parent biotype SA1 share a very similar genome, displaying only 0.0008% variation in protein-coding gene sequences (Burger and Botha 2017). This makes these biotypes an ideal model to study epigenetic mechanisms such as methylation, without concerns regarding the confounding effects of genetic variation (Verhoeven and Preite 2014).

The objective of Chapter 3 was to assess the capacity of different methodologies to detect and quantify RWA methylation. The first technical objective of this chapter was to identify differences in methylation banding patterns between the biotypes using the methylation-sensitive amplification polymorphism (MSAP) technique (Reyna-López *et al*. 1997), to profile these differences, and to relate them to the reported virulence levels of the South African RWA biotypes (Jankielsohn 2014, 2016). The MSAP technique also provided a means for estimating the level of methylation, by dividing the sum of unique MspI and HpaII bands by the total number of bands (Kronforst *et al*. 2008). The second technical objective was to identify trends in methylation of South African RWA biotypes through the use of a novel technique denoted restriction site-specific fluorescent labelling (RSSFL), which was also tested on *Homo sapiens* and *Apis mellifera capensis* DNA as a comparative measure.

The objective of Chapter 4 was to characterise the genes encoding proteins which catalyse methylation (Goll and Bestor 2005) – the DNA methyltransferases (*DNMTs*), in terms of both sequence and expression, and to relate these findings to the observed methylation, hydroxymethylation and virulence levels of the South African RWA biotypes. Four technical objectives were set out, the first being to identify, clone and sequence the *DNMTs* of biotypes SA1 and SAM. Technical objective two was to quantify the baseline expression levels of the *DNMTs* using RT-qPCRs, followed by technical objective three, to quantify the protein activity levels of the DNMTs making use of antibodies. The fourth technical objective was to quantify the relative global methylation and hydroxymethylation levels, the latter providing an indication of RWA demethylation levels for the first time.

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1.2 Thesis layout

Chapter 2 introduces the cereal pest RWA, provides an overview of strategies used to manage its infestation and dispersal, and describes the RWA-wheat interaction and the important role of RWA saliva during feeding. Factors which could contribute to an increase in RWA virulence and biotypification are also reviewed. One of these factors, DNA methylation, is reviewed in detail, as it forms the focus of the research presented in Chapters 3 and 4.

In **Chapter 3** the capability of the techniques MSAP and RSSFL to detect and quantify methylation of RWA, is assessed and reported on.

Appendix A contains figures illustrating the MSAP banding patterns, sequences of adaptors and primers used for MSAP and RSSFL analysis, a table showing the banding patterns obtained using seven MSAP primer combinations, *p* values from statistical tests performed and a supplementary DNA extraction method.

Chapter 4 focuses on the characterisation of the RWA DNA methyltransferases, in terms of sequence and expression, and relates these findings to methylation, hydroxymethylation and virulence levels of the South African RWA biotypes.

Appendix B contains the sequences of primers used for sequencing the *DNMTs*, as well as those used for the *DNMT* expression analysis. Standard curves and melt curves from the expression analysis are also shown. This appendix also includes supplementary figures and tables pertaining to the methods and results of Chapter 4.

Chapter 5 summarises the principle findings of the thesis, discusses the implications thereof, and provides insight into future research directions.

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Chapter 2

Literature review

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2.1 The host: *Triticum aestivum* (L.) (wheat)

In 2014, more than 729 million tonnes of wheat were harvested worldwide from an area of over 220 million hectares (FAOSTAT – http://www.fao.org/faostat/). Additionally, wheat's worldwide export value of US$ 49.4 billion exceeded that of all other cereal crops in 2013 (most recent trade data available – FAOSTAT), highlighting the importance of wheat as a commodity throughout the world. Wheat's importance is attributed to the fact that wheat, together with maize and rice, is a staple grain in many countries including South Africa (McFall and Fowler 2009). Collectively these staples provide more than 60% of the protein and calorie intake of the human population (Gill *et al*. 2004), with wheat also being a substantial source of carbohydrates (Anathakrishnan *et al*. 2014). Being a staple food, wheat is often the main and sometimes only source of nutrients and it is vital that wheat production remains high.

Sustaining a wheat yield high enough to meet the needs of an ever-growing world population is, however, threatened by a number of biotic and abiotic stresses. Wheat is faced with a multitude of adverse environmental conditions including drought, extreme temperatures, insufficient soil nutrients and changes in salinity, among others, all of which constitute abiotic stresses (Cramer *et al*. 2011). Wheat must also deal with biotic stresses, most commonly in the form of pathogen and pest attacks (Botha *et al*. 2014a).

2.2 The pest: *Diuraphis noxia* (Kurdjumov) (Russian wheat aphid)

2.2.1 Morphology and genome

Wheat, barley (*Hordeum vulgare* (L.)) and selective *Bromus* grasses make up the host range of the insect pest, *Diuraphis noxia* (Kurdjumov, Hemiptera: Aphididae – or Russian wheat aphid, RWA) (Botha *et al*. 2005; Porter *et al*. 2009; Botha 2013). These small (<2.3 mm), phloem-feeding insects are spindle-shaped and yellow-green or green-grey in colour (Stoetzel 1987). They possess truncated cornicles, and appear to have a double tail, on account of the supracaudal process on their 8th abdominal tergite (Stoetzel 1987). The draft genome of *D. noxia* is 624 048 Mb in size, which

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places it between that of fellow Hempiterans, *Acyrthosiphon pisum* (541 675 Mb) and *Rhodnius prolixus* (702 643 Mb). The RWA boasts the most AT-rich insect genome and a total of 31 885 protein-coding genes (Burger and Botha 2017).

2.2.2 RWA biotypes and virulence

In the context of this study, a new aphid biotype is a population of aphids which can damage wheat cultivars previously deemed resistant (Smith *et al*. 1992; Botha *et al*. 2010; Botha 2013). Biotypes are morphologically similar and display different levels of virulence, which can be defined by the damage a biotype causes to a differential set consisting of wheat plants containing different *Dn* (*D. noxia*) resistance genes (Weiland *et al*. 2008). To date, 14 genes conferring differential resistance to RWA biotypes have been identified. These genes are denoted *Dn1–Dn9*, *Dnx* and *Dny* (Botha *et al*. 2005; Jankielsohn 2011), *Dn2414* (Peng *et al*. 2007), *Dn626580* (Valdez *et al.* 2012) and *Dn2401* (Fazel-Najafabadi *et al*. 2015).

Rigorous screening led to the identification of four RWA biotypes in the fields of South Africa. Russian wheat aphid SA1 was first recorded in the country in 1978 (Walters *et al*. 1980), followed by RWA SA2 in 2005 (Tolmay *et al*. 2007), RWA SA3 in 2009 (Jankielsohn 2011) and RWA SA4 in 2011 (Jankielsohn 2014, 2016). There is also one laboratory-contained biotype known as the South African Mutant (SAM) biotype, which developed as a result of laboratory-induced *Dn* resistant selective pressure on SA1 (Van Zyl and Botha 2008; Swanevelder *et al*. 2010). Biotype SA1 was force-fed on resistant wheat cultivars until the eventual development of the highly virulent SAM biotype (Van Zyl 2007; Van Zyl and Botha 2008; Botha *et al*. 2014a).

The virulence of the South African RWA biotypes increases from biotype SA1 through SA4 (i.e., SA1 < SA2 < SA3 < SA4), with the more virulent biotypes being able to break down/overcome the resistance of numerous *Dn* genes and feed on a wider variety of wheat cultivars. The least virulent South African biotype, SA1, is only virulent to cultivars that contain the recessive *dn3* gene, resulting in susceptible damage symptoms in these plants (Jankielsohn 2011). Biotypes SA2, SA3 and SA4 are all virulent to cultivars containing *Dn1*, *Dn2*, *dn3* and *Dn9* (Jankielsohn 2011). In

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addition to this, SA3's virulence profile contains *Dn4*, and SA4's profile contains *Dn4* and *Dn5* (Jankielsohn 2011, 2014, 2016). Biotype SAM is the most virulent RWA biotype ever reported and has the ability to overcome the resistance of all the *Dn* genes, including *Dn7* (Swanevelder *et al*. 2010; Botha 2013; Botha *et al*. 2014a). A cultivar is yet to be developed that provides resistance against SAM. This is not, however, a problem as SAM only serves as a genetic model to resolve aphid biotypification and is highly contained.

2.2.3 Symptoms of RWA feeding

The effects of RWA infestation and subsequent feeding are most pronounced in South Africa and the United States of America (USA) (Basky 2003; Botha *et al*. 2005; Porter *et al*. 2009). Symptoms of RWA feeding on resistant and susceptible wheat varieties differ markedly, the former mostly present as necrotic lesions (Figure 2.1A) (Fouché *et al*. 1984; Botha *et al*. 2006). Feeding on susceptible wheat cultivars causes damage to chloroplasts, resulting in chlorophyll degradation, a symptom visible as chlorosis or longitudinal streaking (Figure 2.1B) (Burd and Elliott 1996; Heng-Moss *et al*. 2003; Botha *et al*. 2006). This decrease in chlorophyll content is associated with a reduction in the plant's photosynthetic capacity (Fouché *et al*. 1984; Burd and Burton 1992). The rolling of leaves, both newly developed, and fully extended, also commonly occurs when RWA feed on susceptible wheat varieties (Figure 2.1C). Leaf rolling can result from an aphid-induced reduction in leaf turgor that prevents newly developed leaves from unrolling, sometimes causing head trapping (Burd and Burton 1992). These symptoms culminate in reductions in wheat yield as high as 92% (Hewitt 1988) and in severe cases, plant death.

The consequences of such a high yield reduction for the USA, the world's leading wheat exporter (FAOSTAT), are far-reaching, with a threat to global food security being of great concern. In South Africa, a decrease in wheat yield has widespread effects on the poverty-stricken who depend on a staple diet consisting of wheat and maize (Altman *et al*. 2009), as well as the more affluent population who also consume large amounts of wheat. In an attempt to lessen the impact of aphid

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feeding, there is an inevitable increase in spending on pest management strategies augmenting the financial burden already associated with crop loss.

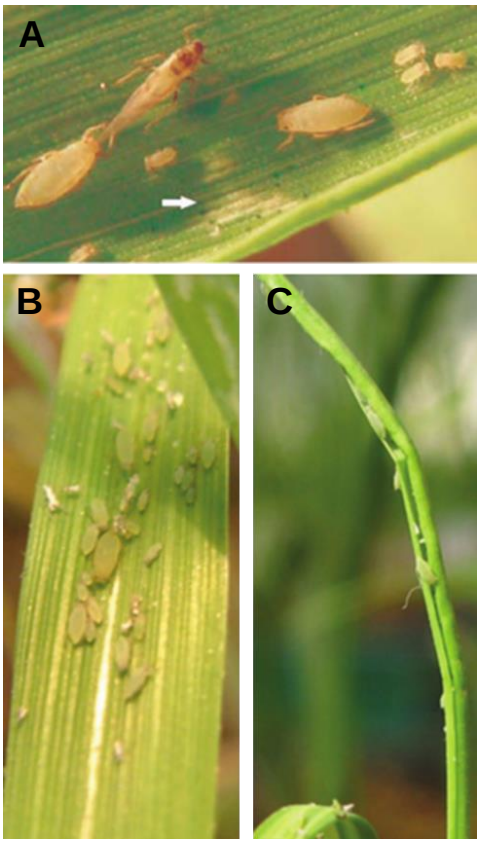

Figure 2.1. Symptoms of RWA feeding on resistant and susceptible wheat varieties. (A) Feeding on resistant varieties results in the expression of necrotic lesions (arrow). Image sourced from Botha *et al*. (2006). Feeding on susceptible varieties causes (B) chlorosis or longitudinal streaking and (C) leaf rolling. Images sourced from Botha *et al*. (2014a).

2.3 The solution: An integrated pest management strategy focusing on host plant resistance

The scientific community has an important role to play in lessening the impact of this cereal pest. A number of strategies including cultural practices, biological and chemical control methods, and genetic approaches, most notably, the introgression of resistance genes into host plants, have been employed in an attempt to minimise and regulate RWA infestation (Smith and Clement 2012). Although these strategies all lead to a reduction in aphid damage, they each have associated shortcomings. Aphid infestation can, however, be effectively controlled by employing two or more strategies simultaneously, in the form of an integrated pest management (IPM) programme, with the most effective strategy forming the foundation of the programme.

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2.3.1 Cultural control

Cultural control involves a number of farming practices, aimed at managing the physical or biological environment of a crop (Wratten *et al*. 2007). Altering the sowing rate (reflected as plant density), sowing date, and fertiliser use, are a few ways in which the crop's physical environment is managed to decrease aphid damage (Wratten *et al*. 2007; Dedryver *et al*. 2010). An increase in plant density for example, creates an environment with a high relative humidity, a factor that discourages aphid infestation. There is a striking contrast in the sowing rate of wheat between countries in which RWA is considered a pest of wheat (South Africa and the USA) and those in which it is not (Hungary). The former countries have a maximum sowing rate of 120 kg/ha whilst the sowing rate in Hungary ranges between 200 kg/ha – 220 kg/ha, resulting in a higher plant density and an unfavourable aphid environment (Basky 2003). Biological environment management entails providing either refuge or resources for natural enemies of the crop pest (Wratten *et al*. 2007). Increasing the population of natural enemies of a pest is intrinsically linked to conservation biological control (Powell and Pell 2007; Dedryver *et al*. 2010), but can fall under cultural control in that it involves habitat management and manipulation.

2.3.2 Biological control

Biological control is the use of natural enemies to decrease a population of pests (Powell and Pell 2007). Enemies used to decrease aphid populations include predators, parasitoids and entomopathogenic fungi (Dedryver *et al*. 2010). Predators are classified as either specialist or generalist based on the prey they consume. Specialist predators are monophagous, preying specifically on the pest, whilst generalist predators are polyphagous and have a variety of prey that includes the pest (Hassell and May 1986). Some specialist predators that have been used to control RWA infestations are ladybugs (Coccinellidae), green lacewings (Chrysopidae) and hover flies (Syrphidae), whilst various spider (Araneae) and beetle (Carabidae and Staphylinidae) species act as generalist predators (Kauffman and LaRoche 1994; Bergeson and Messina 1998; Brewer and Elliott 2004). In addition to the use of predators, *Aphelinus hordei*, an exotic aphid parasitoid from

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Ukraine was released into South Africa to help counter aphid infestation (Prinsloo *et al*. 2002). Entomopathogenic fungi can be used to create mycoinsecticides such as Mycotrol® ES containing the hyphomycete *Beauveria bassiana*, that has been used with some success to control RWA infestation of resistant wheat cultivars in South Africa (Hatting *et al*. 2004).

2.3.3 Chemical control

Imidacloprid and pymetrozine are prominent examples of insecticides used to control RWA infestation (Burd *et al*. 1996; Tolmay *et al*. 1997). The use of imidacloprid, a neonicotinoid, resulted in higher wheat yields of the varieties "Gamtoos" and "Gamtoos DN", when compared with untreated plants (Tolmay *et al*. 1997). Neonicotinoids assert their effect by acting on the post-synaptic nicotinic acetylcholine receptors of the nervous system, causing paralysis and ultimately aphid death (Dewar 2007). Pymetrozine exhibits an irreversible antifeedant effect on aphids, causing them to die of starvation. It does this by affecting the nerves controlling the salivary pump (Dewar 2007). Burd *et al*. (1996) observed that pymetrozine treatment of RWA led to an increase in non-probing aphid activities, and to shorter intervals of ingestion. Methods of insecticide application include directly coating seeds, drenching the soil in which seeds are planted or spraying the host plant at certain growth stages (Burd *et al*. 1996; Hatting *et al*. 2004; Dewar 2007).

2.3.4 Drawbacks of cultural, biological and chemical control

The management of a crop environment is seen as an ecofriendly manner to reduce aphid infestations (Wratten *et al*. 2007), which is often cheaper than chemical, biological or genetic control methods (Rebek *et al*. 2012). It is, however, sometimes difficult to predict the effect that farming practices will have on aphid populations. The effects of such practices may only become clear upon implementation, and monitoring their efficacy is challenging.

The aphid-induced change in plant architecture that manifests as leaf rolling, presents a challenge for both biological and chemical control (Clark and Messina 1998). This phenotypic symptom of aphid feeding makes it difficult for aphids to be reached by either predators or chemical agents, as

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they remain enclosed and protected by the folded wheat leaves (Wraight *et al*. 1993; Kauffman and LaRoche 1994; Tolmay *et al*. 2000; Basky 2003). A further disadvantage of biological control is that the use of predators can result in increased mortality of non-target arthropods, especially if the predators are generalist in nature. The presence of co-occurring, but non-target species can also decrease the effectiveness of the predator as a means of control. For example, Bergeson and Messina (1998) found that the co-occurrence of RWA and *Rhopalosiphum padi* rendered the use of lacewings less effective in controlling RWA, as they consumed more *R. padi*.

Disadvantages associated with chemical control include the detrimental effect of insecticides on non-target and/or beneficial insects, on the environment and human health, and the development of aphid resistance (Wraight *et al*. 1993; Burd *et al*. 1996; Dewar 2007; Dedryver *et al*. 2010). It is also difficult to predict the magnitude of aphid damage at the time of sowing seeds, meaning that insecticides are often used prophylactically, as is the case with seed treatment and soil drenching (Dewar 2007; Dedryver *et al*. 2010). Although sometimes necessary, this incurs large economic costs and poses a greater threat to the environment than, for example, spraying only the plants that become infested with aphids (Dewar 2007; Dedryver *et al*. 2010).

2.3.5 Host plant resistance

The introgression of genes which confer resistance to RWA into host plants (known as host plant resistance or HPR) is an attractive alternative to the former methods of minimising and regulating aphid infestations. Host plant resistance is more cost-effective because aphid control is incorporated into the cost of the seed (Smith and Clement 2012), and safer as it decreases the need for insecticide use (Smith *et al*. 2004; Dedryver *et al*. 2010).

The co-evolution of certain aphid biotypes and grass species (Kellogg 1998; Botha 2013) led to the development of progenitor grass species containing genes (*Dn* genes) which naturally confer resistance to particular aphid biotypes. By selectively crossing these progenitor grass species with locally adapted wheat varieties, wheat cultivars have been established that contain one or a number of *Dn* genes (Du Toit 1989a) and confer resistance to certain biotypes. Host plant resistance has

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been used effectively in both the USA and South Africa to manage and decrease aphid infestations (Porter *et al*. 2009). The main threat to the efficacy of HPR is the relatively fast development of new aphid biotypes that can break down the resistance of *Dn* genes of currently available resistant wheat cultivars, decreasing the period for which these cultivars are effective (Botha *et al*. 2005, 2010; Tagu *et al*. 2008; Sinha and Smith 2014). However, even with the development of new biotypes, HPR still forms the cornerstone of most IPM programs (Tolmay *et al*. 1997; Smith and Clement 2012; Sinha and Smith 2014).

2.4 RWA-wheat interaction

2.4.1 Non-host (basal) resistance of wheat

Plant resistance is broadly divided into two categories, non-host resistance and host resistance (Neu *et al*. 2003). Non-host resistance, also referred to as basal resistance, provides the first line of defence for wheat and is induced by non-specific stimuli, including abiotic stresses and the attack of non-specific pests or pathogens (Neu *et al*. 2003; Botha *et al*. 2005). Attack by pests and pathogens leads to damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) respectively (Chisholm *et al*. 2006; Lotze *et al*. 2007; Botha *et al*. 2014a). Basal resistance mechanisms include the release of preformed allelochemicals into damaged tissues and have either a toxic or an antifeedant effect on the attacking pest/pathogen (Botha *et al*. 2005, 2014a; Smith and Clement 2012). The presence of the cell wall which forms a barrier of protection around the plasma membrane, is also a mechanism of basal resistance (Botha *et al*. 2005, 2014a; Smith and Clement 2012). Together these chemical and structural mechanisms afford wheat plants the ability to negatively affect invaders and to withstand certain abiotic stresses.

2.4.2 Host (specific) resistance of wheat

Host resistance is a specific resistance response that ensues when a wheat resistance (R) protein recognises an effector/avirulence (avr) factor released from a host-specific pest (Neu *et al*. 2003; Botha *et al*. 2005). The interaction between aphids and wheat falls under this category of wheat

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resistance, with aphids releasing/depositing salivary effectors in the form of avr proteins into the plant upon feeding (Walling 2008; Botha *et al*. 2014a). Most aphid avr proteins interact with complementary R proteins, coded for by wheat *Dn* genes, which are constitutively expressed in all cells that could potentially be damaged by aphid feeding (Van der Biezen and Jones 1998). This is known as an incompatible interaction and occurs in resistant cultivars, resulting in the initiation of a signaling cascade that leads to a successful plant defence response (Botha *et al*. 2005, 2006).

In susceptible wheat cultivars however, the host lacks the complementary R protein, which allows the avr protein to act as a virulence factor (Van der Biezen and Jones 1998), and the aphid to overcome the wheat defence response or to avoid the initiation thereof (Botha *et al*. 2005). This is termed a compatible interaction and results in plant disease symptoms and effective manipulation of the plant by the aphid (Botha *et al*. 2005; Smith and Clement 2012).

The long-standing gene-for-gene model (Flor 1971) is one of two models/hypotheses that have been proposed to explain the mechanism of resistance that occurs during incompatible interactions, whereby the protein product of a single *R* gene from wheat recognises the protein from a single aphid *avr* gene. The guard hypothesis, an improved hypothesis, posits that a more complex interaction takes place than that of just two complementary gene products, and that R proteins recognise avr factors indirectly (Van der Biezen and Jones 1998; Dangl and Jones 2001; Botha *et al*. 2005; Jones and Dangl 2006). According to this hypothesis, a large resistance protein complex exists which contains the *R* gene product and effector target proteins (Dangl and Jones 2001). The R protein of this complex has a surveillance role and acts as a mediator to detect the binding of avr proteins to host target proteins, upon which a resistance response is initiated (Van der Biezen and Jones 1998; Dangl and Jones 2001; Botha *et al*. 2005). Owing to the very specific resistance response of some of the *Dn*-containing wheat cultivars (e.g., *Dn2* and *Dn5*) to the effectors of different aphid biotypes, the gene-for-gene hypothesis best describes the wheat-RWA incompatible interaction (Botha *et al*. 2005, Lapitan *et al*. 2007; Tagu *et al*. 2008). However, it is not yet clear if

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all R proteins interact directly with aphid effectors. For example, a study by Zaayman *et al*. (2009) suggests that *Dn7* acts as a surveillance R protein and conforms to the guard hypothesis.

2.4.3 Modes of resistance

A single *Dn* gene can confer one or a combination of the three well-described modes of resistance employed by plants to combat/counter aphid feeding (Smith *et al*. 1992). *Dn1*, *Dn5* and *Dn2*-containing cultivars respectively afford antibiosis, antixenosis and tolerance as their predominant mode of resistance, with *Dn5*-containing cultivars also exhibiting some level of antibiosis (Du Toit 1989b; Budak *et al*. 1999; Wang *et al*. 2004; Botha *et al*. 2014b). Antibiosis occurs when resistant plants have a negative effect on aphid biology, often seen as a decrease in aphid fecundity or an increase in aphid mortality (Painter 1958). Upon recognition of the avr protein in antibiotic-conferring cultivars, the hypersensitive response ensues resulting in symptomatic necrotic lesions (Botha *et al*. 2014b). This involves the increased production of reactive oxygen species that can injure the aphid directly, or indirectly by damaging the dietary compounds the aphids ingest (Botha *et al*. 2005, 2014b). With antixenosis, also known as non-preference, the resistance response of the wheat cultivar makes it undesirable for RWA to feed, seek shelter or reproduce on these cultivars (Painter 1958). This response appears to be linked to an increase in the production of volatile organic compounds (VOC). Evidence for this was provided by Botha *et al*. (2008, 2014b) who found that O-methyltransferase and β-glucosidase, both previously shown to be involved in VOC production (Lam *et al*. 2007; Morant *et al*. 2008), were up-regulated only in the antixenotic conferring *Dn5*-containing cultivar.

Tolerance is the ability of a resistant plant to withstand levels of aphid infestation that would severely harm a susceptible plant (Painter 1958). It is characteristic for tolerant plants to have normal heights despite aphid feeding (Botha *et al*. 2014b). Unlike antibiosis and antixenosis that involve the active production of compounds to lessen or deter aphid feeding, tolerance is a passive resistance mechanism that opposes chlorophyll damage incurred upon aphid feeding (Botha *et al*. 2008, 2014b). Chlorophyll breakdown, and specifically photosystem II damage (Burd and Elliott

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1996; Heng-Moss *et al*. 2003), is a known symptom of aphid feeding, seen as severe chlorosis in susceptible cultivars, and present to a lesser extent in antibiotic and antixenotic cultivars (Heng-Moss *et al*. 2003; Wang *et al*. 2004). Infested tolerant cultivars maintain a stable chlorophyll content (similar to or higher than their uninfested counterparts) (Heng-Moss *et al*. 2003), and display very limited chlorosis (Wang *et al*. 2004). Tolerant cultivars are thus able to compensate for chlorophyll loss/damage by up-regulating photosynthetic machinery genes (Heng-Moss *et al*. 2003; Wang *et al*. 2004; Botha *et al*. 2006, 2012, 2014b).

2.5 RWA feeding and effectors

Russian wheat aphids feed by inserting their stylet mouthpart into the leaves of their host and then manoeuvre their stylet intercellularly until it reaches and punctures the sieve elements of the plant, from which they ingest phloem (Tjallingii 2006; Cooper *et al*. 2010; Carolan *et al*. 2011). Russian wheat aphids attempt to avoid detection by host plants by minimising plant tissue damage during feeding, and if detected, suppress plant wound responses (Tjallingii 2006; Will and van Bel 2006; Botha *et al*. 2014a). A prominent plant defence mechanism in response to sieve element damage is the occlusion of sieve plates, either through the deposition of callose around sieve pores or via protein plugging of these pores (Tjallingii 2006; Will and van Bel 2006; Furch *et al*. 2008). Both occlusion mechanisms are triggered by an increase in calcium in the wounded sieve elements and impede the flow of phloem, in effect depriving aphids of their source of nutrients (Will and van Bel 2006; Will *et al*. 2007).

The composition of aphid saliva however, enables aphids to avoid or suppress sieve plate occlusion by either preventing calcium influx into sieve elements or by chelating free calcium (Will and van Bel 2006). Aphids secrete two types of saliva, namely sheath (gelatinous) and watery saliva. The former is secreted upon stylet insertion into the leaf and quickly hardens to form a protective sheath around the stylet (Tjallingii 2006; Will *et al*. 2007). Another function of gelatinous saliva is to seal the wound site, preventing calcium influx into sieve tubes, and calcium-associated sieve plate occlusion (Will and van Bel 2006; Will *et al*. 2007). Watery saliva is secreted at the beginning of,

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and during what is referred to as the phloem phase, the period of phloem ingestion (Tjallingii 2006). At the start of the phloem phase, watery saliva is secreted into the sieve element, where it binds with free calcium, helping to prevent occlusion (Will and van Bel 2006; Will *et al*. 2007). During phloem sap ingestion watery saliva is continuously secreted and mixed with the phloem that is being ingested, aiding in the digestion of plant defence toxins and preventing the coagulation of phloem proteins in the stylet (Tjallingii 2006; Carolan *et al*. 2011; Nicholson *et al*. 2012).

The success with which aphid biotypes counter mechanical plant defences like occlusion during feeding, is a possible reason for their differential ability to affect wheat hosts (Tjallingii 2006). This is supported by the work of Sinha *et al*. (2016) who found that RWA biotype US2, which is virulent to *Dn4*-containing wheat cultivars, up-regulates transcripts involved in calcium signaling, thereby activating phosphoinositide metabolism and resulting in the removal of free calcium. This was in comparison to RWA biotype US1 which is avirulent to *Dn4*-containing cultivars. Another cause of differing virulence levels could be different compositions of gut peptides, as these are known to be involved in the detoxification of ingested plant toxins (Anathakrishnan *et al*. 2014).

Despite the ability of saliva to prevent aphid detection during feeding, it also contains effectors which, when detected by wheat *Dn* proteins, result in incompatible interactions, and subsequent resistance to aphids (Walling 2008; Botha *et al*. 2014a). Although no aphid effectors have been identified (Botha *et al*. 2005; Smith and Clement 2012), it is possible that distinct biotypes secrete different salivary effectors, causing them to express different levels of virulence and to affect wheat hosts differently. Two important discoveries relating to RWA effectors were made by Lapitan *et al*. in 2007. Firstly, Lapitan and her colleagues determined that RWA effectors are proteinaceous in nature, and secondly, that chitin, a main component of the RWA exoskeleton, does not act as an effector. It thus follows that the aphid saliva, being the only other part of the aphid to come into contact with the host, contains the proteinaceous effectors. A co-evolutionary arms race exists between RWA effectors and wheat resistance genes, in which the effectors evolve to avoid recognition by the *Dn* genes, which themselves evolve to recognise the adapting effectors (Botha

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2013). The effectors of the South African biotype SAM have evolved in such a way that they are not detected by *Dn* genes, and act as virulence factors, allowing SAM to effectively avoid host plant defences and resulting in a continuous supply of phloem (Botha *et al*. 2014a).

2.6 The development of new aphid biotypes

To aid the development of wheat cultivars that are resistant to a greater number of aphid biotypes, thus providing more durable resistance, the molecular mechanism underlying aphid virulence toward their wheat host needs to be elucidated, and the driving factors behind aphid biotypification determined. Possible factors influencing biotypification include, but are not limited to, alterations or mutations that arise in the genome of the aphid itself, sequence variation of the endosymbiont housed by the aphid, and differences in gene regulation brought about by DNA methylation.

Russian wheat aphids are able to reproduce both sexually and asexually, with their mode of reproductive strategy directly affecting the amount of genetic variation that occurs within a population (Ricci *et al*. 2011). Genetic recombination of sexually reproducing populations is a source of genetic variation that could provide the basis for aphid biotypification. South African RWA are, however, wholly anholocyclic with no males present (Ricci *et al*. 2011). They reproduce via a female-driven parthenogenesis, a type of asexual reproduction specifically suited to RWA in regions like South Africa, that have mild winters and are warm all year round (Puterka *et al*. 2012). Owing to the nature of RWA reproduction in South Africa, it is unlikely that biotype development has a large genetic component. Indeed, a recent investigation into genetic variation between SAM, and its parent biotype SA1, revealed only a 0.0008% variation in protein-coding gene sequences between these two biotypes, exemplifying the very limited genetic variation present (Burger and Botha 2017). Furthermore, some of this limited genetic variation may have arisen as a result of chromosome fragmentations, which are known to occur in other aphid species such as the peach potato aphid, *Myzus persicae* (Monti *et al*. 2012).

Russian wheat aphids have a mutualistic symbiotic relationship with the bacterial endosymbiont *Buchnera aphidicola*, which inhabit specialised aphid cells called bacteriocytes (Baumann *et al*.

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1995). The need for this relationship, from the aphid's viewpoint, arises from the fact that nutrient-rich phloem, whilst providing ample amounts of sugar, is a problematic food source due to its unbalanced composition of nitrogen-containing amino acids (Sandström and Moran 1999; Douglas 2006; Tagu *et al*. 2008). Phloem contains both essential and non-essential amino acids, with the latter being present in much higher amounts. Douglas (2006) reported that the ratio of essential to non-essential amino acids of phloem can be as high as 1:20. This poses a challenge for aphids, which require all 20 amino acids for protein synthesis, but are unable to produce the nine essential amino acids present in low amounts in phloem (Douglas 2006). *Buchnera aphidicola* contains genes to produce the essential amino acids, thus providing the aphid access to a full suite of amino acids (Baumann *et al*. 1995; Sandström and Moran 1999; Moran *et al*. 2005). Mutations in the *B. aphidicola* genome could result in the development of aphid populations able to feed on previously resistant wheat cultivars, i.e., new biotypes (Botha 2013). Swanevelder *et al*. (2010) investigated the extent of variation of the *B. aphidicola* genome of various RWA biotypes of South African and American origin, by sequencing *B. aphidicola*'s leucine plasmid. The only sequence difference identified between *B. aphidicola* sequences of the RWA biotypes was a CCC insert in the leucine plasmid of some biotypes, albeit not different between SA1 and SAM. This limited genetic variation has thus not influenced the development of the South African RWA biotypes to date. Although it cannot be ruled out as a cause of future biotype development, the Swanevelder study indicates that it is unlikely to be very influential (Swanevelder *et al*. 2010).

DNA methylation, one of various epigenetic modifications, is a potential mechanism through which biotypification could be mediated. It has been shown to influence evolution in both plants and animals, with natural selection acting on differentially methylated individuals, and presumably selecting for the methylation level that imparts the highest level of fitness (Kalisz and Purugganan 2004; Rapp and Wendel 2005; Xiang *et al*. 2010; Zeng *et al*. 2012). The differential addition/removal of methyl groups affords additional regulation at the level of gene expression, without changing the underlying DNA sequence (Feng *et al*. 2012; Hunt *et al*. 2013b; Schulz *et al*.

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2013). This methylation-mediated regulation of gene expression occurs in response to various external (environmental) and internal signals/stimuli and can result in a competitive advantage for some individuals, leading to regulatory evolution and speciation (Rapp and Wendel 2005; Bonasio *et al*. 2010; Feil and Fraga 2012; Foret *et al*. 2012; Zeng *et al*. 2012; Yan *et al*. 2015).

Given that a number of biotypes have developed over a short evolutionary timespan (SA2, SA3 and SA4 biotypes between 1978 and 2011 (Tolmay *et al*. 2007; Jankielsohn 2011, 2014), and SAM, which developed over 87 generations from SA1 (Van Zyl 2007)), with only limited genetic differences, DNA methylation might be driving regulatory changes/evolution, which better equip aphids to confront or avoid the initiation of plant defences. DNA methylation of numerous insects including bees (Kucharski *et al*. 2008; Lyko *et al*. 2010; Foret *et al*. 2012), locusts (Boerjan *et al*. 2011; Robinson *et al*. 2016), ants (Bonasio *et al*. 2012) and wasps (Weiner *et al*. 2013), is reportedly associated with phenotypic plasticity that can arise despite a fixed genotype. In much the same way, DNA methylation could be associated with biotype development in aphids with limited genetic variation.

2.7 DNA methylation, a widespread epigenetic modification

Epigenetics is defined as the regulation of, or changes in gene expression, which are mediated through DNA methylation, histone modification, chromatin re-modelling and non-coding RNA activity (Jeltsch 2002; Foret *et al*. 2012; Roberts and Gavery 2012; Mukherjee *et al*. 2015). The stable modifications brought about by the aforementioned mechanisms help shape the dynamic epigenome of a cell, are heritable, yet reversible (Drewell *et al*. 2012), and do not involve alterations of the primary nucleotide sequence (Weiner *et al*. 2013). Epigenetic modifications provide an additional layer of regulation and complexity above that which is dictated by the DNA sequence of the genome (Lyko and Maleszka 2011). Epigenomes vary between cells and tissue types, involve a complex interplay between their constituents and environmental cues, and confer a certain amount of flexibility to organisms (Jones and Takai 2001; Suzuki and Bird 2008; Foret *et al*. 2012).

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DNA methylation is a well-studied epigenetic modification present in both prokaryotes and eukaryotes, and occurs at cytosine and adenine residues in the former (Klose and Bird 2006, Bogdanović and Veenstra 2009). There is now clear evidence that adenine methylation also occurs in plants (Vanyushin 2005; Ratel *et al*. 2006; Vanyushin and Ashapkin 2011) and may be present in animals (Vanyushin 2005; Ratel *et al*. 2006). However, taking into account the fact that methylation in animals occurs almost exclusively at cytosines (Glastad *et al*. 2011), DNA methylation for the purposes of this study is defined as follows: the covalent addition of a methyl group, donated by S-adenosyl-L-methionine, to the 5' position of cytosine residues, predominantly, but not exclusively in the CG dinucleotide context (Attwood *et al*. 2002; Glastad *et al*. 2011; Lyko and Maleszka 2011). The resulting 5-methylcytosine (5mC) has been referred to as the fifth base, highlighting the significance of this base modification (Lister and Ecker 2009).

2.8 DNA methyltransferases catalyse DNA methylation

DNA methyltransferases (DNMTs) are the conserved group of proteins responsible for catalysing DNA methylation (Goll and Bestor 2005). They are separated into three subfamilies namely, DNMT1, DNMT2 and DNMT3, on the basis of sequence homology and the nature of their activity (Kunert *et al*. 2003; Glastad *et al*. 2011). Based on an early study of *Apis mellifera* DNMTs (Wang *et al*. 2006), the functions of insect DNMTs are assumed to be the same as the mammalian orthologues, which have been functionally characterised (Goll and Bestor 2005; Glastad *et al*. 2014).

The semiconservative nature of DNA replication results in hemimethylated double-stranded DNA, meaning that only one of the DNA strands, in this case the parental strand, contains methylation (Deobagkar *et al*. 1990; Jeltsch 2002; Goll and Bestor 2005; Fulneček and Kovařík 2014). This hemimethylated DNA acts as a preferential substrate for proteins of the DNMT1 subfamily (Yoder *et al*. 1997; Hermann *et al*. 2004; Goll and Bestor 2005; Glastad *et al*. 2011), known as maintenance methyltransferases, the function of which is to copy the methylation pattern from the parental to the daughter strand (Kunert *et al*. 2003; Goll and Bestor 2005; Schaefer and Lyko 2007; Glastad *et al*.

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2011; Lyko and Maleszka 2011). In this manner, the pre-existing methylation pattern is accurately maintained during cell division (Glastad *et al*. 2011, 2014). In addition to exhibiting substrate specificity, DNMT1 proteins also exhibit sequence specificity, almost always methylating cytosines in the CG dinucleotide context (Araujo *et al*. 2001; Goll and Bestor 2005; Feng *et al*. 2010).

There is some controversy surrounding the function of the DNMT2 subfamily proteins as they have been shown to be involved in both DNA and RNA methylation (Goll *et al*. 2006; Jeltsch *et al*. 2006; Schaefer and Lyko 2007, 2010). Studies on the fruit fly *Drosophila melanogaster*, which contains a *DNMT2* homologue but lacks homologues of *DNMT1* and *DNMT3*, suggest that DNMT2 proteins methylate DNA in a wide variety of sequence contexts, perhaps even all dinucleotide contexts (Kunert *et al*. 2003; Phalke *et al*. 2009). The RNA methyltransferase activity of *Drosophila* DNMT2 proteins, and perhaps DNMT2 proteins of other insects, is specific to cytosine 38 of the aspartate, glycine and valine transfer RNAs (tRNAs) (i.e., $tRNA^{Asp}{}_{GUC}$, $tRNA^{Gly}{}_{GCC}$ and $tRNA^{Val}{}_{AAC}$) (Goll *et al*. 2006; Schaefer *et al*. 2010).

DNA methyltransferase 3 proteins are *de novo* methyltransferases which methylate unmethylated and hemimethylated DNA at equal rates (Okano *et al*. 1998; Goll and Bestor 2005; Jones and Liang 2009), predominantly in the CG context (Okano *et al*. 1998; Goll and Bestor 2005). Yokochi and Robertson (2002), however, claim that DNMT3A (one of the two mammalian *de novo* methyltransferases, the other being DNMT3B (Goll and Bestor 2005)) preferentially methylates unmethylated DNA, and Ramsahoye *et al*. (2000) found that DNMT3A is capable of methylating cytosines in non-CG contexts. DNA methyltransferase 3 proteins play an important role in establishing new methylation patterns during development, and are responsive to environmental stimuli (Goll and Bestor 2005; Schaefer and Lyko 2007; Glastad *et al*. 2011, 2014; Zhang *et al*. 2015; Standage *et al*. 2016).

2.9 Expansion or contraction of *DNMT* gene sets

During the course of evolution the complement of *DNMTs* of different insect lineages has either expanded through the development of certain homologues such that there is more than one

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representative for a *DNMT* subfamily, or contracted through the loss of homologues or entire *DNMT* subfamilies (Goll and Bestor 2005; Glastad *et al*. 2011; Lyko and Maleszka 2011). Invertebrates display a wide range of *DNMT* complements, as evidenced by the fact that no standard set of *DNMTs*, representative of all invertebrates, exists (Lyko and Maleszka 2011). As one would expect, the *DNMT* subfamilies present in an organism affect the overall methylation status of the organism, including the level and sequence specificity of methylation. Traditionally, organisms with at least one *DNMT1* and *DNMT3* representative were considered to have a fully functional methylation system, being able to both establish and maintain DNA methylation patterns (Feliciello *et al*. 2013; Glastad *et al*. 2014).

However, as more insect genomes have been sequenced it has become clear that a number of insect genomes, including that of the silkworm, *Bombyx mori* and the red flour beetle, *Triboleum castaneum*, lack *DNMT3* homologues (Figure 2.2) (Xiang *et al*. 2010; Feliciello *et al*. 2013). The genomes of *D. melanogaster* and the mosquito, *Anopheles gambiae*, represent a particularly interesting case in that they only contain a *DNMT2* homologue (Figure 2.2) (Kunert *et al*. 2003; Marhold *et al*. 2004). Despite this, *D. melanogaster* and *A. gambiae* do exhibit DNA methylation (Gowher *et al*. 2000; Lyko *et al*. 2000; Kunert *et al*. 2003; Marhold *et al*. 2004; Capuano *et al*. 2014; Panikar *et al*. 2015). The presence of DNA methylation in insects without a homologue of each *DNMT* indicates that there are still knowledge gaps in the understanding of the intricacies of DNA methylation. This raises questions such as which gene (genes) mediates maintenance and *de novo* methylation in the absence of *DNMT1* and *DNMT3*? (Feliciello *et al*. 2013; Glastad *et al*. 2014).

DNA methylation in the pea aphid, *A. pisum* was reported in 2007 by Mandrioli and Borsatti. In 2010 the International Aphid Genomics Consortium published the genome sequence of the pea aphid and the confirmation of a functional DNA methylation system followed later that year (Walsh *et al.* 2010). Homologues of all three *DNMT* subfamilies were identified in the pea aphid genome, with two genes each encoding DNMT1 and DNMT3 proteins, and one gene encoding a DNMT2

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protein, illustrated in Figure 2.2. The recent sequencing of the closely related *D. noxia* genome (Nicholson *et al*. 2015; Burger and Botha 2017) will enable the complement of RWA *DNMT* genes to be determined.

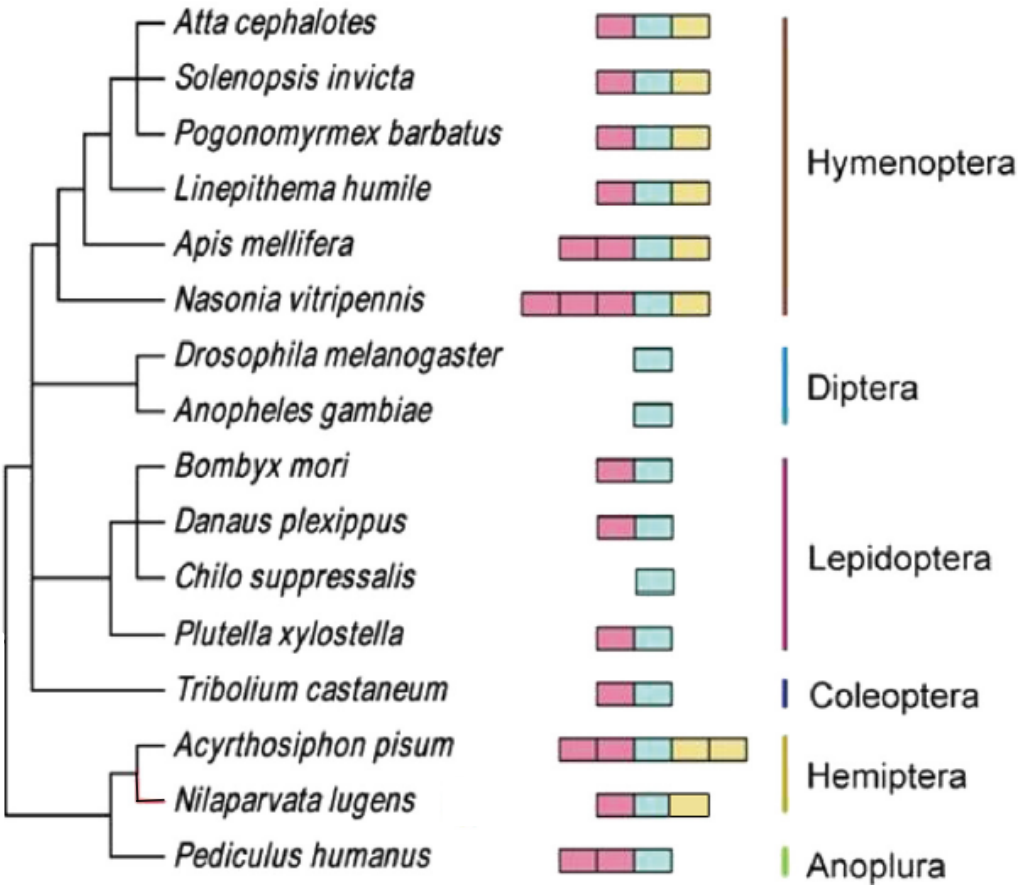

Figure 2.2. Phylogenetic distribution of insect DNA methyltransferases. Red, blue and yellow rectangles indicate the number of *DNMT1*, *DNMT2* and *DNMT3* genes respectively. Figure sourced from Zhang *et al*. (2015).

2.10 Methylation distribution

Although *DNMT* complements differ between insect taxa, the distribution of methylation in most insects studied remains highly conserved, and is markedly different from that of vertebrates (Glastad *et al*. 2014). Vertebrate genomes are globally methylated, meaning that methylation occurs in genes, transposons and intergenic regions (Bird 2002; Suzuki and Bird 2008).

DNA methylation of insects (illustrated in Figure 2.3) is targeted to actively transcribed genes where it is present in both exons and introns (Suzuki and Bird 2008; Zemach *et al*. 2010; Glastad *et al*. 2011). Exons are the principal site of methylation, and those at the 5' region of genes are

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particularly rich in methylation (Elango *et al*. 2009; Feng *et al*. 2010; Zemach *et al*. 2010; Hunt *et al*. 2013a). Introns have lower levels of methylation that is localised near exon-intron boundaries (Feng *et al*. 2010; Glastad *et al*. 2014).

Transposons and other repetitive elements, as well as intergenic regions and promoters, are largely unmethylated (or sparsely methylated) in insects, resulting in a mosaic pattern of methylation characterised by areas of dense methylation interspersed with areas of no methylation (Suzuki and Bird 2008; Zemach *et al*. 2010; Lyko and Maleszka 2011; Glastad *et al*. 2014). Methylation has also been shown to increase in a linear fashion with increasing distance from transposable elements (Zemach *et al*. 2010). Although repetitive elements of most insects are not methylated, those of locusts (*Schistocerca gregaria* and *Locusta migratoria*), ants (*Camponotus floridanus* and *Harpegnathos saltator*) and the stick insect *Medauroidea extradentata*, exhibit methylation (Krauss *et al*. 2009; Robinson *et al*. 2011, 2016; Bonasio *et al*. 2012; Falckenhayn *et al*. 2013).

Insect genes are not uniformly targeted for DNA methylation and fall into two categories in this regard. Genes that are ubiquitously (broadly) expressed across cell and tissue types or different phenotypes are preferential targets of insect methylation (Elango *et al*. 2009; Foret *et al*. 2009; Hunt *et al*. 2010; Xiang *et al*. 2010; Glastad *et al*. 2011, 2014). In contrast, genes that are narrowly, or differentially expressed in specific cells, tissues, phenotypes or developmental stages are less likely to be methylated, or have low levels of methylation (Foret *et al*. 2009; Hunt *et al*. 2010; Glastad *et al*. 2011, 2014).

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(Mandrioli and Volpi 2003), insects tend to have very low levels of overall methylation. These levels sit around 0.1% for *A. mellifera* (Lyko *et al*. 2010), *B. mori* (Xiang *et al*. 2010) and the ants *C. floridanus* and *H. saltator* (Bonasio *et al*. 2012), around 1.3% for *S. gregaria* (Falckenhayn *et al*. 2013) and 2.1% for the ant *Cerapachys biroi* (Libbrecht *et al*. 2016) (all quantified using BS-Seq). *Drosophila melanogaster's* levels range between 0% and approximately 0.5% depending on its life stage (quantified using a multitude of methods – Gowher *et al*. 2000; Lyko *et al*. 2000; Marhold *et al*. 2004; Zemach *et al*. 2010; Raddatz *et al*. 2013; Capuano *et al*. 2014; Panikar *et al*. 2015), and *Nasonia vitripennis'* have been reported as 0.18% and 1.45% by two different research groups using BS-Seq (Wang *et al*. 2013; Beeler *et al*. 2014).

2.12 Methylation sequence context

Cytosine methylation in vertebrates (Field *et al*. 2004) and invertebrates (Su *et al*. 2011), and in insects in particular (Lyko and Maleszka 2011) occurs predominantly in the CG dinucleotide context, in which a cytosine is followed immediately downstream by a guanine (Hunt *et al*. 2013b). Methylation does also occur in other nucleotide contexts, albeit less frequently. CC methylation has been detected within the 5' CCGG 3' nucleotide context in *M. brassicae* and there is a small amount of CA methylation in *A. pisum* (Field *et al*. 2004; Walsh *et al*. 2010). CA and CT methylation also occur in *M. extradentata*, and in *T. castaneum* and *Pogonomyrmex barbatus*, methylation is found in all dinucleotide contexts (Krauss *et al*. 2009; Smith *et al*. 2012; Feliciello *et al*. 2013). Russian wheat aphid methylation has been identified in multiple sequence contexts including CG, CHG, and CHH (H is A, C or T) (Gong *et al*. 2012). *Drosophila melanogaster*, unlike most insects, preferentially methylates its DNA in the non-CG dinucleotide context including both CT (the most common sequence context for *D. melanogaster* methylation) and CA dinucleotides (Bird 2002; Kunert *et al*. 2003; Field *et al*. 2004).

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2.13 Functions of methylation

2.13.1 Promoter methylation

A well-established function of DNA methylation is that of gene silencing, which forms the basis of a number of vertebrate gene regulatory systems, including X chromosome inactivation and chromosome imprinting (Bird 2002; Feng *et al*. 2010). The detrimental effect of transposable element activity is also repressed via gene silencing in both vertebrates and plants (Bird 2002; Zemach *et al*. 2010; Glastad *et al*. 2011).

When CpG islands (stretches of CGs) present in promoter regions are methylated, the transcription of the downstream gene is repressed in one of two ways. Firstly, the presence of methylation inhibits the binding of transcription factors to their recognition sequences in promoters, thus directly reducing gene expression (Attwood *et al*. 2002; Klose and Bird 2006). A more complex and indirect mechanism leading to gene silencing is the establishment of a repressive chromatin environment in the vicinity of DNA methylation (Bogdanović and Veenstra 2009). This occurs through the recruitment of repressive regulatory methyl-CpG-binding proteins, which through associations with other proteins including histone deacetylases, modify chromatin from a transcriptionally active to inactive form (Attwood *et al*. 2002; Klose and Bird 2006; Bogdanović and Veenstra 2009; Glastad *et al*. 2011).

2.13.2 Intragenic methylation

Gene body methylation is an evolutionarily conserved feature that is found in plants and animals (both vertebrates and invertebrates) (Feng *et al*. 2010; Zemach *et al*. 2010). Despite its conservation, the functions of intragenic methylation are not as apparent or well-defined as that of promoter methylation. The prevention of spurious transcription (and regulation of alternative promoters) (Bird 1995; Simmen *et al*. 1999; Mandrioli 2007; Suzuki *et al*. 2007; Zilberman *et al*. 2007; Maunakea *et al*. 2010; Jones 2012; Hunt *et al*. 2013b) and the regulation of alternative splicing (Lyko and Maleszka 2011; Shukla *et al*. 2011; Bonasio *et al*. 2012; Maunakea *et al*. 2013;

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Glastad *et al*. 2014; Yan *et al*. 2015) have garnered support as the two main functions of intragenic methylation.

The presence of promoters within genes, in addition to their canonical location upstream/5' of genes, is not uncommon (Bird 1995; Ayoubi 2003; Jones 2012), and the aberrant/spurious initiation of transcription from these alternative promoters can result in unwanted background transcriptional noise (Bird 1995). Intragenic methylation in mammals has been shown to regulate the use of alternative promoters (Maunakea *et al*. 2010), which may be mediated through a DNA methylation-induced increase of nucleosome compaction (Hunt *et al*. 2013a, 2013b). RNA polymerase II's traversal of nucleosomes can cause nucleosome eviction/turnover which exposes previously histone-associated DNA to transcription factors (Zilberman *et al*. 2007; Hunt *et al*. 2013a, 2013b). This could give rise to aberrant transcription, if an alternative promoter or cryptic binding site lies in the exposed regions (Zilberman *et al*. 2007; Hunt *et al*. 2013a). Intragenic methylation thus prevents spurious transcription by causing tighter winding of DNA around histones, making nucleosomes harder to evict (Hunt *et al*. 2013b).

Strong evidence exists for a role of intragenic methylation in preventing spurious transcription in insects, and stems from the overlapping localities/distributions of methylation and RNA polymerase II (Hunt *et al*. 2013a). RNA polymerase II is most prevalent in exons and its concentration peaks just after the transcription start site (Yin *et al*. 2011; Hunt *et al*. 2013a). Similarly, exons are the predominant site of insect methylation, with those closer to the 5' region of genes being especially rich in methylation (Elango *et al*. 2009; Feng *et al*. 2010; Zemach *et al*. 2010; Hunt *et al*. 2013a). Methylation in these areas of high RNA polymerase II concentration may aid in increasing nucleosome compaction, thereby lessening nucleosome eviction and the exposure of cryptic binding sites or alternative promoters.

As mentioned in section 2.10, ubiquitously expressed genes, a category which includes house-keeping genes (Hunt *et al*. 2013a; Mejía-Guerra *et al*. 2015), are preferentially targeted by methylation. This makes sense, given the importance of the regulation of house-keeping gene

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expression, and the prevention of aberrant transcription at alternative promoters by DNA methylation. Hunt *et al*. (2010) refers to "enhanced negative effects" that are likely associated with spurious transcription in broadly expressed genes. Conversely, the presence of spurious transcripts arising from narrowly expressed genes (generally less methylated) may not be as detrimental, and it is possible that differences in gene expression between tissues or phenotypes could be attributed to these transcripts.

Intragenic DNA methylation has been linked to alternative mRNA splicing by directly affecting the function and binding of factors that are associated with splicing, by altering polymerase transit and by influencing exon definition (Lyko and Maleszka 2011; Shukla *et al*. 2011; Hunt *et al*. 2013a, 2013b; Glastad *et al*. 2014). Intragenic methylation can paradoxically cause either the inclusion or exclusion of exons by recruiting or interfering with different DNA-binding proteins, thus influencing the production of splice variants (Bonasio *et al*. 2012; Glastad *et al*. 2014; Yan *et al*. 2015).

RNA polymerase II pausing leads to the inclusion of exons, and is promoted by the binding of certain *trans*-acting factors such as the human CCCTC-binding factor (CTCF) (Figure 2.4A) (Luco *et al*. 2011; Shukla *et al*. 2011; Yan *et al*. 2015). Similarly, the binding of human methyl-CpG-binding protein 2 (MeCP2) is believed to decrease RNA polymerase II-mediated transcriptional elongation efficiency, again resulting in exon inclusion (Figure 2.4D) (Maunakea *et al*. 2013; Yan *et al*. 2015). However, these two binding factors bind DNA under different circumstances, with CTCF binding to unmethylated DNA and MeCP2 binding to methylated DNA. In the presence of methylation, CTCF cannot bind to the DNA, RNA polymerase II's traversal is not inhibited, and the exon is excluded (Figure 2.4B). The same is true for MeCP2 in the absence of methylation (Figure 2.4C).

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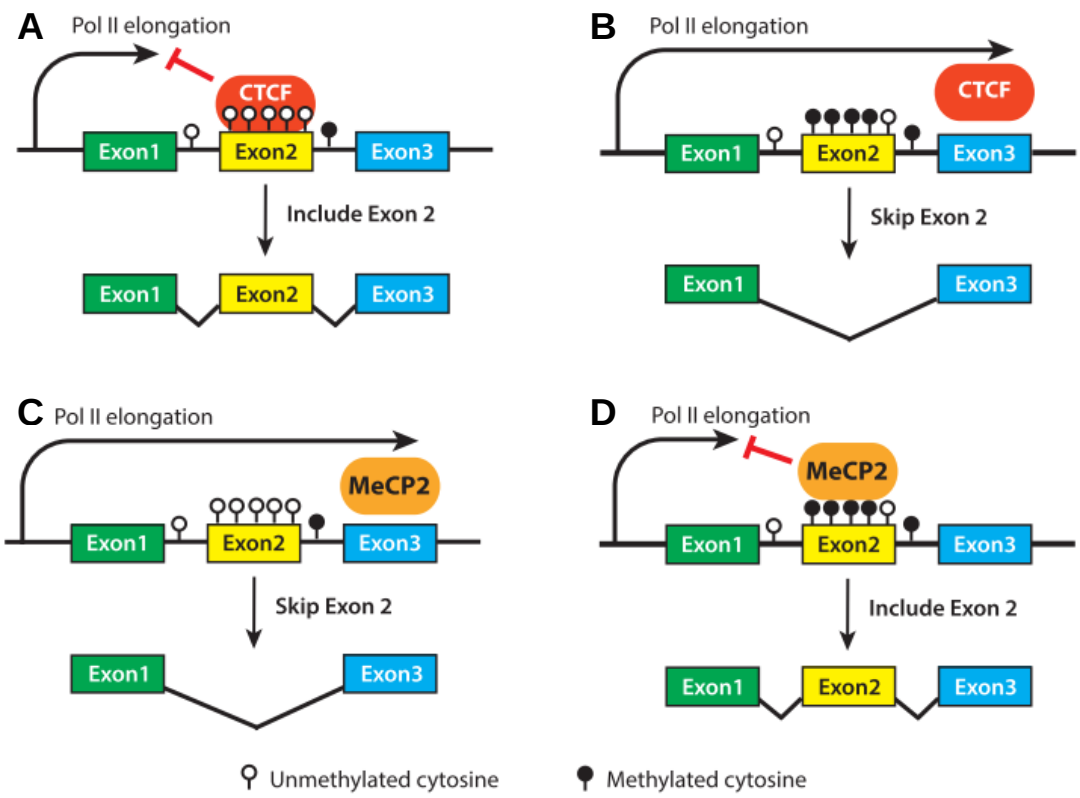

Figure 2.4. The effects of methylation on exon inclusion/exclusion. (A and B) Methylation interferes with CTCF binding. (A) CTCF binds to unmethylated exons causing RNA polymerase II stalling and exon inclusion. (B) CTCF is unable to bind to methylated exons, RNA polymerase II traverses the DNA uninhibited, and the exon is excluded. (C and D) Methylation recruits MeCP2. (C) In the absence of methylation, MeCP2 is not recruited to DNA, RNA polymerase II traverses the DNA uninhibited, and the exon is excluded. (D) Methylation recruits MeCP2 and RNA polymerase II-mediated transcriptional elongation efficiency is reduced, resulting in the inclusion of the exon. Figure sourced from Yan *et al*. (2015).

The effect of methylation on alternative splicing has been shown in the honey bee, where the knockdown of *DNMT3* via interfering RNA led to significant differences in alternative splicing (Li-Byarlay *et al*. 2013). Flores *et al*. (2012) also studied the relationship between methylation and alternative splicing in the honey bee and found that included exons were methylated to a higher degree than excluded/skipped exons, suggesting that the mediation of exon inclusion in the honey bee may be MeCP2 related. However, when alternative splicing of the honey bee *anaplastic lymphoma kinase* gene was investigated, hypomethylation, and possibly CTCF-binding, resulted in exon inclusion (Foret *et al*. 2012). Cingolani *et al*.'s (2013) findings are in line with the latter, where methylation is associated with exon skipping. Methylation has also been associated with alternative splicing in ants, termites and wasps (Park *et al*. 2011; Bonasio *et al*. 2012; Terrapon *et*

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al. 2014). In ants there are instances of both hypo- and hypermethylation leading to exon inclusion (Bonasio *et al*. 2012), and in the termite, *Zootermopsis nevadensis* and the honey bee, methylated genes were found to be enriched for alternative splicing (Flores *et al*. 2012; Terrapon *et al*. 2014).

2.13.3 Other functions of methylation

Early evidence of methylation in actively transcribed insect genes came from a study on *M. persicae*, in which resistance to various insecticides has been linked to the amplification of esterase genes. These amplified genes were shown to be methylated whilst the single copy of the gene in non-resistant aphids was unmethylated (Field *et al*. 1989, 2004).

Oppold *et al*. (2015) provided further evidence for a role of methylation in insecticide resistance by showing that changes in global methylation levels of the mosquito *Aedes albopictus* affect insecticide sensitivity. These two examples highlight the possible involvement of DNA methylation, either at the level of a single gene, or genome-wide, in adaptation to insecticides (Oppold *et al*. 2015).

Methylation also plays a role in *A. mellifera* memory and learning, *N. vitripennis* early embryonic development, *Nilaparvata lugens* (brown planthopper) fecundity and *A. pisum* pigmentation, growth rate and morph distribution (Dombrovsky *et al*. 2009; Lockett *et al*. 2010; Zwier *et al*. 2012; Zhang *et al*. 2015). In the citrus mealy bug *Planococcus citri*, the parental origin of chromosomes is marked by DNA (hypo)methylation, with paternally and maternally inherited chromosomes exhibiting different methylation levels (Bongiorni *et al*. 1999; Bongiorni and Prantera 2003).

Methylation is a mediator of phenotypic plasticity in numerous insects including locusts, where it regulates behavioural and neuronal differences associated with phenotypic plasticity in the form of phase polyphenism (Boerjan *et al*. 2011; Robinson *et al*. 2016). The finding that silencing *DNMT3* expression through RNA interference alters the developmental trajectory of honey bee larvae, implicates DNA methylation in the reproductive caste determination of honey bees (Kucharski *et al*. 2008). An involvement of methylation in caste determination is also seen in the ants *C. floridanus*

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and *H. saltator*, where certain genes are differentially methylated based on their caste (Bonasio *et al*. 2012). In the ant *C. biroi* however, no phase-related differentially methylated genes were detected, leading to the conclusion that methylation is not involved in behaviour or reproduction in this species (Libbrecht *et al*. 2016). The contribution of methylation to phenotypic plasticity in wasps is also disputed (Wang *et al*. 2013; Weiner *et al*. 2013; Standage *et al*. 2016).

2.14 DNA demethylation – restoring DNA to its unmodified state

Despite being a heritable epigenetic alteration, methylated DNA can be reverted to the unmethylated state through the process of demethylation (Gowher *et al*. 2000; Jair *et al*. 2006; Branco *et al*. 2012). Demethylation can occur both passively and actively (Wu and Zhang 2010; Branco *et al*. 2012; Kohli and Zhang 2013; Piccolo and Fisher 2014). Passive demethylation occurs during DNA replication if the pattern of methylation is not copied from the parent to the daughter strand through the action of the maintenance methyltransferase, DNMT1. Active demethylation involves the enzymatic modification or removal of the methyl group (Kohli and Zhang 2013; Piccolo and Fisher 2014). Numerous avenues of active demethylation have been identified (Figure 2.5), and have been studied more thoroughly in mammals and plants, than in insects (Wu and Zhang 2010; Branco *et al*. 2012; Piccolo and Fisher 2014). It was only in 2013 when a single ten-eleven translocation (TET) homologue (important for one of the active demethylation pathways) was identified in various insects (Cingolani *et al*. 2013; Dunwell *et al*. 2013; Feliciello *et al*. 2013). The active demethylation pathways mentioned below are thus described largely from a mammalian viewpoint.

Methylcytosine can be deaminated to thymine by the activation-induced cytidine deaminase (AID)/apolipoprotein B mRNA editing enzyme, catalytic polypeptide (APOBEC) family of cytidine deaminases (Morgan *et al*. 2004; Branco *et al*. 2012). Glycosylases such as thymine DNA glycosylase (TDG) and methyl-CpG-binding domain protein 4 (MBD4) recognise the deamination-induced T:G mismatch and remove the thymine base, following which, base excision repair (BER) machinery is employed to repair the resulting abasic site (Figure 2.5 – pathway 1) (Hendrich *et al*.

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1999; Morgan *et al*. 2004; Zhu 2009; Cortellino *et al*. 2011; Branco *et al*. 2012). It is interesting to note that MBD4 has a dual function, playing a role in both demethylation and methylation. Methyl-CpG-binding domain protein 4 functions as a glycosylase in the demethylation pathway, but by virtue of its ability to bind methylated CG sites, is also involved in establishing a repressive chromatin environment (Hendrich *et al*. 1999; Kondo *et al*. 2005; Bogdanović and Veenstra 2011; Branco *et al*. 2012).

Methylcytosine can also be hydroxylated by the TET enzymes to form 5-hydroxymethylcytosine (5hmC) (Figure 2.5 – pathway 2) (Tahiliani *et al*. 2009; Ito *et al*. 2010; Shen *et al*. 2014). The mammalian TET proteins (TET1, TET2 and TET3) were identified in 2009 (Tahiliani *et al*.), followed by the identification of a single TET orthologue in the insects *A. mellifera* (Cingolani *et al*. 2013; Wojciechowski *et al*. 2014), *D. melanogaster* (Dunwell *et al*. 2013), *T. castaneum* (Feliciello *et al*. 2013) and *N. vitripennis* (Pegoraro *et al*. 2016). Wojciechowski *et al*. (2014) found that like its mammalian counterpart, the single honey bee TET orthologue is able to hydroxylate 5mC to form 5hmC.

Hydroxymethylcytosine, like 5mC, is amenable to deamination by AID/APOBEC resulting in 5-hydroxymethyluracil (5hmU), which is acted on by glycosylases and BER (Figure 2.5 – pathway 2a) (Cortellino *et al*. 2011; Guo *et al*. 2011; Branco *et al*. 2012; Hashimoto *et al*. 2012). Alternatively, 5hmC can be further oxidised by TET enzymes to form 5-formylcytosine (5fC) and then 5-carboxylcytosine (5caC), both of which are removed through the action of glycosylases and BER (Figure 2.5 – pathway 2b) (He *et al*. 2011; Maiti and Drohat 2011; Branco *et al*. 2012). The mammalian *de novo* methyltransferases, DNMT3A and DNMT3B, have been shown to act as redox-dependent dehydroxymethylases *in vitro*, providing a third potential mechanism for 5hmC removal (Figure 2.5 – pathway 2c) (Chen *et al*. 2012).

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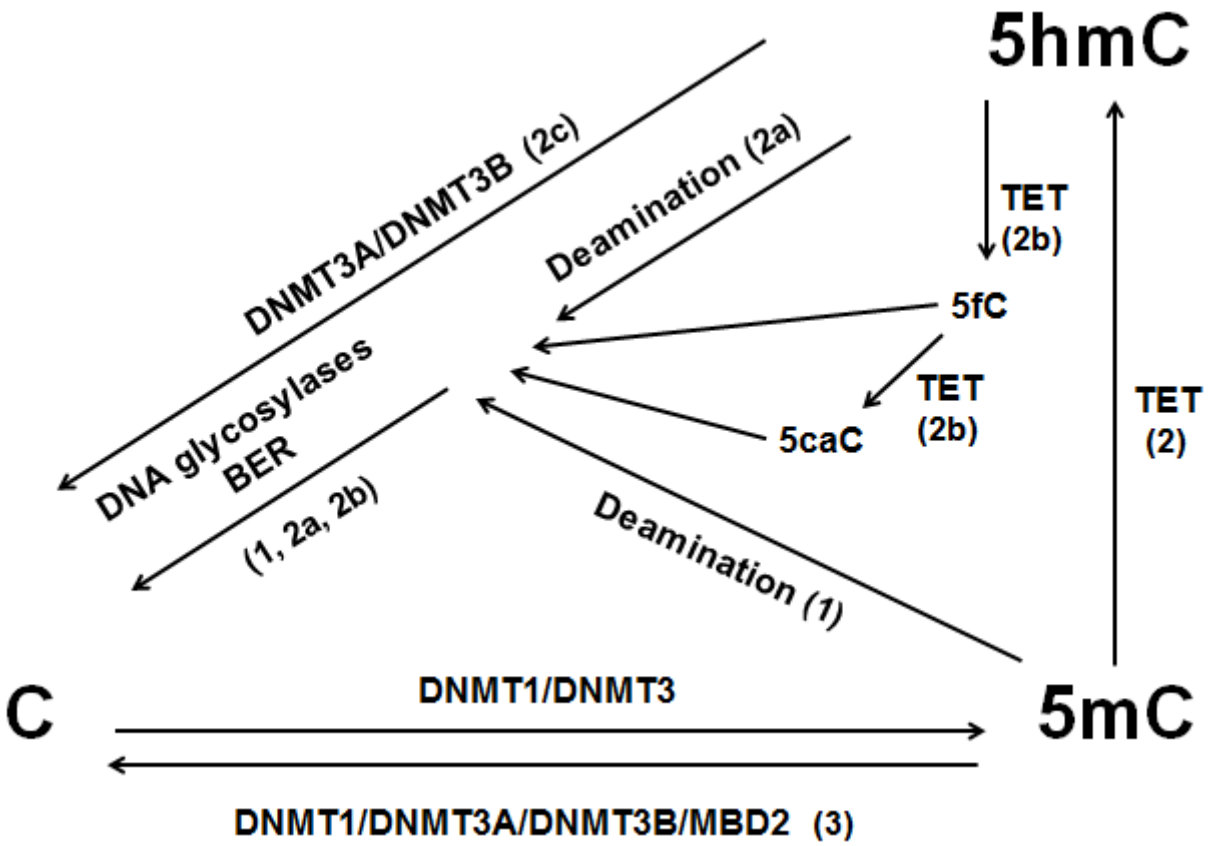

Figure 2.5. Active DNA demethylation pathways. Methylcytosine can be deaminated to thymine by the AID/APOBEC family of cytidine deaminases. The T:G mismatch is recognised by DNA glycosyases (TDG or MBD4), thymine is removed, and BER machinery repairs the abasic site (pathway 1). Methylcytosine can be converted to 5hmC by TET, and then to 5hmU by AID/APOBEC deaminases. Glycosylases and BER then act to replace 5hmU with cytosine (pathway 2a). Hydroxymethylcytosine can also undergo sequential oxidation to 5fC and 5caC by TET. These modified bases are recognised and replaced by TDG and BER (pathway 2b). Hydroxymethylcytosine can also be directly removed through the dehydroxymethylation activities of DNMT3A and DNMT3B (pathway 2c). Methylcytosine can be directly removed by the demethylase activities of DNMT1, DNMT3A, DNMT3B or MBD2 (pathway 3). Figure adapted from Chen *et al*. (2012).

In addition to being deaminated or hydroxylated, 5mC can also be directly converted back to cytosine through the activity of demethylases (Figure 2.5 – pathway 3). Methyl-CpG-binding domain protein 2 (MBD2) is believed to function dually as both a transcriptional repressor (Ng *et al*. 1999; Boeke *et al*. 2000; Feng and Zhang 2001; Sekimata *et al*. 2001) and a demethylase, capable of removing the methyl group from fully or hemimethylated DNA (Bhattacharya *et al*. 1999; Ramchandani *et al*. 1999; Detich *et al*. 2002). Although MBD2 has been shown to demethylate certain promoters, resulting in their transcriptional activation (Detich *et al*. 2002), its demethylase activity has been contested as it could not be reproduced by other research groups (Ng *et al*. 1999; Wade *et al*. 1999; Boeke *et al*. 2000). In addition to MBD2, the mammalian DNA

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methyltransferases, DNMT1, DNMT3A and DNMT3B, also possess demethylase activity under certain calcium and redox conditions *in vitro* (Figure 2.5) (Chen *et al*. 2013).

2.15 Hydroxymethylcytosine, an epigenetic characteristic

Hydroxymethylcytosine, in addition to being an intermediate of an active demethylation pathway, has been proposed as an independent epigenetic mark/characteristic, following the discovery of proteins which bind specifically to this base modification (Spruijt *et al*. 2013). Hydroxymethylcytosine has only been detected in a few insects including *A. mellifera*, *N. vitripennis*, *T. castaneum* and *D. melanogaster* (Cingolani *et al*. 2013; Feliciello *et al*. 2013; Wojciechowski *et al*. 2014; Delatte *et al*. 2016; Pegoraro *et al*. 2016; Rasmussen *et al*. 2016). It has been studied most extensively in *A. mellifera*, where it is found predominantly at intronic non-CG sites (Cingolani *et al*. 2013). On account of 5hmC's intronic location, and a correlation detected between 5hmC and alternative splicing, it has been suggested that 5hmC could play a role in alternative splicing by defining the location of introns (Cingolani *et al*. 2013). Interestingly, in *D. melanogaster*, 5hmC has been detected not only in DNA (Rasmussen *et al*. 2016), but also in RNA (Delatte *et al*. 2016), the latter present mostly in coding sequences of polyadenylated RNA and found at highest levels in the brain.

2.16 Detecting and quantifying DNA methylation

Techniques for the detection and quantification of DNA methylation are broadly divided into three categories namely, i) methods that exploit the differential restriction capabilities of methylation-sensitive restriction enzymes, ii) methods that are based on the selective affinity of antibodies or proteins for methylated cytosine, and iii) methods which utilise chemicals that react differently with methylated and unmethylated cytosines (Fouse *et al*. 2010; Jin *et al*. 2010).

2.16.1 Methylation-sensitive restriction enzyme-based techniques

Restricting DNA with isoschizomers, pairs of restriction enzymes that display differential sensitivity to the methylation status of the cytosines of their common recognition sequence, is a

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technique that has been used for many years (McClelland *et al*. 1994). It was, in fact, used to identify methylation in the amplified esterase genes of *M. persicae* in 1989 (Field *et al*. 1989). A frequently used isoschizomer pair is HpaII and MspI, which share the recognition sequence 5' CCGG 3' and restrict DNA between the two cytosines. Methylation-sensitive restriction fragment length polymorphism (MS-RFLP) (Jaligot *et al*. 2002; Rival *et al*. 2009) and methylation-sensitive amplification polymorphism (MSAP or MS-AFLP) (Reyna-López *et al*. 1997), the latter being a modification of the amplified fragment length polymorphism (AFLP) technique (Vos *et al*. 1995), are just two of numerous methods that employ the HpaII/MspI isoschizomer pair.

Methylation-sensitive amplification polymorphism, the more widely used technique, involves two separate double digestion reactions using EcoRI and either HpaII or MspI. Double-stranded EcoRI and HpaII/MspI adaptors, the sticky ends of which are complementary to the respective restricted recognition sequences, are then ligated to the DNA. This is followed by two rounds of amplification (pre-amplification and selective amplification) using primers with selective nucleotides on their 3' ends. The amplification products are electrophoresed on polyacrylamide gels, revealing differential separation patterns or fragment profiles of DNA digested with HpaII and MspI, and thus providing insight into the presence and/or extent of methylation in different samples (Reyna-López *et al*. 1997; Xu *et al*. 2000).

Methylation-sensitive restriction enzymes are not always used in tandem, a case in point being the use of McrBC (Mcr stands for modified cytosine restriction), a GTP-requiring, modification-dependent restriction enzyme of *Escherichia coli* K-12 (Raleigh 1992; Stewart *et al*. 2000). The preference of McrBC for restricting methylated DNA makes it useful for depleting samples of their methylated portion, known as McrBC depletion, and thus reveals regions of the genome which are unmethylated (Fouse *et al*. 2010; Huang *et al*. 2010a). By differentially labelling and cohybridising an McrBC-treated and untreated (reference) sample to a microarray, the degree of methylation of genes present on said array can be quantified and is indicated by the ratio of hybridisation intensities (Lippman *et al*. 2004; Nouzova *et al*. 2004; Fouse *et al*. 2010; Huang *et al*. 2010a).

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2.16.2 Affinity-based techniques

Affinity-based techniques exploit the specific binding capabilities that certain antibodies and proteins harbour for methylated cytosines, resulting in the enrichment of the methylated portion of the genome. Methylated DNA immunoprecipitation (MeDIP), first demonstrated by Weber and colleagues in 2005, makes use of a 5-methylcytosine antibody which, after binding to single-stranded methylated DNA, is immunoprecipitated. The enriched DNA can then be subjected to next-generation sequencing (MeDIP-seq) to yield information about which regions of the genome are methylated (Down *et al*. 2008; Pomraning *et al*. 2009). Alternatively, the enriched fraction as well as the original, non-enriched sample are labelled with different fluorescent dyes and cohybridised to microarrays (MeDIP-chip) allowing relative methylation levels at specific loci to be determined (Weber *et al*. 2005; Fouse *et al*. 2010; Huang *et al*. 2010a; Laird 2010). Antibodies which bind specifically to 5-methylcytosine can also be used to quantify genome-wide methylation levels, by using an ELISA assay to measure (colourimetrically or fluorometrically) the relative amount of antibody that binds to the DNA. The use of such antibodies is advantageous as they enable detection of methylation in all sequence contexts (Suzuki and Bird 2008; Fouse *et al*. 2010; Laird 2010).

Methyl-CpG immunoprecipitation (MCIp) also enriches methylated DNA by using a recombinant MBD-Fc fusion protein that binds double-stranded DNA at methylated CG sites (Gebhard *et al*. 2006; Sonnet *et al*. 2013). Because the affinity of DNA fragments towards MBDs increases with increasing amounts of CG methylation (Gebhard *et al*. 2006), differing salt concentrations are used to elute bound DNA containing different degrees of methylation. Other chromatography techniques such as the methylated CpG island recovery assay (MIRA) do not involve the use of antibodies. The MBD proteins are instead glutathione-S-transferase (GST)-tagged, facilitating their binding to a membrane. Methylated DNA bound to MBD proteins is recovered and gene-specific PCRs are performed to determine if the gene (or CpG island) of interest was methylated (Rauch and Pfeifer 2005).

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2.16.3 Chemical treatment-based techniques

Sodium bisulphite, hydrazine and permanganate all react differently with methylated and unmethylated cytosines, allowing sites of methylation to be detected (Oakeley 1999; Fraga and Esteller 2002). However, the labour-intensive nature of sequencing reactions following DNA treatment with hydrazine and permanganate, as well as the low sensitivity of these methods, has seen the use of these chemicals phased out (Fraga and Esteller 2002). There has been a concurrent rise in the popularity of sodium bisulphite treatment, with many commercially available kits.

Treatment of DNA with sodium bisulphite causes the preferential deamination of unmethylated cytosines, which are then chemically converted to uracil upon desulphonation (shown in Figure 2.6B) (Frommer *et al*. 1992; Clark *et al*. 2006; Darst *et al*. 2010). Methylated cytosines are deaminated at a much slower rate than their unmethylated counterparts (Frommer *et al*. 1992; Darst *et al*. 2010; Huang *et al*. 2010b). Subsequent PCR amplification of treated DNA results in newly formed uracils being replaced with thymines. The treated and amplified DNA is then sequenced, and the sequence is compared to that of untreated, reference DNA, allowing the exact location and percentage of methylated cytosines (i.e., methylation level) to be determined. A cytosine base in the same position of both sequences indicates methylation was present at that base and prevented deamination, whilst a cytosine in one sequence and a thymine in the other (at the same position) indicates the presence of an unmodified cytosine that underwent deamination. In this manner BS-Seq translates an epigenetic mark/characteristic into a quantifiable genetic one (Lister and Ecker 2009; Huang *et al*. 2010a).

Bisulphite sequencing is thus a powerful technique that enables methylation of the genome, or smaller regions thereof, to be mapped at a single nucleotide resolution (Lister and Ecker 2009; Huang *et al*. 2010b; Sun *et al*. 2014). Furthermore, because all unmethylated cytosines are converted to uracils, the methylation status of all cytosines, in any sequence context can be determined (Clark *et al*. 2006). This is particularly advantageous when investigating insect methylation which, although occurring predominantly in the CG context, is also present in other

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sequence contexts. Bisulphite sequencing relies on the availability of a reference genome or at the very least, sequence information on the regions of interest being investigated (Laird 2010; Krueger *et al*. 2012). This prerequisite renders BS-Seq an ineffective technique for many initial investigations into methylation (including those presented in Chapters 3 and 4), for which restriction enzyme and affinity-based techniques are better suited.

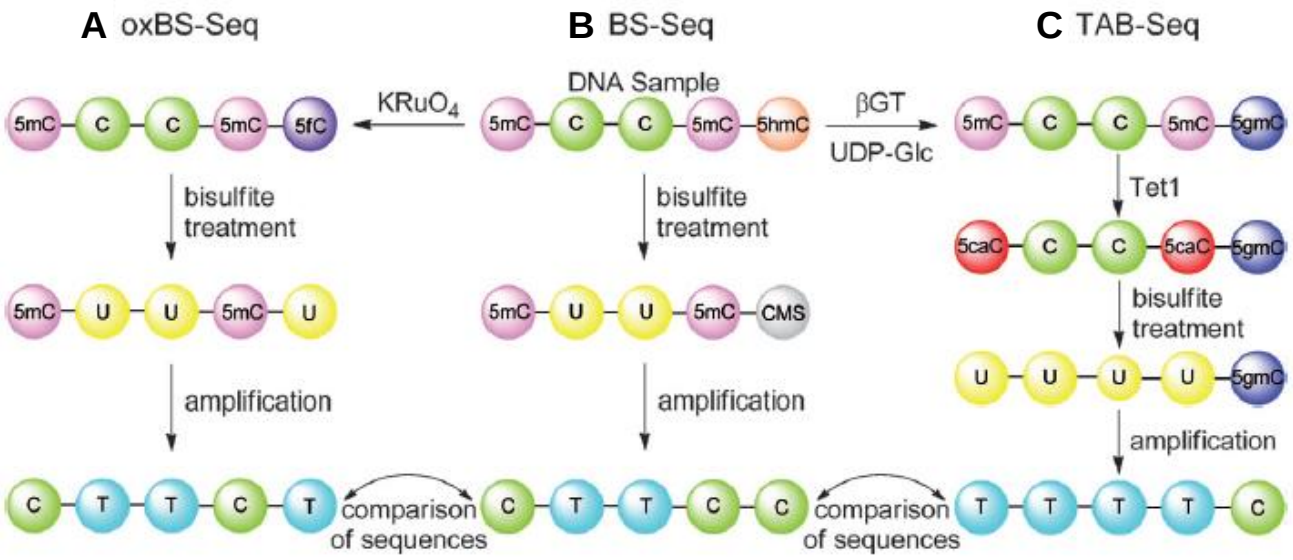

Figure 2.6. A schematic representation of BS-Seq and its variations. (A) Oxidative bisulphite sequencing: $KRuO_4$ oxidises 5hmC to 5fC which along with unmodified C is deaminated during bisulphite treatment. (B) Traditional bisulphite sequencing: unmodified C is deaminated to U. Hydroxymethylcytosine forms CMS during bisulphite treatment, and, like 5mC, is resistant to deamination. (C) TET-assisted bisulphite sequencing: βGT glucosylates 5hmC to protect it from oxidation. Methylcytosine is oxidised to 5caC by TET1 and is deaminated along with unmodified C. Figure sourced from Schüler and Miller (2012).

2.17 Detecting and quantifying hydroxymethylation

Methods for detecting and quantifying hydroxymethylation are mostly based on modifications of the BS-Seq technique or on the selective affinity of antibodies for 5hmC and cytosine-5-methylenesulphonate (CMS), the product formed upon treating 5hmC with sodium bisulphite (Figure 2.6B). With the functions of 5hmC still emerging in current studies, the ability to map this epigenetic modification at single nucleotide resolution has become a necessity. The inability of traditional bisulphite sequencing to distinguish between 5mC and 5hmC (as neither become deaminated) (Huang *et al*. 2010b; Jin *et al*. 2010), has led to the development of two modifications

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of BS-Seq technology, namely oxidative bisulphite sequencing (oxBS-Seq) and TET-assisted bisulphite sequencing (TAB-Seq), both of which have a high resolving capacity (Booth *et al*. 2012; Yu *et al*. 2012).

Oxidative bisulphite sequencing takes cognisance of the fact that 5hmC's oxidised derivative, 5fC, is amenable to deamination by bisulphite treatment, and includes a DNA treatment prior to this with potassium perruthenate ($KRuO_4$) to oxidise all 5hmC residues (Figure 2.6A) (Booth *et al*. 2012; Schüler and Miller 2012). Oxidised and bisulphite-treated DNA is then sequenced and compared to both an untreated DNA sample, and a bisulphite-treated sample to reveal the positions of unmodified cytosine, 5mC and 5hmC. TET-assisted bisulphite sequencing, the second BS-Seq modification, exploits the ability of the TET1 enzyme to sequentially oxidise 5mC to 5hmC, 5fC and ultimately 5caC, which undergoes deamination during bisulphite treatment. β-glucosyltransferase (βGT) is first added to the DNA, generating β-glucosyl-5-hydroxymethylcytosine (5gmC) and thereby protecting existing 5hmC residues from becoming oxidised during subsequent treatment with TET1 (Figure 2.6C) (Schüler and Miller 2012; Yu *et al*. 2012). TET1-treated DNA is then bisulphite-treated and sequenced, and a comparison with an untreated DNA sample, and a bisulphite-treated sample will again reveal the positions of unmodified cytosine, 5mC and 5hmC.

Antibodies specific to 5hmC are used to enrich for 5hmC-containing DNA via immunoprecipitation, in a technique referred to as hydroxymethylated DNA immunoprecipitation (HMeDIP) (Ito *et al*. 2010; Ficz *et al*. 2011; Xu *et al*. 2011). Hydroxymethylated DNA immunoprecipitation can be used in conjunction with sequencing analyses (HMeDIP-seq) to identify regions of the genome that are hydroxymethylated (i.e., contain hydroxymethylation) (Ficz *et al*. 2011; Xu *et al*. 2011). Hydroxymethylcytosine-specific antibodies can also be used to quantify genome-wide 5hmC levels, through the use of an ELISA assay to measure (colourimetrically or fluorometrically) the relative amount of antibody that binds to the DNA.

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Hydroxymethylation can also be detected by using a combination of sodium bisulphite treatment of DNA, and an antibody specific to CMS.

There are a few methods of 5hmC detection that involve the use of restriction enzymes. For example, MspI restricts hydroxymethylated DNA, but is unable to restrict glucosylated hydroxymethylated DNA, which is formed by treating DNA with βGT (Davis and Vaisvila 2011; Ficz *et al*. 2011). Performing quantitative PCRs with primers designed flanking MspI recognition sites allows the detection and quantification of hydroxymethylation (Davis and Vaisvila 2011; Ficz *et al*. 2011). The presence of a product would indicate the sequence was not restricted and hydroxymethylation was present. Additionally, the enzyme PvuRts1I, which recognises and restricts DNA close to hydroxymethylated sites, has been employed in a technique coined Pvu-Seq to detect honey bee hydroxymethylation (Szwagierczak *et al*. 2011; Wang *et al*. 2011; Cingolani *et al*. 2013). Other enzymes of the PvuRts1I family such as AbaSI can also be coupled with sequencing technologies (Aba-Seq) (Wang *et al*. 2011; Sun *et al*. 2013; Plongthongkum *et al*. 2014).

The aim of the current study was to determine if methylation plays a role in the process of RWA biotypification, by investigating methylation and demethylation, using several of the aforementioned techniques for methylation and hydroxymethylation detection and quantification. To detect and quantify methylation, both MSAP (restriction enzyme-based technique), and a 5-methylcytosine-specific antibody (affinity-based technique) were used, whilst methylation trends were detected using a novel technique denoted restriction site-specific fluorescent labelling (RSSFL, restriction enzyme-based technique). Hydroxymethylation was detected and quantified using a 5-hydroxymethylcytosine-specific antibody (affinity-based technique). The DNA methyltransferases which catalyse methylation (Goll and Bestor 2005) were also investigated, to add to the growing body of knowledge on insect DNMTs, and to provide the first sequence and expression information relating to the South African RWA *DNMTs*.

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2.18 References

2.18.1 Journal articles

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2.18.2 Websites

FAOSTAT http://www.fao.org/faostat/

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Chapter 3

Detection and quantification of methylation levels in *Diuraphis noxia* using methylation-sensitive restriction enzymes

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3.1 Introduction

Epigenetics refers to the regulation of, or changes in gene expression, which do not involve alterations of the DNA sequence, and are mediated through DNA methylation, non-coding RNA activity, chromatin complexes and histone modification (Jeltsch 2002; Foret *et al*. 2012; Roberts and Gavery 2012; Mukherjee *et al*. 2015). These stable epigenetic modifications, which are heritable, yet reversible (Drewell *et al*. 2012), could provide the impetus for aphid biotype development that a lack of genetic variation, owing to an anholocyclic reproduction strategy of parthenogenesis, fails to do (Ricci *et al*. 2011). DNA methylation is known to be a driving factor for plant (Kalisz and Purugganan 2004; Rapp and Wendel 2005; Xiang *et al*. 2010) and animal (Xiang *et al*. 2010; Zeng *et al*. 2012) evolution and therefore could also be influential in the development of new aphid biotypes. Aphid biotypes, in the context of this study, are populations of morphologically similar aphids which damage hosts previously deemed resistant, with each biotype displaying a different level of virulence (Smith *et al*. 1992; Botha *et al*. 2010; Botha 2013). In turn, aphid virulence is defined by the damage caused to a differential set of wheat plants containing different *Dn* (*Diuraphis noxia*) resistance genes (Weiland *et al*. 2008).

During DNA methylation, a methyl group donated by S-adenosyl-L-methionine, is covalently added to the fifth carbon of the pyrimidine ring of cytosine residues (Jeltsch 2002; Glastad *et al*. 2011; Lyko and Maleszka 2011; Yan *et al*. 2015). In insects this occurs predominantly, but not exclusively in the CG dinucleotide context (Lyko and Maleszka 2011; Su *et al*. 2011), with reports of non-CG methylation in the following insects: *Drosophila melanogaster* (Bird 2002; Kunert *et al*. 2003; Field *et al*. 2004), *Medauroidea extradentata* (Krauss *et al*. 2009), *Triboleum castaneum* (Feliciello *et al*. 2013), *Mamestra brassicae* (Field *et al*. 2004), *Pogonomyrmex barbatus* (Smith *et al*. 2012), *Acyrthosiphon pisum* (Walsh *et al*. 2010) and *D. noxia* (Gong *et al*. 2012).

Diuraphis noxia (Kurdjumov, Hemiptera: Aphididae – or Russian wheat aphid, RWA) was first reported an invasive pest in 1978 in South Africa, and then in 1986 in the United States of America (USA), where it causes widespread and significant yield penalties to wheat and barley production

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(Walters *et al*. 1980; Morrison and Peairs 1998; Basky 2003; Botha *et al.* 2005; Porter *et al*. 2009). Russian wheat aphid infestation and subsequent damage were curbed by farmers through the planting of resistant varieties. However, in 2003 the development of a new aphid biotype in the USA – denoted RWA US2 – led to the breakdown of *Dn4* resistance (Haley *et al*. 2004; Lapitan *et al*. 2007; Shufran *et al*. 2007; Porter *et al*. 2009). The development of new RWA biotypes was also reported for South Africa (Tolmay *et al*. 2007; Jankielsohn 2011, 2014, 2016) and Argentina (Clua *et al*. 2004).

There are currently five biotypes in South Africa, four of which (SA1, SA2, SA3 and SA4) are naturally occurring. The highly virulent South African mutant biotype (SAM) developed from the least virulent biotype, SA1, after pressuring the latter biotype by feeding on resistant wheat varieties (Van Zyl and Botha 2008; Swanevelder *et al*. 2010). Despite the fact that the existence of biotypes is well-documented in literature (Clua *et al*. 2004; Haley *et al*. 2004; Botha *et al*. 2005, 2010, 2014a, 2014b; Burd *et al*. 2006; Lapitan *et al*. 2007; Tolmay *et al*. 2007; Randolph *et al*. 2009; Smith 2009; Jankielsohn 2011, 2014, 2016; Botha 2013; Burger and Botha 2017), the mechanism underlying aphid biotypification and the associated increase in virulence remains unknown.

The genetic similarity between the related biotypes SA1 and SAM, which display only 0.0008% variation in protein-coding gene sequences (Burger and Botha 2017), points to a mechanism for increasing virulence that is independent of the DNA sequence – i.e., an epigenetic modification. Various aphid processes are regulated by epigenetic mechanisms/modifications including aphid wing polyphenism (Srinivasan and Brisson 2012), whereby aphids of a single biotype (and thus having identical genomes) can exist as winged or wingless morphs (Tagu *et al*. 2008). Other examples are insecticide resistance in *Myzus persicae* (Field *et al*. 1989, 2004), and the molecular variability among asexually reproducing *A. pisum*, which is an important contributing factor towards morph distribution, growth rate and pigmentation (Dombrovsky *et al*. 2009). Both insecticide resistance and molecular variability are influenced specifically by the epigenetic modification of methylation.

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The presence of DNA methylation has been detected in the aphid species *M. persicae* (Field *et al*. 1989, 2004), *A. pisum* (Dombrovsky *et al*. 2009; Walsh *et al*. 2010) and *D. noxia* (Gong *et al*. 2012). Previously, Gong *et al*. (2012) investigated the methylation of a small set of genes encoding salivary proteins between RWA biotypes US1 and US2, and found that the selected set of genes was more highly methylated in the less virulent biotype (RWA US1). This study provided some evidence that DNA methylation, or alterations thereof, may be involved in biotypification, and was followed by the sequencing of the RWA genome (Nicholson *et al*. 2015; Burger and Botha 2017), which revealed a complete set of DNA methylation genes. Thus, the potential for methylation as a driving factor of biotypification and increased virulence exists, but remains largely unexplored.

In order to explore methylation as a possible contributing factor to the observed difference in virulence between two aphid biotypes with documented shared genealogy (SA1 and SAM; Burger and Botha 2017) and another two biotypes, the genealogy of which is unknown (SA2 and SA3), a well-established method, namely methylation-sensitive amplification polymorphism (MSAP or MS-AFLP) (Reyna-López *et al*. 1997; Xu *et al*. 2000; Weiner *et al*. 2013), was applied, allowing a comparison of the methylation states of various loci. Additionally, a novel technique denoted restriction site-specific fluorescent labelling (RSSFL) was used to identify differences, if any, in methylation trends between the biotypes. This technique is based on the separate restriction of DNA using the isoschizomers HpaII and MspI, whereafter an adaptor containing a fluorophore is ligated to restricted DNA, thus allowing for measurement of fluorescence intensity as a means of quantifying the extent of restriction.

The principle reason for the choice of MSAP and RSSFL techniques is their common use of the restriction enzymes HpaII and MspI. As the assembly of the sequenced RWA genome was only recently completed (Nicholson *et al*. 2015; Burger and Botha 2017), methods that do not rely on extensive sequence information provide a good alternative to, for example, bisulphite sequencing, as a means to assess methylation. The isoschizomers HpaII and MspI have a common recognition site, 5’ CCGG 3’, but restrict DNA differently depending on the methylation state within this site

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(i.e., fully, hemi- or unmethylated at the external, internal or both cytosines – Figure 3.1) (McClelland *et al*. 1994).

	Methylation status	MspI	HpaII	Type of information
C C G G G G C C	No methylation	+	+	Condition I
C C G G G G C C	Full methylation of internal cytosine	+	-	Condition II
C C G G G G C C	Hemimethylation of internal cytosine	+	-	Condition II
C C G G G G C C	Hemimethylation of external cytosine	-	+	Condition III
C C G G G G C C	Full methylation of external cytosine	-	-	Condition IV
C C G G G G C C	Full methylation of both cytosines	-	-	Condition IV
C C G G G G C C	Hemimethylation of both cytosines	-	-	Condition IV
C C G G MUTATION G G C C	Unknown	-	-	Condition IV

Figure 3.1. An illustration of all possible methylation states within the common HpaII/MspI recognition site. HpaII and MspI's sensitivity to the methylation state, and ability to restrict the DNA is indicated by a + (can restrict) or a – (cannot restrict). The presence and absence of bands on the MSAP gels was recorded as one of four conditions. Figure adapted from Schulz *et al*. (2013).

When scoring MSAP gels, these states can be divided into four 'conditions' based on the presence or absence of bands (fragments) in the lanes containing DNA restricted with HpaII and MspI.

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Condition I, the presence of a band of a certain size in both lanes, occurs when the recognition site is unmethylated, allowing both enzymes to restrict the DNA (Figure 3.1). Condition II, the presence of a band in only the MspI lane (i.e., only MspI restricted the DNA), arises when the internal cytosine is fully or hemimethylated (Figure 3.1). HpaII, but not MspI, restricts DNA that is externally hemimethylated, giving rise to Condition III, a band in only the HpaII lane (Figure 3.1). Finally, condition IV, the absence of a band in the HpaII and MspI lanes, indicating that neither HpaII nor MspI restricted the DNA, occurs when the external cytosine is fully methylated or when both cytosines are fully or hemimethylated (Figure 3.1).

With no prior investigations of South African RWA biotype methylation having been performed, the study sought to assess the capacity of different techniques to detect and quantify RWA methylation. Two technical objectives were set out for this chapter, the first being to identify differences in methylation profiles (banding patterns) between the RWA biotypes using MSAP, to score these differences, to relate these differences to the reported virulence levels of the South African RWA biotypes (Jankielsohn 2014, 2016), and to use the banding patterns to quantify the methylation levels of the biotypes (Kronforst *et al*. 2008). The second technical objective was to assess the methylation trends of the South African RWA biotypes using the RSSFL technique and *Homo sapiens* and *Apis mellifera capensis* DNA as internal controls.

3.2 Methods

3.2.1 Russian wheat aphid rearing

Colonies of parthenogenetic apterous female aphids of South African RWA biotypes SA1, SA2, SA3 and SAM, expressing different levels of virulence, were separately reared in BugDorm cages (MegaView Science Education Services Co. Ltd, Taiwan) in an insectary with the following conditions: 22.5°C ± 2.5°C, 35%–40% relative humidity, and continuous artificial lighting from high pressure sodium lamps. The RWA biotypes rank in virulence as follows: SA1 < SA2 < SA3 < SAM (Botha 2013; Botha *et al*. 2014a; Jankielsohn 2014, 2016). For the MSAP experiment, biotype SA1 (Hewitt *et al*. 1984) was maintained on the susceptible “Tugela” wheat cultivar, while SA2,

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SA3 (Jankielsohn 2011) and SAM (Swanevelder *et al*. 2010) were maintained on "TugelaDN", a wheat cultivar containing the *Dn1* resistance gene. For the RSSFL experiment, colonies of all biotypes were maintained on the "SST 356" wheat cultivar, obtained from SENSAKO (Pty) Ltd (South Africa). Cultivars were planted in sand-filled pots and watered daily with a fertiliser 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 l of water.

3.2.2 DNA extraction

3.2.2.1 *Diuraphis noxia*

DNAzol® Reagent (Thermo Fisher Scientific, USA) was used to extract genomic DNA from aphids of the four South African RWA biotypes SA1, SA2, SA3 and SAM, following a modified protocol. Aphids (n=50) were collected using a soft-bristled brush, homogenised in extraction reagent, and centrifuged at 10 000 rpm for 15 min to pellet the cell debris. The resulting supernatant was transferred to a clean 1.5 ml Eppendorf tube, 500 µl ice cold 100% (v/v) ethanol was added, and the tubes were left overnight at -20°C to precipitate the DNA. Precipitated DNA was transferred to a new Eppendorf tube and washed with 75% (v/v) ethanol to remove excess salts. The DNA was then collected through centrifugation at 10 000 rpm for 5 min. An additional wash and centrifugation step were carried out and the resulting pellets were air-dried. DNA was resuspended in 50 µl low Tris-Ethylenediaminetetraacetic acid (EDTA) (TE, 10 mM Tris-HCl and 0.1 mM EDTA, pH 8) buffer and was quantified using the Qubit® 2.0 fluorometer (Thermo Fisher Scientific, USA) at the Central Analytical Facility (CAF) of Stellenbosch University. DNA quality was visually assessed through gel electrophoresis on a 1.5% Tris/Acetic acid/EDTA (TAE, 40 mM Tris, 20 mM Acetic acid and 1 mM EDTA, pH 8) agarose gel post-stained with ethidium bromide (2.5 µg/ml).

3.2.2.2 *Apis mellifera capensis*

Apis mellifera capensis (Cape honeybee) specimens were kindly provided by Professor Wossler (Department of Botany and Zoology, Stellenbosch University). The thoracic and abdominal

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sections of individual bees (n=2) were separated using a sterile scalpel, and the abdomen was discarded. The bee head and thorax were homogenised in DNAzol® Reagent using a handheld homogeniser (Labotec, South Africa). All steps following homogenisation were performed as described in 3.2.2.1, and DNA was quantified at CAF using the Qubit® 2.0 fluorometer. Although multiple extractions were performed, only two yielded a high enough DNA concentration for use in the RSSFL experiment.

3.2.2.3 *Homo sapiens*

Homo sapiens blood samples (n=3) were graciously provided by Professor Anna-Mart Engelbrecht (Department of Physiological Sciences, Stellenbosch University), and extracted by Mrs Lundi Korkie (Human Genetics Laboratory, Department of Genetics, Stellenbosch University) using the methodology outlined in Appendix A (Method A1). DNA quantification was performed at CAF using the Qubit® 2.0 fluorometer.

3.2.3 Methylation-sensitive amplification polymorphism analysis

3.2.3.1 Restriction digestion of genomic DNA

Two separate restriction enzyme double digestion reactions were performed for each aphid biotype, using 8 U of either HpaII or MspI (isoschizomers both from Thermo Fisher Scientific, USA) and 2.4 U EcoRI (Promega, USA). The 12.5 µl reactions also contained 200 ng genomic DNA and 1x Tango buffer (Thermo Fisher Scientific, USA). The reactions were incubated for 2 hours at 37°C and the enzymes were inactivated through heating at 80°C for 15 min.

3.2.3.2 Ligation of adaptors

A double-stranded HpaII/MspI adaptor was designed using Primer3 (http://bioinfo.ut.ee/primer3/, Rozen and Skaletsky 2000), to ligate to the overhangs of HpaII and MspI restricted DNA (Appendix A, Table A1). This adaptor was used in conjunction with a double-stranded EcoRI adaptor (Vos *et al*. 1995) (Appendix A, Table A1; both adaptors ordered from Integrated DNA Technologies (IDT), USA). The 25 µl ligation reaction consisted of 12.5 µl of the restriction

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reaction, 60 pmoles of both strands of both adaptors, 1 U T4 DNA Ligase (Thermo Fisher Scientific, USA) and 1x T4 DNA Ligase buffer. The reaction was incubated for 2 hours at 20°C and the T4 DNA Ligase was heat-inactivated at 65°C for 10 min.

3.2.3.3 Pre-amplification

The pre-amplification reaction consisted of 2.5 µl of a 1:10 TE (10 mM Tris-HCl and 1 mM EDTA, pH 8) buffer diluted ligation mixture, 300 pmoles EcoRI (+1) pre-amplification primer (Vos *et al*. 1995) (Table A1; IDT, USA), 300 pmoles HpaII/MspI pre-amplification primer designed to be complementary to the HpaII/MspI adaptor (Table A1; IDT, USA), 0.5 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 U Taq DNA polymerase (Thermo Fisher Scientific, USA) and 1x amplification buffer, made up to a final volume of 25.5 µl. An initial 3 min denaturation step at 94°C was followed by 40 cycles of 94°C for 30 sec, 56°C for 1 min and 72°C for 1 min. A final 5 min elongation step was performed at 72°C.

3.2.3.4 Selective amplification

Selective amplification was carried out in low light conditions, using seven primer pair combinations (ACG/T; ACT/T; AGC/T; ACG/A; AGC/A; AGG/A; ACC/A). IRDye® 700-labelled EcoRI (+3) primers (Table A1; LI-COR Biosciences, USA), and HpaII (+1)/MspI (+1) primers designed with one selective nucleotide on the 3' end (Table A1; IDT, USA), were used. Each 11 µl selective amplification reaction contained 2 µl pre-amplification product, 0.5 pmoles selective IRDye® 700-labelled EcoRI primer, 27 pmoles selective HpaII/MspI primer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.75 U Taq DNA polymerase and 1x amplification buffer. The following PCR conditions were used: 13 cycles of 94°C for 30 sec, 65°C (minus 0.7°C/cycle) for 30 sec and 72°C for 1 min. A further 23 cycles of 94°C for 30 sec, 56°C for 30 sec and 72°C for 1 min were carried out.

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3.2.3.5 Gel electrophoresis and visualisation

The methylation profiles of each biotype (n=50 aphids/sample) were visualised by loading selective amplification products (1 µl) into two adjacent lanes (n=2 technical replicates) of denaturing polyacrylamide gels, containing 8% Long Ranger™ gel solution (Lonza, Switzerland) and 7 M urea (Myburg *et al*. 2001), as well as 1x Tris/Boric acid/EDTA (TBE, 89 mM Tris, 89 mM Boric acid and 2 mM EDTA, pH 8) buffer. The DNA was resolved in the LI-COR DNA Analyzer (Model 4300, USA) for 3.5 hours at 45°C and 1 500 V as previously described (Zaayman *et al*. 2009). The IRDye® 700-labelled 50–700 bp sizing standard was used (LI-COR Biosciences, USA).

3.2.3.6 Scoring and analysis of bands

Unambiguous bands were scored manually on a hit versus no-hit basis. For each primer combination, polymorphisms were quantified by scoring the presence or absence of HpaII and MspI bands within, and between the respective biotypes. Identified polymorphic loci were designated as one of four conditions per biotype (condition I–IV, Figure 3.1), based on the sensitivity of HpaII and MspI to the methylation state of the loci.

3.2.3.7 Quantification of methylation level

The overall methylation level, which takes into account both fully and hemimethylated sites, was calculated for each biotype using the methodology of Kronforst *et al*. (2008), whereby the sum of the number of unique MspI and unique HpaII bands was divided by the total number of bands. A unique MspI or HpaII band refers to a fragment of a certain size that is present in only the lane containing DNA restricted with MspI and EcoRI, or only the lane containing DNA restricted with HpaII and EcoRI respectively. Methylation in the CG context, termed ‘internal methylation’ as the methylation is at the internal cytosine of the HpaII/MspI recognition site, was calculated by dividing the number of unique MspI bands by the total number of bands. Hemimethylation in the CC context, termed ‘external methylation’ was calculated by dividing the number of unique HpaII bands by the total number of bands.

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3.2.4 Restriction site-specific fluorescent labelling analysis

3.2.4.1 Restriction digestion of genomic DNA

Two separate restriction reactions were carried out, one using HpaII and the other, MspI, for each of the three biological repeats per RWA biotype (n=3), the three human samples (n=3) and the two bee samples (n=2). Each reaction consisted of 200 ng DNA, 1 U of either HpaII or MspI, and 1x Tango buffer in a total volume of 30 µl. After 1 hour incubation at 37°C, enzyme activity was terminated through heat-inactivation at 80°C for 5 min.

3.2.4.2 Ligation of adaptor

For ligation, a double-stranded oligonucleotide adaptor was designed using Primer3, based on the common HpaII/MspI restriction site (Appendix A, Table A1). This adaptor contained a tetramethylrhodamine fluorophore (excitation: 559 nm and emission: 583 nm, https://eu.idtdna.com/Site/Catalog/Modifications/Dyes) attached to the 3' end of one of the two strands. Both strands of the adaptor (200 pmoles each) were ligated to the restricted DNA using 1 U T4 DNA Ligase and 1x T4 DNA Ligase buffer, in a final volume of 40 µl. The ligation was carried out overnight at 4°C and the enzyme was inactivated through heating at 70°C for 5 min.

3.2.4.3 Removal of unbound adaptor

Excess unbound adaptor was removed using the MinElute® Reaction Cleanup Kit (Qiagen, Germany) following the manufacturer's protocol, resulting in 9 µl eluate. To this, 191 µl dH_2O was added to bring the assay volume to a total of 200 µl.

3.2.4.4 Fluorescence readings

The restricted and adaptor-ligated DNA, as well as dH_2O (200 µl) which was used as a blank, were loaded into a black 96 well plate. Fluorescence was measured at an emission of between λ 580 nm and 640 nm using the green optical kit (which detects Rhodamine-containing fluorophores) of the Glomax®-Multi Detection System (Promega, USA).

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3.2.4.5 Statistical analysis

Statistical analysis was performed in Microsoft Excel (2010)/XLSTAT Premium (Addinsoft Inc., USA), and graphs were plotted in SigmaPlot (2001) based on the average sample readings and standard deviation. The Shapiro-Wilk test was employed to test for the normality of the residuals (significance set at $p \leq 0.05$), whereafter dependent *t*-tests were performed to test for significant differences between the HpaII and MspI readings within each species and aphid biotype, with the level of significance set at $p \leq 0.05$.

The fluorescence readings resulting from both the HpaII and MspI digestions were also compared across/between the biotypes. An ANOVA, with the level of significance set at $p \leq 0.05$, was first conducted to test for significant differences between the biotype/species readings. The model assumptions of normality and homoscedasticity were tested for using the Shapiro-Wilk test and Levene's test respectively (significance set at $p \leq 0.05$ for both tests). In cases where the ANOVA null hypothesis – that the means of the treatment groups are equal – was rejected, a Fisher's LSD test was performed with Bonferroni adjustment for Type I error.

3.3 Results

3.3.1 Methylation-sensitive amplification polymorphism analysis

3.3.1.1 Detection of polymorphisms

Using the seven primer combinations, a total of 637 loci were amplified (Appendix A, Table A2). MspI/EcoRI restricted DNA resulted in 631 amplified loci (total # bands subtract instances where only HpaII/EcoRI restriction resulted in the presence of a band in the four biotype profiles), whilst HpaII/EcoRI restricted DNA yielded 625 loci (total # bands subtract instances where only MspI/EcoRI restriction resulted in the presence of a band in the four biotype profiles). A total of 41 polymorphic loci were detected between the four biotypes (refer to Appendix A, Figure A1 for an example of an MSAP gel, and to Figures A2 and A3 for an example of conditions I, II and III).

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When comparing the polymorphisms between the RWA biotypes with documented shared genealogy – SAM and its parent biotype, SA1 – the progression of methylation gain/loss at these loci during SAM's development could be inferred (Figure 3.2). A total of 22 changes in methylation status were identified, of which 16 changed from an unmethylated state in SA1 (condition I) to a hemi- (condition II – internal cytosine, condition III – external cytosine and condition IV – both cytosines), or fully methylated (condition II – internal cytosine and condition IV – external or both cytosines) state in SAM. A further two methylation sites changed from being externally hemimethylated (condition III) to fully methylated at both or only the external cytosine, or hemimethylated at both cytosines (condition IV), which also indicated a gain in methylation.

The remaining four changes are sites in SA1 that have lost methylation during SAM's development. Two sites that were fully methylated (at the external or both cytosines) or hemimethylated (at both cytosines) (condition IV) in SA1 lost methylation to become externally hemimethylated (condition III). Another site changed from an internally fully or hemimethylated state (condition II) to an unmethylated state (condition I). The last locus changed from condition IV in SA1 to II in SAM, and it is thus likely that it lost methylation during this process. However, as condition IV can be due to full methylation of the external cytosines, and condition II can be full methylation of the internal cytosine, one cannot exclude the possibility that the external methylation from condition IV was removed followed by a gain in methylation at the internal cytosines to attain condition II.

3.3.1.2 Quantification of methylation levels

The overall methylation level, as well as the level of internal methylation (encompassing full and hemimethylation in the CG dinucleotide context), and external methylation (hemimethylation in the CC dinucleotide context) can be estimated based on the number of loci in a biotype that are restricted by only MspI or only HpaII. The proportion of methylated sites (unique MspI plus unique HpaII bands divided by the total number of bands) provides an indication of the overall methylation level (Table 3.1). Likewise, the proportion of unique MspI or unique HpaII bands enables the

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internal and external methylation level to be estimated (Table 3.1). Biotype SAM exhibited the highest levels of overall, internal and external methylation (Table 3.1).

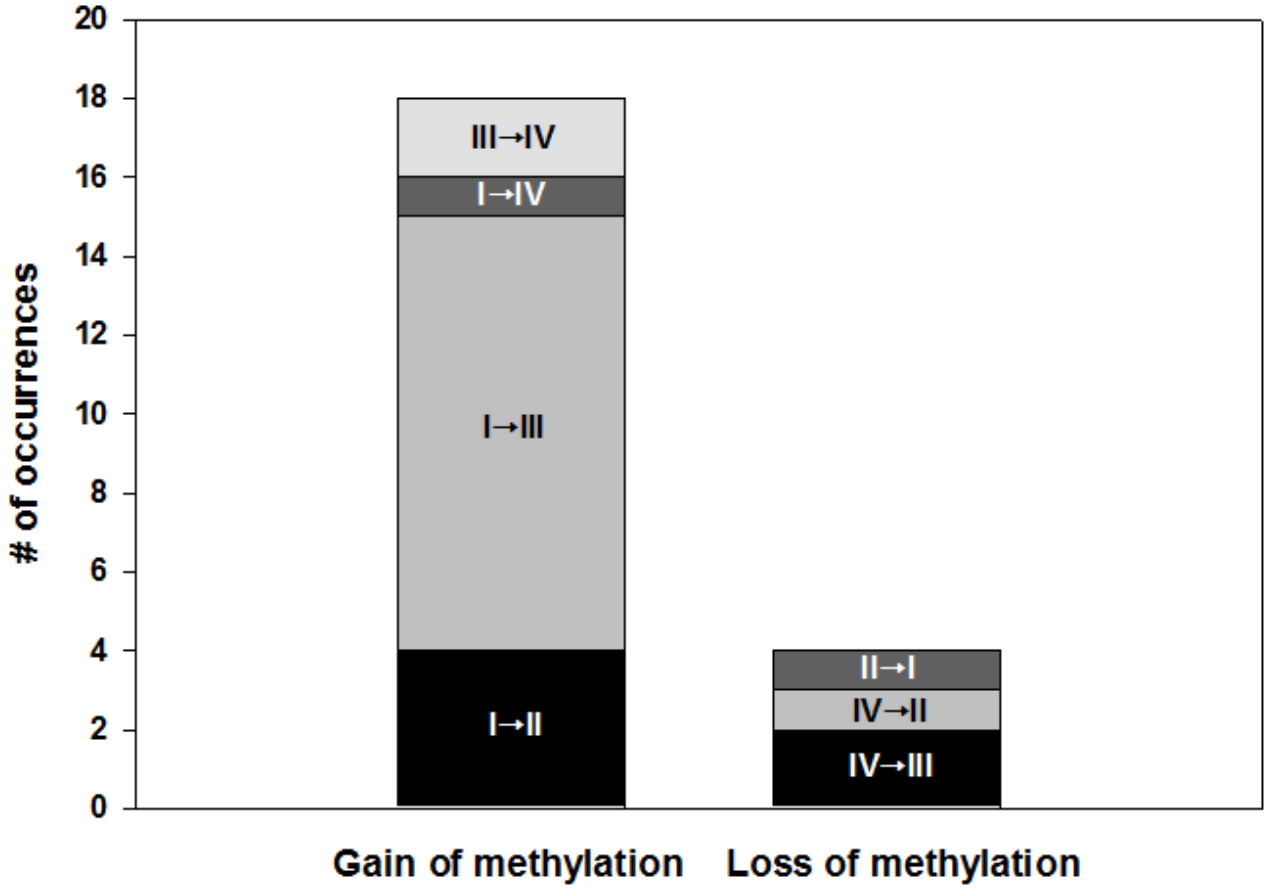

Figure 3.2. Methylation changes during the evolution of SA1 to SAM that resulted in 22 polymorphic loci. Loci are divided into gain and loss of methylation. The arrows indicate the progression of methylation condition from biotype SA1 to SAM. Conditions I to IV are visually explained in Figure 3.1.

Table 3.1. Overall, internal and external methylation levels of the RWA biotypes, estimated using the proportion of unique MspI and HpaII bands, identified through MSAP analysis.

	Formula	SA1	SA2	SA3	SAM
Overall methylation level (%)	(# unique MspI + # unique HpaII bands)/ # total bands	5.70	4.41	3.33	8.23
Internal full/hemimethylation (%)	# unique MspI bands/ # total bands	2.22	2.52	2.38	2.85
External hemimethylation (%)	# unique HpaII bands/ # total bands	3.48	1.89	0.95	5.38

3.3.2 Detection of methylation trends using restriction site-specific fluorescent labelling

The within-species (for human and bee) and within-biotype (for RWA) dependent *t*-tests did not reveal any significant differences between the HpaII and MspI readings (Figure 3.3; Appendix A,

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Table A3). The average fluorescence readings after HpaII and MspI restriction, and thus the methylation present, in the three less virulent biotypes, SA1, SA2 and SA3, displayed a similar trend to that of the human samples, whilst the fluorescence (and methylation) of the highly virulent SAM biotype mirrored that of the bees.

When looking at the MspI results (Figure 3.4; Appendix A, Table A4), the average level of fluorescence (437 RFU), and thus amount of restriction of biotype SAM DNA, was significantly lower than the average fluorescence level of the human (593 RFU), SA1 (553 RFU), SA2 (573 RFU) and SA3 (542 RFU) samples (Figure 3.4), with p values of 0.0001, 0.001, 0.0004 and 0.003 respectively. Furthermore, although the average SAM and bee fluorescence levels did not differ significantly (p=0.513), the average bee fluorescence (417 RFU), as with the average SAM fluorescence, was significantly lower than that of the human, SA1, SA2 and SA3 samples (Figure 3.4), with respective p values of 0.0001, 0.001, 0.0003 and 0.002. The average fluorescence readings of the human, SA1, SA2 and SA3 samples did not differ significantly from each other (Appendix A, Table A4).

Regarding the HpaII results (Figure 3.5; Appendix A, Table A5), the only significant difference in average fluorescence readings was between the related SA1 (559 RFU) and SAM (446 RFU) biotypes, with SA1's average fluorescence reading and amount of restriction being significantly higher than that of SAM (p=0.002).

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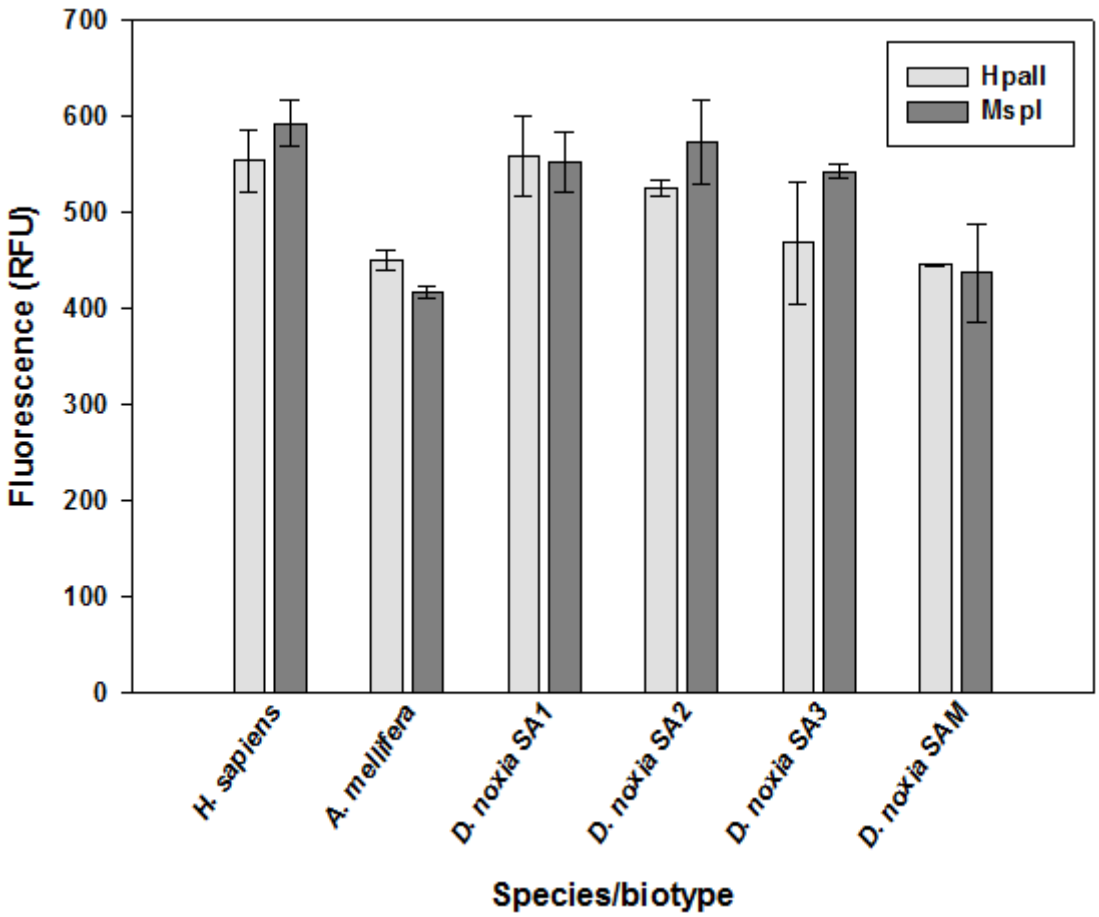

Figure 3.3. Bar chart illustrating the comparison of the average fluorescence readings of DNA from *Homo sapiens* (n=3), *Apis mellifera capensis* (n=2) and *Diuraphis noxia* (n=3), restricted with HpaII and MspI. Error bars representing the standard deviation of the sample readings for each species/biotype are also shown. RFU = Relative Fluorescence Units.

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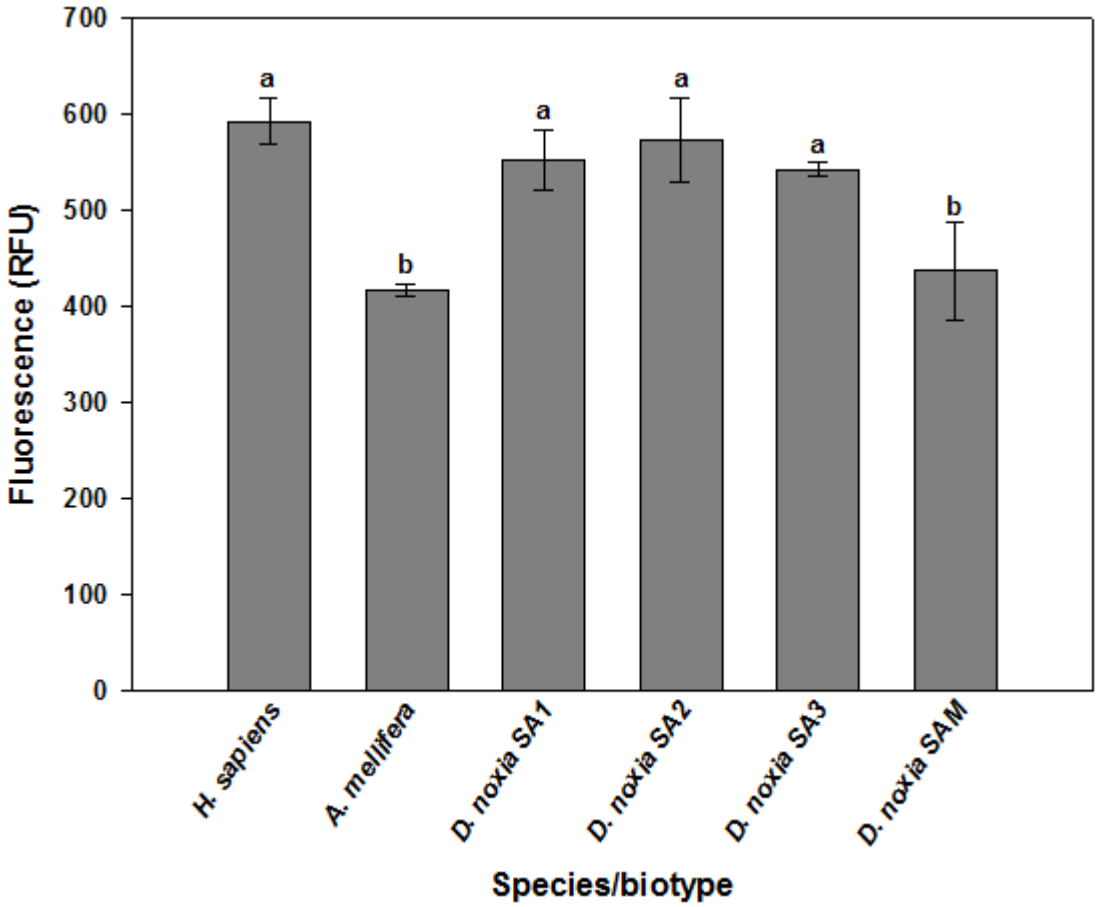

Figure 3.4. Bar chart illustrating the comparison of the average fluorescence readings of DNA from *Homo sapiens* (n=3), *Apis mellifera capensis* (n=2) and *Diuraphis noxia* (n=3), restricted with MspI. Error bars representing the standard deviation of the sample readings for each species/biotype are also shown. Different alphabetic letters indicate significant differences ($p \leq 0.05$). RFU = Relative Fluorescence Units.

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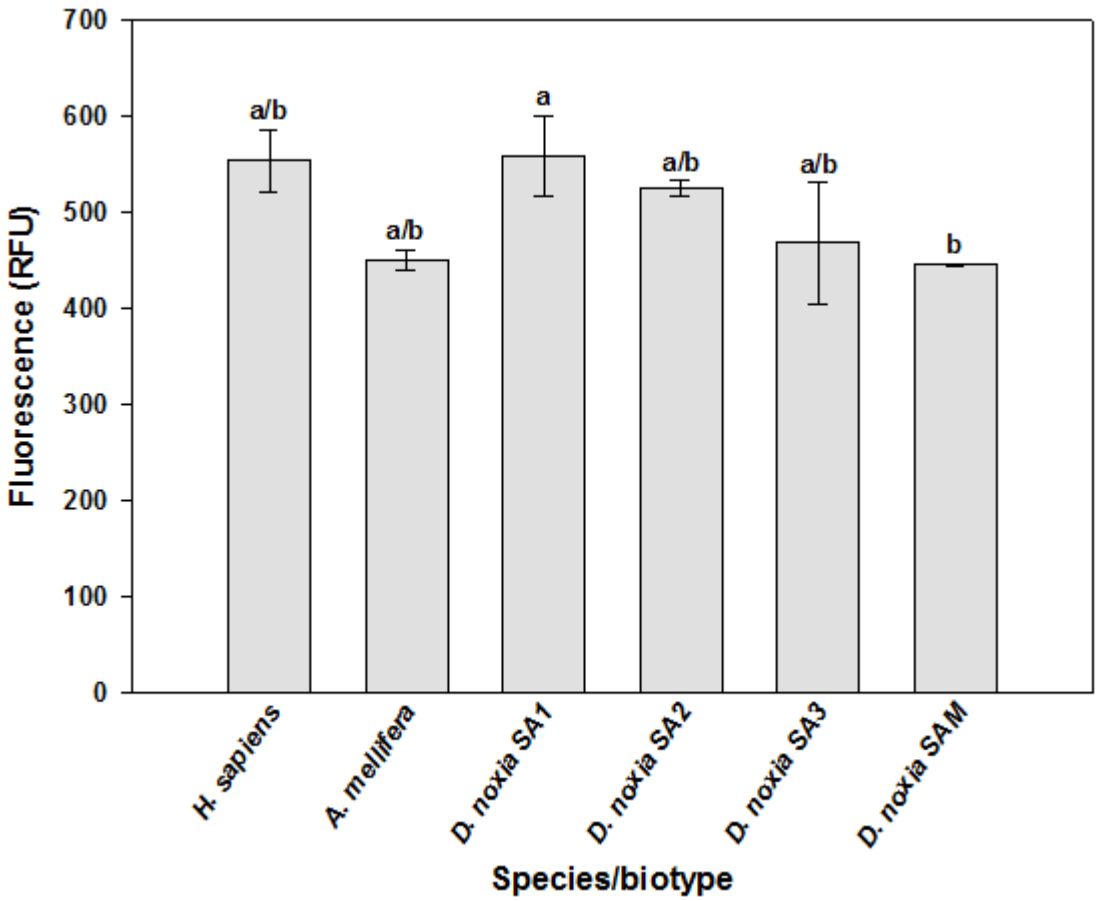

Figure 3.5. Bar chart illustrating the comparison of the average fluorescence readings of DNA from *Homo sapiens* (n=3), *Apis mellifera capensis* (n=2) and *Diuraphis noxia* (n=3), restricted with HpaII. Error bars representing the standard deviation of the sample readings for each species/biotype are also shown. Different alphabetic letters indicate significant differences ($p \leq 0.05$). RFU = Relative Fluorescence Units.

3.4 Discussion

In South Africa, the presence of four naturally occurring RWA biotypes threatens the durability of available resistant wheat cultivars (Botha *et al*. 2005, 2010; Tagu *et al*. 2008; Sinha and Smith 2014; Jankielsohn 2016). Although the SA1 biotype is only virulent to cultivars that contain the recessive *dn3* gene (Jankielsohn 2011), biotypes SA2, SA3 and SA4 are virulent towards cultivars containing a larger variety of resistance genes (Jankielsohn 2014, 2016), and their infestation renders these cultivars susceptible. The potential development of new biotypes, more virulent than SA1–SA4, highlights the need to understand the mechanism underlying biotypification and the associated increase in virulence, about which little is currently known (Shufran and Payton 2009; Botha *et al*. 2014a). The availability of the highly virulent model biotype SAM (Van Zyl and Botha

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2008; Swanevelder *et al*. 2010) provides a unique opportunity to gain insight into the currently enigmatic mechanism. With this information at hand, scientists can attempt to develop more durable cultivars that are resistant to a larger number of biotypes, based on the molecular mechanisms aphids use to overcome plant resistance (Sinha and Smith 2014).

In the present study the ability of the MSAP and RSSFL techniques to detect and quantify RWA methylation were assessed. The MSAP technique was used to identify differences in methylation banding patterns of RWA biotypes expressing different levels of virulence, and to quantify the overall, internal and external methylation levels of the biotypes, whilst RSSFL enabled the identification of methylation trends of the RWA biotypes.

3.4.1 Methylation-sensitive amplification polymorphism analysis

The 2015 release of the RWA genome (Nicholson *et al*. 2015) now makes this species amenable to bisulphite sequencing at a genome-wide level, the "gold standard" methodology for investigating methylation level and sequence context (Huang *et al*. 2010), which has been used for numerous insects including *A. mellifera* (Feng *et al*. 2010; Lyko *et al*. 2010; Zemach *et al*. 2010; Foret *et al*. 2012), *Harpegnathos saltator* and *Camponotus floridanus* (Bonasio *et al*. 2012), *Bombyx mori* (Xiang *et al*. 2010; Zemach *et al*. 2010), *Nasonia vitripennis* (Wang *et al*. 2013; Beeler *et al*. 2014), *Schistocerca gregaria* (Falckenhayn *et al*. 2013) and *D. melanogaster* (Zemach *et al*. 2010; Raddatz *et al*. 2013). However, as the RWA genome had not been released at the commencement of the current study, the MSAP technique, which requires no *a priori* sequence information and identifies methylation at a large number of anonymous 5' CCGG 3' sites throughout the genome, was employed (Meudt and Clarke 2007; Kronforst *et al*. 2008; Schulz *et al*. 2013; Weiner *et al*. 2013).

Interpreting the methylation profiles generated by MSAP requires knowledge of which recognition site (5' CCGG 3') methylation states enable HpaII and MspI restriction, and which states prohibit such activity. As mentioned by Schulz *et al*. (2013) there is some debate with regards to these methylation states, and the restriction enzyme database REBASE (http://rebase.neb.com/rebase/rebase.html) provides the best indication of when these enzymes can

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and cannot restrict DNA. According to REBASE (and shown in Figure 3.1), HpaII restricts both unmethylated DNA and DNA that is hemimethylated at the external cytosine, and MspI, in addition to restricting unmethylated DNA, also restricts DNA that is fully or hemimethylated at the internal cytosine.

The fact that amplified fragment length polymorphism (AFLP) techniques and their variations do not require sequence information of the organism under study, is simultaneously an advantage and a drawback. Whilst it greatly facilitates the study of non-model organisms, or organisms which have not had their genomes sequenced, the anonymity of the sites surveyed also means that one does not know which regions of the genome (e.g., intra- or intergenic) are methylated (Meudt and Clarke 2007; Weiner *et al*. 2013). However, as insect DNA methylation is found predominantly within genes (Zemach *et al*. 2010; Glastad *et al*. 2011; Lyko and Maleszka 2011), it is likely that this is also the case with the current methylated MSAP fragments.

3.4.1.1 Detection of polymorphisms

When comparing the DNA methylation profiles of the RWA biotypes with documented shared genealogy, SA1 and SAM, an interesting perspective on the development of virulence during SAM's 'evolution' is presented. Of the 22 polymorphisms detected specifically between these biotypes, 18 individual loci gained methylation during SAM's development from SA1 (Figure 3.2). It is tempting to speculate that the newly methylated loci reside on genes that encode RWA effectors. The effector proteins present in RWA saliva are released into the plant upon feeding, and if recognised by wheat resistance (R) proteins, result in a plant defence response (Walling 2008; Botha *et al*. 2014a). The SAM biotype, however, is able to avoid recognition by the plant during feeding (through unknown mechanisms) and evade plant defences (Botha *et al*. 2014a). SAM's effective feeding strategy might be accomplished, or at least aided by an increase in methylation of its effector genes, in one of two ways.

As methylation is involved in the prevention of spurious transcripts emanating from intragenic promoters or cryptic binding sites, and in the regulation of alternative promoter usage (Hunt *et al*.

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2010, 2013a, 2013b; Maunakea *et al*. 2010), an increase in methylation of effector genes would result in tighter transcriptional regulation of these genes in SAM, leading to fewer spurious transcripts and proteins available for recognition by plant R proteins. The same genes in SA1 would undergo less or no methylation-mediated transcriptional regulation (based on the scored polymorphism) and the proteins encoded by the spurious transcripts produced could be recognised by plant R proteins. Intragenic DNA methylation also regulates alternative mRNA splicing by either recruiting or interfering with different DNA-binding proteins and thereby affecting the inclusion of exons into produced transcripts (Bonasio *et al*. 2012; Glastad *et al*. 2014; Yan *et al*. 2015). An increase in methylation of SAM effector genes could thus influence the production of splice variants, such that the variant produced by SA1 is recognised by R proteins initiating a defence response, whilst the variant produced by SAM is not recognised, thus enabling SAM's avoidance of plant detection.

The possibility of the newly methylated sites being located in genes other than effectors cannot be ignored. It is, however, clear that whatever these genes are, an increase in their methylation is beneficial to SAM. Whether this is as a result of tighter transcriptional regulation and less spurious transcription, or due to methylation-mediated alternative splicing, remains unknown.

3.4.1.2 Quantification of methylation levels

Kronforst *et al*. (2008) were the first to use the MSAP technique to estimate the overall methylation levels of insects. Since 2008, this methodology has also been adopted by Lo *et al*. (2012), Smith *et al*. (2012), Weiner *et al*. (2013), Zhou *et al*. (2013), Zhang *et al*. (2014), and Libbrecht *et al*. (2016) to investigate the methylation of various insects. As applied in the current study, the overall, internal and external methylation levels of four South African RWA biotypes were quantified and compared (Table 3.1).

The first interesting finding is that there is a higher proportion of external hemimethylation (which occurs in the CC dinucleotide context) than internal methylation (found in the CG dinucleotide context) in biotypes SA1 and SAM (Table 3.1), which highlights the similar characteristics of the

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methylation system in these two related biotypes. It is, however, unusual for insects to exhibit a higher level of non-CG, as opposed to CG methylation, because, with the exception of *D. melanogaster* (Bird 2002; Kunert *et al*. 2003; Field *et al*. 2004), most documented insect methylation occurs in the CG context (Lyko and Maleszka 2011). For example, although CA methylation has been identified in the Hemipteran species, *A. pisum* (Walsh *et al*. 2010), CA and CT methylation in the Phasmatodean species, *M. extradentata* (Krauss *et al*. 2009), and CA, CT and CC methylation in the Hymenopteran species, *P. barbatus* (Smith *et al*. 2012), CG remained the most common context for methylation in these insects.

Despite this, it is not the first time that RWA methylation in a non-CG context has been reported as more prevalent than in the CG context. In 2012, Gong *et al*. found more CHG methylation (where H=A, C or T) than CG methylation in the two US biotypes investigated. It is, however, important to consider both the approach and techniques used by Gong *et al*. (2012) and the present study, as these could provide insight into this observation. In Gong *et al*.'s (2012) study, a targeted approach was adopted whereby the methylation of only four genes of interest was investigated. The distribution of methylation between CG and non-CG dinucleotides could be somewhat different when the genome as a whole is assayed. Likewise, due to the use of certain primer combinations during the MSAP analysis, only a subset of genomic sequences was surveyed for methylation.

Regarding the methylation levels, it is clear that SAM's methylation increased during its development from SA1, as SAM and SA1 exhibit overall methylation levels of 8.23% and 5.7% respectively, despite having very similar genome sequences (Burger and Botha 2017). Of particular interest is that whilst the internal methylation levels of SA1 and SAM are quite similar (2.22% and 2.85% respectively), there was an increase of 1.9% external hemimethylation in SAM. This raises the possibility that it is an increase in external hemimethylation that is related to increased aphid virulence, at least for SA1 and SAM. The methylation levels of biotypes SA2 and SA3 were also reported on, but since their genealogy is unknown, there was no point of comparison for these levels.

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3.4.2 Detection of RWA methylation trends using restriction site-specific fluorescent labelling

The differential ability of HpaII and MspI to restrict DNA containing differing methylation states makes it difficult to draw conclusions regarding the methylation levels of the RWA biotypes using the RSSFL technique. The HpaII reading consists of all 5' CCGG 3' sites in the genome where there is no methylation or where there is external hemimethylation, whilst the MspI reading consists of unmethylated sites as well as internally fully or hemimethylated sites. Despite this drawback, trends in methylation among the RWA biotypes could still be identified by comparing the average fluorescence readings of aphid DNA, restricted with HpaII and MspI, to that of the internal controls (human and bee DNA), restricted using the same enzymes.

As the aphid DNA fluorescence levels, obtained after HpaII and MspI restriction, were comparable to those of the human and bee samples, and it is known that both human and bee DNA exhibit methylation, it can be concluded that all the aphid biotypes were methylated. Human blood has a level of methylation ranging from 0.31%–5.04% which depends on, among other factors, the method of detection used, the population under study, the age of the individual and their state of health. Lower methylation levels have been reported using ELISA-based methods (i.e., 0.32% (Tellez-Plaza *et al*. 2014), 0.41% (Figueroa-Romero *et al*. 2012) and 0.9% (Tellez-Plaza *et al*. 2014)), and higher levels (3.14%–5.04%) using an HPLC/MS/MS method (Ma *et al*. 2009). The methylation level of bee brain tissue, as measured using bisulphite sequencing, is approximately 0.11% (Lyko *et al*. 2010).

The methylation levels of the aphid biotypes could not be directly inferred from those of the human and bee methylation levels for three reasons. Firstly, the methods used to quantify methylation of the bee and human DNA differed, secondly, the different methods used for quantifying human blood methylation resulted in a range of methylation levels spanning over four percent, and thirdly, because the current study made use of bee head and thoracic regions, as opposed to brain tissue. To avoid detecting methylation that was from sources other than the bee itself, the bee abdomen was

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not used. However, as whole aphids were used, the possible effects of plant DNA methylation, present in the aphid gut was not accounted for.

The similar fluorescence levels obtained after restriction, present between the human DNA and that of the three less virulent aphid biotypes, SA1, SA2 and SA3, suggests that these biotypes are likely more methylated than SAM, which has similar fluorescence levels to the bee DNA. By comparing the fluorescence levels of the related SA1 and SAM biotypes, it was revealed that there is a definitive change in these patterns, and thus in methylation, during biotypification. This provides some evidence that methylation is a good candidate factor influencing virulence development and biotypification.

3.5 Conclusion

The MSAP technique was successful in both detecting and quantifying RWA methylation in the CG and CC sequence context. Methylation could not, however, be quantified in all sequence contexts owing to the recognition site of HpaII and MspI. Use of the RSSFL technique enabled the detection of trends in RWA methylation, when used in conjunction with appropriate controls. Although differences in methylation trends were detected between the biotypes, the amount/level of methylation present in the biotypes could not be concluded with certainty using this technique.

The RSSFL technique is thus best suited to introductory studies of methylation, and can be used to detect trends in methylation, as shown here. The MSAP technique is a good follow-up technique to investigate methylation, as it provides more information regarding the sequence context (CC, CG or both) and level of methylation. In the current study, the most important information gleaned from the RSSFL experiment was the clear difference in methylation trends between biotypes SA1 and SAM. The MSAP results confirmed that there was a difference in methylation level between these biotypes. However, the methylation level of biotype SAM was higher than that of biotype SA1 when calculated using Kronforst *et al.*'s (2008) methodology, despite biotype SA1 exhibiting similar fluorescence levels, and thus methylation, to the more highly methylated human blood samples. This discrepancy could be explained by the use of only certain HpaII/MspI selective

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amplification primers during the MSAP experiment (Appendix A, Table A1). Whilst the RSSFL technique took into account all 5' CCGG 3' sites, only 5' CCGG 3' sites followed immediately downstream by an A or a T were surveyed in the MSAP experiment.

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3.6.1 Journal articles

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3.6.2 Websites

IDT dyes https://eu.idtdna.com/Site/Catalog/Modifications/Dyes

Primer3 http://bioinfo.ut.ee/primer3/

REBASE http://rebase.neb.com/rebase/rebase.html

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3.7 Appendix A

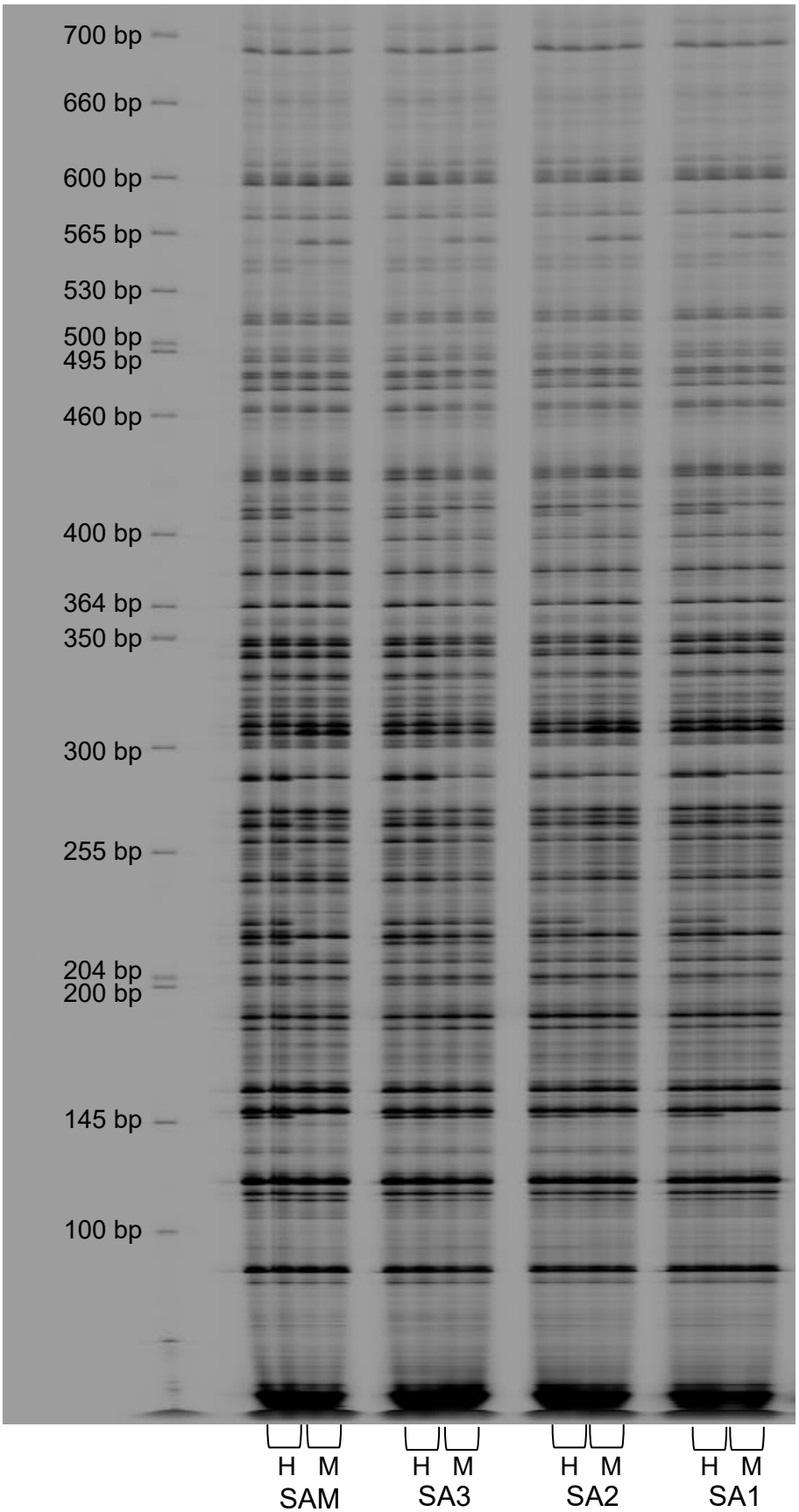

Figure A1. An example of selective amplification products (primer set AGC/T) resolved on a polyacrylamide gel. H = HpaII and M = MspI.

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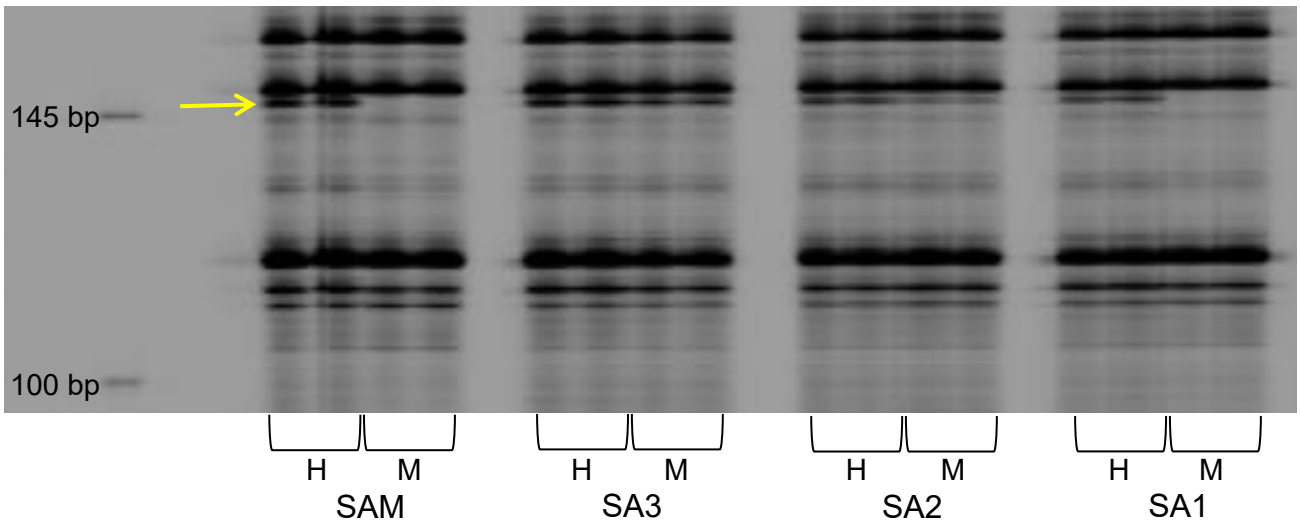

Figure A2. Example of a polymorphism (see arrow) where SA1 and SAM exhibit condition III (a band in only the HpaII lane), and SA2 and SA3 exhibit condition I (a band in both lanes). This is an enlarged version of Figure A1 (see ladder for fragment sizes). H = HpaII and M = MspI.

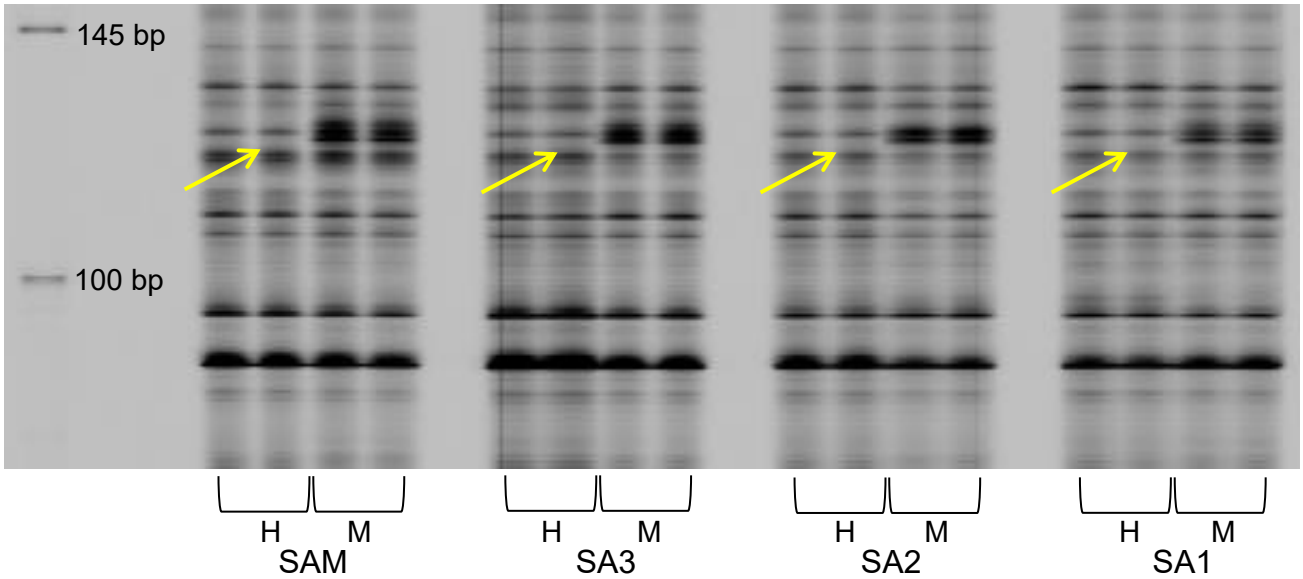

Figure A3. Example of a polymorphism (see arrows) where all biotypes exhibit condition II (a band in only the MspI lane). Selective amplification products of primer set ACC/A. H = HpaII and M = MspI.

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Table A1. Sequences of adaptors and primers used for MSAP and RSSFL experiments. Selective nucleotides are highlighted. The red circle represents the tetramethylrhodamine fluorophore.

Adaptor /primer	Sequence	Reference
MSAP HpaII/MspI adaptor	5'-GATCATGAGTCCTGCT-3' 3'-TACTCAGGACGAGC-5'	Current study
MSAP HpaII/MspI pre-amplification primer	5'-CATGAGTCCTGCTCG-3'	Current study
MSAP HpaII/MspI selective amplification primers	5'-CATGAGTCCTGCTCGGA-3' 5'-CATGAGTCCTGCTCGGT-3'	Current study
MSAP EcoRI adaptor	5'-CTCGTAGACTGCGTACC-3' 3'-CATCTGACGCATGGTTAA-5'	Vos *et al*. (1995)
MSAP EcoRI pre-amplification primer	5'-GACTGCGTACCAATTCA-3'	Vos *et al*. (1995)
MSAP EcoRI selective amplification primers**	5'-GACTGCGTACCAATTCACT-3' 5'-GACTGCGTACCAATTCACC-3' 5'-GACTGCGTACCAATTCACG-3' 5'-GACTGCGTACCAATTCAGC-3' 5'-GACTGCGTACCAATTCAGG-3'	Zhou *et al*. (2013)* Zhang *et al*. (2014)*
RSSFL tetramethylrhodamine-labelled HpaII/MspI adaptor	5'-CGGCGATCATGAGTCCTGCT-3' 3'-CGCTAGTACTCAGGAC-5'	Current study

*Insect study in which these primers have been used.

**EcoRI selective amplification primers are available in the AFLP® selective amplification kit from LI-COR Biosciences (USA).

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Table A2. Scoring of MSAP fragments after selective amplification of restricted DNA extracted from RWA biotypes SA1, SA2, SA3 and SAM. Primer set and fragment sizes are indicated. The total number of bands per primer set, as well as the number of bands present (+) or absent (-) for each enzyme (MspI or HpaII) per biotype is also noted.

			SA1		SA2		SA3		SAM	
Primer set	**Band number**	**Size (bp)**	**MspI**	**HpaII**	**MspI**	**HpaII**	**MspI**	**HpaII**	**MspI**	**HpaII**
ACG/T	1	85	+	+	+	+	+	+	+	+
ACG/T	2	105	+	+	+	+	+	+	+	+
ACG/T	3	106	+	+	+	+	+	+	+	+
ACG/T	4	119	+	+	+	+	+	+	+	+
ACG/T	4.1	122	+	+	+	+	+	+	+	+
ACG/T	5	154	+	+	+	+	+	+	+	+
ACG/T	6	170	+	+	+	+	+	+	+	+
ACG/T	8	179	+	+	+	+	+	+	-	+
ACG/T	9	182	+	-	+	-	+	-	+	-
ACG/T	10	186	+	+	+	+	+	+	+	+
ACG/T	11	198	+	+	+	+	+	+	+	+
ACG/T	12	200	+	+	+	+	+	+	+	+
ACG/T	13	206	+	+	+	+	+	+	+	+
ACG/T	14	209	+	+	+	+	+	+	+	+
ACG/T	15	214	+	+	+	+	+	+	+	+
ACG/T	17	224	+	+	+	+	+	+	+	+
ACG/T	18	232	+	+	+	+	+	+	+	+
ACG/T	19	247	+	+	+	+	+	+	+	+
ACG/T	20	250	+	+	+	+	+	+	+	+
ACG/T	21	260	+	-	+	-	+	-	+	-
ACG/T	22	268	+	+	+	+	+	+	+	+
ACG/T	23	269	+	+	+	+	+	+	+	+
ACG/T	24	271	+	+	+	+	+	+	+	+
ACG/T	25	277	+	+	+	+	+	+	+	+
ACG/T	26	283	+	+	+	+	+	+	+	+
ACG/T	27	287	+	+	+	+	+	+	+	+
ACG/T	29	297	+	+	+	+	+	+	+	+
ACG/T	30	308	+	+	+	+	+	+	+	+
ACG/T	31	311	+	+	+	+	+	+	+	+
ACG/T	32	315	+	+	+	+	+	+	+	+
ACG/T	34	323	+	+	+	+	+	+	+	+
ACG/T	35	327	+	+	+	+	+	+	+	+
ACG/T	36	336	+	+	+	+	+	+	+	+
ACG/T	37	344	+	+	+	+	+	+	+	+
ACG/T	38	350	+	+	+	+	+	+	+	+
ACG/T	39	354	+	+	+	+	+	+	+	+
ACG/T	40	356	+	+	+	+	+	+	+	+
ACG/T	41	358	+	+	+	+	+	+	+	+

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ACG/T	42	402	+	+	+	+	+	+	+	+
ACG/T	43	408	+	+	+	+	+	+	+	+
ACG/T	44	433	+	+	+	+	+	+	+	+
ACG/T	45	447	+	+	+	+	+	+	+	+
ACG/T	46	526	+	+	+	+	+	+	+	+
Total # bands per primer set = 43			**43+ / 0-**	**41+ / 2-**	**43+ / 0-**	**41+ / 2-**	**43+ / 0-**	**41+ / 2-**	**42+ / 1-**	**41+ / 2-**
ACT/T	1	104	+	+	+	+	+	+	+	+
ACT/T	2	115	+	+	+	+	+	+	+	+
ACT/T	3	116	+	+	+	+	+	+	+	+
ACT/T	4	121	-	+	-	+	+	+	-	+
ACT/T	5	124	+	+	+	+	+	+	+	+
ACT/T	6	126	+	+	+	+	+	+	+	+
ACT/T	7	127	+	+	+	+	+	+	+	+
ACT/T	8	133	+	+	+	+	+	+	+	+
ACT/T	9	136	+	+	+	+	+	+	+	+
ACT/T	10	144	+	+	+	+	+	+	+	+
ACT/T	11	147	+	+	+	+	+	+	+	+
ACT/T	12	158	+	+	+	+	+	+	+	+
ACT/T	13	162	+	+	+	+	+	+	+	+
ACT/T	14	165	+	+	+	+	+	+	+	+
ACT/T	15	169	+	+	+	+	+	+	+	+
ACT/T	16	170	+	+	+	+	+	+	+	+
ACT/T	17	171	+	+	+	+	+	+	+	+
ACT/T	18	176	+	+	+	+	+	+	+	+
ACT/T	19	185	+	+	+	+	+	+	+	+
ACT/T	20	190	+	-	+	-	+	-	+	-
ACT/T	22	199	+	+	+	+	+	+	+	+
ACT/T	23	205	+	+	+	+	+	+	+	+
ACT/T	23.1	207	+	+	+	+	+	+	+	+
ACT/T	23.2	208	+	+	+	+	+	+	+	+
ACT/T	24.1	214	+	+	+	+	+	+	+	+
ACT/T	24	215	+	+	+	+	+	+	+	+
ACT/T	24.2	217	+	+	+	+	+	+	+	+
ACT/T	25	224	+	+	+	+	+	+	+	+
ACT/T	26	227	-	+	+	+	+	+	-	+
ACT/T	27	232	+	+	+	+	+	+	+	+
ACT/T	28	242	+	+	+	+	+	+	+	+
ACT/T	29	250	+	+	+	+	+	+	+	+
ACT/T	30	256	+	+	+	+	+	+	+	+
ACT/T	31	258	+	+	+	+	+	+	+	+
ACT/T	32	258	+	+	+	+	+	+	+	+
ACT/T	33	262	+	+	+	+	+	+	+	+
ACT/T	34	264	+	+	+	+	+	+	+	+
ACT/T	35	267	+	+	+	+	+	+	+	+
ACT/T	36	268	+	+	+	+	+	+	+	+
ACT/T	37	272	+	+	+	+	+	+	+	+

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ACT/T	38	273	+	+	+	+	+	+	+	+
ACT/T	39	280	+	+	+	+	+	+	+	+
ACT/T	39.1	284	+	+	+	+	+	+	+	+
ACT/T	40	284	+	+	+	+	+	+	+	+
ACT/T	41	287	+	+	+	+	+	+	+	+
ACT/T	42	290	+	+	+	+	+	+	+	+
ACT/T	43	293	+	+	+	+	+	+	+	+
ACT/T	44	299	+	+	+	+	+	+	+	+
ACT/T	45	301	+	+	+	+	+	+	+	+
ACT/T	46	302	+	+	+	+	+	+	+	+
ACT/T	47	315	+	+	+	+	+	+	+	+
ACT/T	48	315	+	+	+	+	+	+	+	+
ACT/T	49	318	+	+	+	+	+	+	+	+
ACT/T	50	321	+	+	+	+	+	+	+	+
ACT/T	51	326	+	+	+	+	+	+	+	+
ACT/T	52	326	+	+	+	+	+	+	+	+
ACT/T	53	328	+	+	+	+	+	+	+	+
ACT/T	53.1	334	+	+	+	+	-	-	+	-
ACT/T	54	336	+	+	+	+	+	+	+	+
ACT/T	55	339	+	+	+	+	+	+	+	+
ACT/T	57	345	+	+	+	+	+	+	+	+
ACT/T	58	347	+	+	+	+	+	+	+	+
ACT/T	60	355	+	+	+	+	+	+	+	+
ACT/T	61	357	-	+	-	+	+	+	-	+
ACT/T	62	359	+	+	+	+	+	+	+	+
ACT/T	63	365	+	+	+	+	+	+	+	+
ACT/T	64	373	+	+	+	+	+	+	+	+
ACT/T	65	376	+	+	+	+	+	+	+	+
ACT/T	66	383	+	+	+	+	+	+	+	+
ACT/T	67	387	+	+	+	+	+	+	+	+
ACT/T	68	392	+	+	+	+	+	+	+	+
ACT/T	69	399	+	+	+	+	+	+	+	+
ACT/T	70	415	+	+	+	+	+	+	+	+
ACT/T	71	417	+	-	+	-	+	-	+	-
ACT/T	72	420	+	+	+	+	+	+	+	+
ACT/T	73	422	+	+	+	+	+	+	+	+
ACT/T	74	425	+	+	+	+	+	+	+	+
ACT/T	75	429	+	+	+	+	+	+	+	+
ACT/T	76	432	+	+	+	+	+	+	+	+
ACT/T	77	438	+	+	+	+	+	+	+	+
ACT/T	78	447	+	+	+	+	+	+	+	+
ACT/T	79	449	+	+	+	+	+	+	+	+
ACT/T	80	455	+	+	+	+	+	+	+	+
ACT/T	81	462	+	+	+	+	+	+	+	+
ACT/T	82	468	+	+	+	+	+	+	+	+
ACT/T	83	479	+	+	+	+	+	+	+	+

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ACT/T	84	487	+	+	+	+	+	+	+	+
ACT/T	85	505	+	+	+	+	+	+	+	+
ACT/T	86	512	+	+	+	+	+	+	+	+
ACT/T	87	530	+	+	+	+	+	+	+	+
ACT/T	88	567	+	+	+	+	+	+	+	+
Total # bands per primer set = 91			**88+ / 3-**	**89+ / 2-**	**89+ / 2-**	**89+ / 2-**	**90+ / 1-**	**88+ / 3-**	**88+ / 3-**	**88+ / 3-**
AGC/T	a	77	+	+	+	+	+	+	+	+
AGC/T	b	78	+	+	+	+	+	+	+	+
AGC/T	c	83	+	+	+	+	+	+	+	+
AGC/T	d	84	+	+	+	+	+	+	+	+
AGC/T	1	106	+	+	+	+	+	+	+	+
AGC/T	2	113	+	+	+	+	+	+	+	+
AGC/T	3	116	+	+	+	+	+	+	+	+
AGC/T	4	117	+	+	+	+	+	+	+	+
AGC/T	5	118	+	+	+	+	+	+	+	+
AGC/T	6	121	+	+	+	+	+	+	+	+
AGC/T	7	123	+	+	+	+	+	+	+	+
AGC/T	8	125	+	+	+	+	+	+	+	+
AGC/T	9	132	+	+	+	+	+	+	+	+
AGC/T	10	134	+	+	+	+	+	+	+	+
AGC/T	11	144	+	+	+	+	+	+	+	+
AGC/T	12	147	-	+	+	+	+	+	-	+
AGC/T	13	149	+	+	+	+	+	+	+	+
AGC/T	14	154	+	+	+	+	+	+	+	+
AGC/T	15	158	+	+	+	+	+	+	+	+
AGC/T	16	161	+	+	+	+	+	+	+	+
AGC/T	17	165	+	+	+	+	+	+	+	+
AGC/T	18	169	+	+	+	+	+	+	+	+
AGC/T	19	172	+	+	+	+	+	+	+	+
AGC/T	20	177	+	+	+	+	+	+	+	+
AGC/T	21	184	+	+	+	+	+	+	+	+
AGC/T	22	188	+	+	+	+	+	+	+	+
AGC/T	23	192	+	+	+	+	+	+	+	+
AGC/T	24	201	+	+	+	+	+	+	+	+
AGC/T	25	204	+	+	+	+	+	+	+	+
AGC/T	26	209	+	+	+	+	+	+	+	+
AGC/T	27	210	+	+	+	+	+	+	+	+
AGC/T	28	213	+	+	+	+	+	+	+	+
AGC/T	29	216	+	+	+	+	+	+	-	+
AGC/T	29.1	217	-	+	+	+	+	+	-	+
AGC/T	30	220	+	+	+	+	+	+	+	+
AGC/T	31	221	+	+	+	+	+	+	+	+
AGC/T	32	224	+	+	+	+	+	+	+	+
AGC/T	33	224	+	+	+	+	+	+	+	+
AGC/T	34	225	+	+	+	+	+	+	+	+
AGC/T	35	228	+	+	+	+	+	+	+	+

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AGC/T	36	236	+	+	+	+	+	+	+	+
AGC/T	37	240	+	+	+	+	+	+	+	+
AGC/T	38	240	+	+	+	+	+	+	+	+
AGC/T	39	242	+	+	+	+	+	+	+	+
AGC/T	40	245	+	+	+	+	+	+	+	+
AGC/T	41	249	+	+	+	+	+	+	+	+
AGC/T	42	250	+	+	+	+	+	+	+	+
AGC/T	43	252	+	+	+	+	+	+	+	+
AGC/T	44	255	+	+	+	+	+	+	+	+
AGC/T	45	260	+	+	+	+	+	+	+	+
AGC/T	46	262	+	+	+	+	-	-	+	+
AGC/T	47	263	+	+	+	+	+	+	+	+
AGC/T	48	267	+	+	+	+	+	+	+	+
AGC/T	49	270	+	+	+	+	+	+	+	+
AGC/T	50	285	-	+	-	+	-	+	-	+
AGC/T	51	286	+	+	+	+	+	+	+	+
AGC/T	52	291	+	+	+	+	+	+	+	+
AGC/T	53	300	+	+	+	+	+	+	+	+
AGC/T	54	303	+	+	+	+	+	+	+	+
AGC/T	55	304	+	+	+	+	+	+	+	+
AGC/T	56	307	+	+	+	+	+	+	+	+
AGC/T	57	308	+	+	+	+	+	+	+	+
AGC/T	58	310	+	+	+	+	+	+	+	+
AGC/T	59	314	+	+	+	+	+	+	+	+
AGC/T	60	319	+	+	+	+	+	+	+	+
AGC/T	61	321	+	+	+	+	+	+	+	+
AGC/T	62	323	+	+	+	+	+	+	+	+
AGC/T	63	326	+	+	+	+	+	+	+	+
AGC/T	64	331	+	+	+	+	+	+	+	+
AGC/T	65	333	+	+	+	+	+	+	+	+
AGC/T	66	339	+	+	+	+	+	+	+	+
AGC/T	67	341	+	+	+	+	+	+	+	+
AGC/T	68	343	+	+	+	+	+	+	+	+
AGC/T	69	347	+	+	+	+	+	+	+	+
AGC/T	70	349	+	+	+	+	+	+	+	+
AGC/T	71	354	+	+	+	+	+	+	+	+
AGC/T	72	356	+	+	+	+	+	+	+	+
AGC/T	73	366	+	+	+	+	+	+	+	+
AGC/T	74	373	+	+	+	+	+	+	+	+
AGC/T	75	378	+	+	+	+	+	+	+	+
AGC/T	76	380	+	+	+	+	+	+	+	+
AGC/T	77	391	+	+	+	+	+	+	+	+
AGC/T	78	397	+	+	+	+	+	+	+	+
AGC/T	79	401	+	+	+	+	+	+	+	+
AGC/T	80	408	-	+	-	+	-	+	-	+
AGC/T	81	410	+	+	+	+	+	+	-	+

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AGC/T	82	413	+	+	+	+	+	+	-	+
AGC/T	83	417	+	+	+	+	+	+	+	+
AGC/T	84	420	+	+	+	+	+	+	+	+
AGC/T	85	424	-	+	-	+	-	+	-	+
AGC/T	86	427	+	+	+	+	+	+	+	+
AGC/T	87	429	+	+	+	+	+	+	+	+
AGC/T	88	431	+	+	+	+	+	+	+	+
AGC/T	89	462	+	+	+	+	+	+	+	+
AGC/T	90	464	+	+	+	+	+	+	+	+
AGC/T	91	474	+	+	+	+	+	+	+	+
AGC/T	92	478	+	+	+	+	+	+	+	+
AGC/T	93	480	+	+	+	+	+	+	+	+
AGC/T	94	482	+	+	+	+	+	+	+	+
AGC/T	95	489	+	+	+	+	+	+	+	+
AGC/T	96	492	+	+	+	+	+	+	+	+
AGC/T	97	496	+	+	+	+	+	+	+	+
AGC/T	98	513	+	+	+	+	+	+	+	+
AGC/T	99	516	+	+	+	+	+	+	+	+
AGC/T	100	543	+	+	+	+	+	+	-	+
AGC/T	101	548	+	+	+	+	+	+	+	+
AGC/T	102	559	+	-	+	-	+	-	+	-
AGC/T	103	574	+	+	+	+	+	+	+	+
AGC/T	104	596	+	+	+	+	+	+	+	+
AGC/T	105	609	+	+	+	+	+	+	+	+
AGC/T	106	689	+	+	+	+	+	+	+	+
Total # bands per primer set = 111			**106+ / 5-**	**110+ / 1-**	**108+ / 3-**	**110+ / 1-**	**107+ / 4-**	**109+ / 2-**	**102+ / 9-**	**110+ / 1-**
ACG/A	1	106	+	+	+	+	+	+	+	+
ACG/A	2	120	+	+	+	+	+	+	+	+
ACG/A	3	135	+	+	+	+	+	+	+	+
ACG/A	4	136	+	+	+	+	+	+	+	+
ACG/A	5	138	+	+	+	+	+	+	+	+
ACG/A	6	140	+	+	+	+	+	+	+	+
ACG/A	7	141	+	+	+	+	+	+	+	+
ACG/A	8	144	+	+	+	+	+	+	+	+
ACG/A	9	155	+	+	+	+	+	+	+	+
ACG/A	9.1	157	+	+	+	+	+	+	+	+
ACG/A	10	163	+	+	+	+	+	+	+	+
ACG/A	11	164	+	+	+	+	+	+	+	+
ACG/A	12	168	+	+	+	+	+	+	+	+
ACG/A	14	177	-	-	+	-	+	-	-	-
ACG/A	15	184	+	+	+	+	+	+	+	+
ACG/A	15.1	186	+	+	+	+	+	+	+	+
ACG/A	15.2	188	+	+	+	+	+	+	+	+
ACG/A	16	196	+	+	+	+	+	+	+	+
ACG/A	17	198	+	+	+	+	+	+	+	+
ACG/A	18	206	+	+	+	+	+	+	+	+

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ACG/A	19	208	+	+	+	+	+	+	+	+
ACG/A	20	210	+	+	+	+	+	+	+	+
ACG/A	21	213	+	+	+	+	+	+	+	+
ACG/A	22	216	+	+	+	+	+	+	+	+
ACG/A	23	217	-	+	+	+	+	+	-	+
ACG/A	24	219	+	+	+	+	+	+	+	+
ACG/A	25	220	+	+	+	+	+	+	+	+
ACG/A	26	229	+	+	+	+	+	+	+	+
ACG/A	27	232	+	+	+	+	+	+	+	+
ACG/A	28	235	+	+	+	+	+	+	+	+
ACG/A	29	236	+	+	+	+	+	+	+	+
ACG/A	30	240	+	+	+	+	+	+	+	+
ACG/A	31	246	+	+	+	+	+	+	+	+
ACG/A	32	248	+	+	+	+	+	+	+	+
ACG/A	33	250	+	+	+	+	+	+	+	+
ACG/A	34	260	+	+	+	+	+	+	+	+
ACG/A	35	263	+	+	+	+	+	+	+	+
ACG/A	36	265	+	+	+	+	+	+	+	+
ACG/A	37	266	+	+	+	+	+	+	+	+
ACG/A	38	267	+	+	+	+	+	+	+	+
ACG/A	38.1	269	+	+	+	+	+	+	+	+
ACG/A	39	279	+	+	+	+	+	+	+	+
ACG/A	40	283	+	+	+	+	+	+	+	+
ACG/A	41	286	+	+	+	+	+	+	+	+
ACG/A	42	290	+	+	+	+	+	+	+	+
ACG/A	42.1	292	+	+	+	+	+	+	+	+
ACG/A	42.2	294	+	+	+	+	+	+	+	+
ACG/A	43	296	+	+	+	+	+	+	+	+
ACG/A	43.1	298	+	+	+	+	+	+	+	+
ACG/A	44	301	+	+	+	+	+	+	+	+
ACG/A	44.1	303	+	+	+	+	+	+	+	+
ACG/A	45	307	+	+	+	+	+	+	+	+
ACG/A	45.1	309	+	+	+	+	+	+	+	+
ACG/A	45.2	310	+	+	+	+	+	+	+	+
ACG/A	46	314	+	+	+	+	+	+	+	+
ACG/A	47	316	+	+	+	+	+	+	+	+
ACG/A	48	318	+	+	+	+	+	+	+	+
ACG/A	49	336	+	+	+	+	+	+	+	+
ACG/A	50	338	+	+	+	+	+	+	+	+
ACG/A	51	354	+	+	+	+	+	+	+	+
ACG/A	52	366	+	+	+	+	+	+	+	+
ACG/A	53	368	+	+	+	+	+	+	+	+
ACG/A	54	375	+	+	+	+	+	+	+	+
ACG/A	55	377	+	+	+	+	+	+	+	+
ACG/A	56	381	+	+	+	+	+	+	+	+
ACG/A	57	412	+	+	+	+	+	+	+	+

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ACG/A	58	439	+	+	+	+	+	+	+	+
ACG/A	59	448	+	+	+	+	+	+	+	+
ACG/A	60	525	+	+	+	+	+	+	+	+
Total # bands per primer set = 69			**67+ / 2-**	**68+ / 1-**	**69+ / 0-**	**68+ / 1-**	**69+ / 0-**	**68+ / 1-**	**67+ / 2-**	**68+ / 1-**
AGC/A	a	89	+	+	+	+	+	+	+	+
AGC/A	b	90	+	+	+	+	+	+	+	+
AGC/A	c	92	+	+	+	+	+	+	+	+
AGC/A	1	98	+	+	+	+	+	+	+	+
AGC/A	2	99	+	+	+	+	+	+	+	+
AGC/A	3	101	+	+	+	+	+	+	+	+
AGC/A	4	104	+	+	+	+	+	+	+	+
AGC/A	5	109	+	+	+	+	+	+	+	+
AGC/A	6	117	+	+	+	+	+	+	+	+
AGC/A	7	121	+	+	+	+	+	+	+	+
AGC/A	8	122	+	+	+	+	+	+	+	+
AGC/A	9	123	+	+	+	+	+	+	+	+
AGC/A	10	124	+	+	+	+	+	+	+	+
AGC/A	11	125	+	+	+	+	+	+	+	+
AGC/A	12	127	+	+	+	+	+	+	+	+
AGC/A	13	128	+	+	+	+	+	+	+	+
AGC/A	14	130	+	+	+	+	+	+	+	+
AGC/A	15	138	+	+	+	+	+	+	+	+
AGC/A	16	139	+	+	+	+	+	+	+	+
AGC/A	17	144	-	+	+	+	+	+	-	+
AGC/A	18	145	-	-	-	+	+	+	-	+
AGC/A	19	147	+	+	+	+	+	+	+	+
AGC/A	19.1	149	+	+	+	+	+	+	+	+
AGC/A	19.2	149	+	+	+	+	+	+	+	+
AGC/A	20	151	+	+	+	+	+	+	+	+
AGC/A	21	152	+	-	-	-	-	-	+	-
AGC/A	22	152	+	+	+	+	+	+	+	+
AGC/A	23	154	+	+	+	+	+	+	+	+
AGC/A	24	157	+	+	+	+	+	+	+	+
AGC/A	24.1	158	+	+	+	+	+	+	+	+
AGC/A	25	181	+	+	+	+	+	+	+	+
AGC/A	26	196	-	-	+	-	-	-	-	-
AGC/A	27	196	-	+	+	-	-	-	-	-
AGC/A	28	199	+	+	+	+	+	+	+	+
AGC/A	29	202	+	+	+	+	+	+	+	+
AGC/A	30	203	+	+	+	+	+	+	+	+
AGC/A	31	205	+	+	+	+	+	+	+	+
AGC/A	32	206	+	+	+	+	+	+	+	+
AGC/A	33	208	+	+	+	+	+	+	+	+
AGC/A	34	210	+	+	+	+	+	+	+	+
AGC/A	35	213	+	+	+	+	+	+	+	+
AGC/A	36	216	+	+	+	+	+	+	+	+

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AGC/A	37	222	+	+	+	+	+	+	+	+
AGC/A	38	223	-	+	-	+	-	+	-	+
AGC/A	39	225	-	+	-	+	-	+	-	+
AGC/A	40	227	+	+	+	+	+	+	+	+
AGC/A	41	232	+	+	+	+	+	+	+	+
AGC/A	42	236	+	+	+	+	+	+	+	+
AGC/A	43	238	+	+	+	+	+	+	+	+
AGC/A	44	256	+	+	+	+	+	+	+	+
AGC/A	45	258	+	+	+	+	+	+	+	+
AGC/A	46	260	+	+	+	+	+	+	+	+
AGC/A	47	268	+	+	+	+	+	+	-	+
AGC/A	48	273	+	+	+	+	+	+	+	+
AGC/A	49	274	+	+	+	+	+	+	+	+
AGC/A	51	279	+	+	+	+	+	+	+	+
AGC/A	52	284	+	+	+	+	+	+	+	+
AGC/A	53	288	+	+	+	+	+	+	+	+
AGC/A	54	294	+	+	+	+	+	+	-	+
AGC/A	55	299	+	+	+	+	+	+	+	+
AGC/A	56	301	+	+	+	+	+	+	+	+
AGC/A	57	303	+	+	+	+	+	+	+	+
AGC/A	58	304	+	+	+	+	+	+	+	+
AGC/A	59	306	+	+	+	+	+	+	+	+
AGC/A	60	308	+	+	+	+	+	+	+	+
AGC/A	61	310	+	+	+	+	+	+	+	+
AGC/A	62	312	+	+	+	+	+	+	+	+
AGC/A	63	313	+	+	+	+	+	+	+	+
AGC/A	64	315	+	+	+	+	+	+	+	+
AGC/A	65	318	+	+	+	+	+	+	+	+
AGC/A	66	325	+	+	+	+	+	+	+	+
AGC/A	67	327	+	+	+	+	+	+	+	+
AGC/A	68	328	+	+	+	+	+	+	+	+
AGC/A	69	332	+	+	+	+	+	+	+	+
AGC/A	70	338	+	+	+	+	+	+	+	+
AGC/A	71	340	+	+	+	+	+	+	+	+
AGC/A	72	342	+	+	+	+	+	+	+	+
AGC/A	73	344	+	-	+	-	+	-	+	-
AGC/A	74	348	+	+	+	+	+	+	+	+
AGC/A	75	352	+	+	+	+	+	+	+	+
AGC/A	76	357	+	+	+	+	+	+	+	+
AGC/A	78	365	+	+	+	+	+	+	+	+
AGC/A	79	368	+	+	+	+	+	+	+	+
AGC/A	80	370	+	+	+	+	+	+	+	+
AGC/A	81	379	+	+	+	+	+	+	+	+
AGC/A	82	385	+	+	+	+	+	+	+	+
AGC/A	83	390	+	+	+	+	+	+	+	+
AGC/A	84	394	+	+	+	+	+	+	+	+

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AGC/A	85	405	+	+	+	+	+	+	+	+
AGC/A	86	374	+	+	+	+	+	+	+	+
AGC/A	87	379	+	+	+	+	+	+	+	+
AGC/A	88	383	+	+	+	+	+	+	-	+
AGC/A	89	387	+	+	+	+	+	+	+	+
AGC/A	90	392	+	+	+	+	+	+	+	+
AGC/A	91	398	+	+	+	+	+	+	+	-
AGC/A	92	403	+	+	+	+	+	+	+	+
AGC/A	93	408	+	+	+	+	+	+	+	+
AGC/A	94	412	+	+	+	+	+	+	+	+
AGC/A	95	434	+	+	+	+	+	+	+	+
AGC/A	96	478	+	+	+	+	+	+	+	+
AGC/A	97	485	+	+	+	+	+	+	+	+
AGC/A	98	500	+	+	+	+	+	+	+	+
AGC/A	99	502	+	+	+	+	+	+	+	+
AGC/A	100	506	+	+	+	+	+	+	+	-
AGC/A	101	511	+	+	+	+	+	+	+	+
AGC/A	102	513	+	+	+	+	+	+	+	+
AGC/A	103	536	+	+	+	+	+	+	+	+
AGC/A	104	539	+	+	+	+	+	+	+	+
AGC/A	105	689	+	+	+	+	+	+	+	+
Total # bands per primer set = 109			**103+ / 6-**	**105+ / 4-**	**105+ / 4-**	**105+ / 4-**	**104+ / 5-**	**105+ / 4-**	**100+ / 9-**	**103+ / 6-**
AGG/A	1	84	-	+	+	+	+	+	-	+
AGG/A	2	85	+	+	+	+	+	+	+	+
AGG/A	3	87	+	+	+	+	+	+	+	+
AGG/A	4	89	+	+	+	+	+	+	+	+
AGG/A	5	93	+	+	+	+	+	+	+	+
AGG/A	6	101	+	+	+	+	+	+	+	+
AGG/A	7	103	-	-	-	+	+	+	-	+
AGG/A	8	106	+	+	+	+	+	+	+	+
AGG/A	9	113	+	+	+	+	+	+	+	+
AGG/A	10	115	+	+	+	+	+	+	+	+
AGG/A	11	120	-	+	+	+	+	+	-	+
AGG/A	12	131	+	+	+	+	+	+	+	+
AGG/A	13	133	+	+	+	+	+	+	+	+
AGG/A	14	138	+	+	+	+	+	+	+	+
AGG/A	15	142	+	+	+	+	+	+	+	+
AGG/A	16	156	+	+	+	+	+	+	+	+
AGG/A	17	163	+	+	+	+	+	+	+	+
AGG/A	18	169	+	-	+	+	+	-	+	+
AGG/A	19	174	+	+	+	+	+	+	+	+
AGG/A	20	178	+	+	+	+	+	+	+	+
AGG/A	21	184	+	+	+	+	+	+	+	+
AGG/A	22	186	+	+	+	+	+	+	+	+
AGG/A	23	188	+	+	+	+	+	+	+	+
AGG/A	24	190	+	+	+	+	+	+	+	+

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AGG/A	25	195	+	+	+	+	+	+	+	+
AGG/A	26	199	+	+	+	+	+	+	+	+
AGG/A	27	201	+	+	+	+	+	+	-	-
AGG/A	28	205	+	+	+	+	+	+	+	+
AGG/A	29	207	+	+	+	+	+	+	+	+
AGG/A	30	208	+	+	+	+	+	+	+	+
AGG/A	31	211	+	+	+	+	+	+	+	+
AGG/A	32	215	+	+	+	+	+	+	+	+
AGG/A	33	219	+	+	+	+	+	+	+	+
AGG/A	34	223	+	+	+	+	+	+	+	+
AGG/A	35	225	+	+	+	+	+	+	+	+
AGG/A	36	227	+	+	+	+	+	+	+	+
AGG/A	37	236	+	+	+	+	+	+	+	+
AGG/A	38	239	+	+	+	+	+	+	+	+
AGG/A	39	241	+	+	+	+	+	+	+	+
AGG/A	40	246	-	+	+	+	+	+	-	+
AGG/A	41	247	-	+	+	+	+	+	-	+
AGG/A	42	261	+	-	+	-	+	-	+	-
AGG/A	43	262	+	-	+	-	+	-	+	-
AGG/A	44	263	+	+	+	+	+	+	+	+
AGG/A	45	265	+	+	+	+	+	+	+	+
AGG/A	46	266	+	+	+	+	+	+	+	+
AGG/A	47	268	+	+	+	+	+	+	+	+
AGG/A	48	275	+	+	+	+	+	+	+	+
AGG/A	49	278	+	+	+	+	+	+	+	+
AGG/A	50	280	+	+	+	+	+	+	+	+
AGG/A	51	285	+	+	+	+	+	+	+	+
AGG/A	51.1	288	+	+	+	+	+	+	+	+
AGG/A	51.2	290	+	+	+	+	+	+	+	+
AGG/A	51.3	293	+	+	+	+	+	+	+	+
AGG/A	52	300	+	+	+	+	+	+	+	+
AGG/A	53	314	+	+	+	+	+	+	+	+
AGG/A	54	319	+	+	+	+	+	+	+	+
AGG/A	55	320	+	+	+	+	+	+	+	+
AGG/A	56	323	+	+	+	+	+	+	+	+
AGG/A	57	326	+	+	+	+	+	+	+	+
AGG/A	58	328	+	+	+	+	+	+	+	+
AGG/A	59	333	+	+	+	+	+	+	+	+
AGG/A	60	340	+	+	+	+	+	+	+	+
AGG/A	61	348	+	+	+	+	+	+	+	+
AGG/A	62	349	+	+	+	+	+	+	+	+
AGG/A	63	353	+	+	+	+	+	+	+	+
AGG/A	64	360	+	+	+	+	+	+	+	+
AGG/A	65	362	+	+	+	+	+	+	+	+
AGG/A	66	375	+	+	+	+	+	+	+	+
AGG/A	67	378	+	+	+	+	+	+	+	+

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AGG/A	68	380	+	+	+	+	+	+	+	+
AGG/A	69	387	+	+	+	+	+	+	+	+
AGG/A	70	391	+	+	+	+	+	+	+	+
AGG/A	71	396	+	+	+	+	+	+	+	+
AGG/A	72	416	+	+	+	+	+	+	+	+
AGG/A	73	428	+	+	+	+	+	+	+	+
AGG/A	74	433	+	+	+	+	+	+	+	+
AGG/A	75	435	+	+	+	+	+	+	+	+
AGG/A	76	438	+	+	+	+	+	+	+	+
AGG/A	77	444	+	+	+	+	+	+	+	+
AGG/A	78	461	+	+	+	+	+	+	+	+
AGG/A	79	479	+	+	+	+	+	+	+	+
AGG/A	80	482	+	+	+	+	+	+	+	+
AGG/A	81	493	-	+	+	+	+	+	-	+
AGG/A	82	504	+	+	+	+	+	+	+	+
AGG/A	83	513	+	+	+	+	+	+	+	+
AGG/A	84	520	+	+	+	+	+	+	+	+
AGG/A	85	577	+	+	+	+	+	+	+	+
AGG/A	86	601	+	+	+	+	+	+	+	+
Total # bands per primer set = 89			**83+ / 6-**	**85+ / 4-**	**88+ / 1-**	**87+ / 2-**	**89+ / 0-**	**86+ / 3-**	**82+ / 7-**	**86+ / 3-**
ACC/A	1	75	+	+	+	+	+	+	+	+
ACC/A	2	78	+	+	+	+	+	+	+	+
ACC/A	3	83	+	+	+	+	+	+	+	+
ACC/A	4	84	+	+	+	+	+	+	+	+
ACC/A	5	93	+	+	+	+	+	+	+	+
ACC/A	6	94	+	+	+	+	+	+	+	+
ACC/A	7	96	-	+	+	-	-	-	-	-
ACC/A	8	103	+	+	+	+	+	+	+	+
ACC/A	9	106	+	+	+	+	+	+	+	+
ACC/A	10	108	+	+	+	+	+	+	+	+
ACC/A	11	112	+	+	+	+	+	+	+	+
ACC/A	12	112	+	+	-	+	+	+	+	+
ACC/A	13	114	-	+	-	+	+	+	-	+
ACC/A	14	115	+	+	+	+	+	+	+	+
ACC/A	15	116	+	+	+	+	+	+	+	+
ACC/A	16	121	+	+	+	+	+	+	+	+
ACC/A	17	123	+	+	+	+	+	+	+	+
ACC/A	18	124	+	-	+	-	+	-	+	-
ACC/A	19	125	+	-	+	-	+	-	+	-
ACC/A	20	127	+	+	+	+	+	+	+	+
ACC/A	21	127	+	-	+	-	+	-	+	-
ACC/A	22	128	+	-	+	-	+	-	+	-
ACC/A	22.1	129	-	-	-	-	+	-	+	-
ACC/A	23	131	+	+	+	+	+	+	+	+
ACC/A	24	135	+	+	+	+	+	+	+	+
ACC/A	25	142	+	+	+	+	+	+	+	+

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ACC/A	26	147	+	+	+	+	+	+	+	+
ACC/A	27	150	+	+	+	+	+	+	+	+
ACC/A	28	152	+	+	+	+	+	+	+	+
ACC/A	29	156	+	+	+	+	+	+	+	+
ACC/A	30	159	+	+	+	+	+	+	+	+
ACC/A	31	163	+	+	+	+	+	+	+	+
ACC/A	32	164	+	+	+	+	+	+	+	+
ACC/A	33	169	+	+	+	+	+	+	+	+
ACC/A	34	171	+	+	+	+	+	+	+	+
ACC/A	35	180	+	+	+	+	+	+	+	+
ACC/A	36	182	+	+	+	+	+	+	+	+
ACC/A	37	186	+	+	+	+	+	+	+	+
ACC/A	38	188	+	+	+	+	+	+	+	+
ACC/A	39	190	+	+	+	+	+	+	+	+
ACC/A	40	192	+	+	+	+	+	+	+	+
ACC/A	41	199	+	+	+	+	+	+	+	+
ACC/A	42	201	+	+	+	+	+	+	+	+
ACC/A	43	201	+	+	+	+	+	+	+	+
ACC/A	44	205	+	+	+	+	+	+	+	+
ACC/A	45	207	+	+	+	+	+	+	+	+
ACC/A	46	209	+	+	+	+	+	+	+	+
ACC/A	47	211	+	+	+	+	+	+	+	+
ACC/A	48	216	+	+	+	+	+	+	+	+
ACC/A	49	220	+	+	+	+	+	+	+	+
ACC/A	50	221	+	+	+	+	+	+	+	+
ACC/A	51	223	+	+	+	+	+	+	+	+
ACC/A	52	225	+	+	+	+	+	+	+	+
ACC/A	53	227	+	+	+	+	+	+	+	+
ACC/A	54	230	+	+	+	+	+	+	+	+
ACC/A	55	231	+	+	+	+	+	+	+	+
ACC/A	56	234	+	+	+	+	+	+	+	+
ACC/A	57	235	+	+	+	+	+	+	+	+
ACC/A	58	237	+	+	+	+	+	+	+	+
ACC/A	59	238	+	+	+	+	+	+	+	+
ACC/A	60	241	+	+	+	+	+	+	+	+
ACC/A	61	243	+	+	+	+	+	+	+	+
ACC/A	62	249	+	+	+	+	+	+	+	+
ACC/A	63	250	+	+	+	+	+	+	+	+
ACC/A	64	252	+	+	+	+	+	+	+	+
ACC/A	65	252	+	+	+	+	+	+	+	+
ACC/A	66	253	+	+	+	+	+	+	+	+
ACC/A	67	254	+	+	+	+	+	+	+	+
ACC/A	68	258	+	+	+	+	+	+	+	+
ACC/A	69	260	+	+	+	+	+	+	+	+
ACC/A	70	270	+	+	+	+	+	+	+	+
ACC/A	71	276	+	+	+	+	+	+	+	+

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ACC/A	72	279	+	+	+	+	+	+	+	+
ACC/A	73	282	+	+	+	+	+	+	+	+
ACC/A	74	283	+	+	+	+	+	+	-	+
ACC/A	75	283	+	+	+	+	+	+	-	+
ACC/A	76	285	-	+	+	+	+	+	-	+
ACC/A	77	287	+	+	+	+	+	+	+	+
ACC/A	78	288	+	+	+	+	+	+	+	+
ACC/A	79	290	+	+	+	+	+	+	+	+
ACC/A	80	290	+	+	+	+	+	+	+	+
ACC/A	81	295	+	+	+	+	+	+	+	+
ACC/A	82	299	+	+	+	+	+	+	+	+
ACC/A	83	303	+	+	+	+	+	+	+	+
ACC/A	84	304	+	+	+	+	+	+	+	+
ACC/A	85	308	+	+	+	+	+	+	+	+
ACC/A	86	312	+	+	+	+	+	+	+	+
ACC/A	87	313	+	+	+	+	+	+	+	+
ACC/A	88	318	+	+	+	+	+	+	+	+
ACC/A	89	319	+	+	+	+	+	+	+	+
ACC/A	90	321	+	+	+	+	+	+	+	-
ACC/A	91	322	+	+	+	+	+	+	+	+
ACC/A	92	329	+	+	+	+	+	+	+	+
ACC/A	93	338	+	+	+	+	+	+	+	+
ACC/A	94	344	+	+	+	+	+	+	+	+
ACC/A	95	351	+	+	+	+	+	+	+	+
ACC/A	96	360	+	+	+	+	+	+	+	+
ACC/A	97	363	+	+	+	+	+	+	+	+
ACC/A	98	365	+	+	+	+	+	+	+	+
ACC/A	99	366	+	+	+	+	+	+	-	+
ACC/A	100	368	+	+	+	+	+	+	+	+
ACC/A	101	369	+	+	+	+	+	+	+	+
ACC/A	102	386	+	+	+	+	+	+	+	+
ACC/A	103	388	+	+	+	+	+	+	+	+
ACC/A	104	399	+	+	+	+	+	+	+	+
ACC/A	105	407	+	+	+	+	+	+	+	+
ACC/A	106	409	+	+	+	+	+	+	+	+
ACC/A	107	415	+	+	+	+	+	+	+	+
ACC/A	108	421	+	+	+	+	+	+	+	+
ACC/A	109	429	+	+	+	+	+	+	+	+
ACC/A	110	433	+	+	+	+	+	+	+	+
ACC/A	111	444	+	+	+	+	+	+	-	+
ACC/A	112	449	+	+	+	+	+	+	+	+
ACC/A	113	455	+	+	+	+	+	+	+	+
ACC/A	114	468	+	+	+	+	+	+	+	+
ACC/A	115	471	+	+	+	+	+	+	+	+
ACC/A	116	474	+	+	+	+	+	+	+	+
ACC/A	117	497	+	+	+	+	+	+	+	+

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ACC/A	118	515	+	+	+	+	+	+	+	+
ACC/A	119	521	+	+	+	+	+	+	+	+
ACC/A	120	547	+	+	+	+	+	+	+	+
ACC/A	121	604	+	+	+	+	+	+	+	+
ACC/A	122	617	-	+	-	+	-	+	-	+
ACC/A	123	621	+	+	+	+	+	+	+	+
ACC/A	124	635	+	+	+	+	+	+	+	+
Total # bands per primer set = 125			**120+ / 5-**	**120+ / 5-**	**121+ / 4-**	**119+ / 6-**	**123+ / 2-**	**119+ / 6-**	**117+ / 8-**	**118+ / 7-**
Total # bands across all primer sets = 637			**SA1**		**SA2**		**SA3**		**SAM**	
			MspI	**HpaII**	**MspI**	**HpaII**	**MspI**	**HpaII**	**MspI**	**HpaII**
			610+ / 27-	**618+ / 19-**	**623+ / 14-**	**619+ / 18-**	**625+ / 12-**	**616+ / 21-**	**598+ / 39-**	**614+ / 23-**

Table A3. Probability values obtained from the Shapiro-Wilk test for normality, and from dependent *t*-test comparisons of within-species and within-biotype HpaII and MspI fluorescence readings. The level of significance for both tests was set at $p \leq 0.05$.

Comparison	Shapiro-Wilk *p* value	Dependent *t*-test *p* value
Human MspI vs human HpaII	0.059	0.300
Bee MspI vs bee HpaII	0.536	0.056
SA1 MspI vs SA1 HpaII	0.550	0.675
SA2 MspI vs SA2 HpaII	0.548	0.147
SA3 MspI vs SA3 HpaII	0.399	0.205
SAM MspI vs SAM HpaII	0.191	0.802

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Table A4. Probability values obtained from the ANOVA, Shapiro-Wilk and Levene's test (level of significance of $p \leq 0.05$ for these three tests) using the MspI fluorescence readings. Probability values from Fisher's LSD test with Bonferonni adjustment are also shown (modified significance level of 0.003). Probability values indicating a significant difference are in boldface.

Test	***p* value**
ANOVA	**0.0003**
Shapiro-Wilk	0.962
Levene	**0.032***
Fisher's LSD comparison	
SA1 MspI vs SA2 MspI	0.467
SA1 MspI vs SA3 MspI	0.715
SA1 MspI vs SAM MspI	**0.001**
SA2 MspI vs SA3 MspI	0.283
SA2 MspI vs SAM MspI	**0.0004**
SA3 MspI vs SAM MspI	**0.003**
Human MspI vs SA1 MspI	0.168
Human MspI vs SA2 MspI	0.486
Human MspI vs SA3 MspI	0.091
Human MspI vs SAM MspI	**0.0001**
Human MspI vs Bee MspI	**0.0001**
Bee MspI vs SA1 MspI	**0.001**
Bee MspI vs SA2 MspI	**0.0003**
Bee MspI vs SA3 MspI	**0.002**
Bee MspI vs SAM MspI	0.513

*The ANOVA was performed despite a Levene's test *p* value of less than 0.05 because according to McDonald (2008), "parametric tests are not particularly sensitive to violations" of the assumption of homoscedasticity.

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Table A5. Probability values obtained from the ANOVA, Shapiro-Wilk and Levene's test (level of significance of $p \leq 0.05$ for these three tests) using the HpaII fluorescence readings. Probability values from Fisher's LSD test with Bonferonni adjustment are also shown (modified significance level of 0.003). Probability values indicating a significant difference are in boldface.

Test	***p* value**
ANOVA	**0.007**
Shapiro-Wilk	0.322
Levene	0.061
Fisher's LSD comparison	
SA1 HpaII vs SA2 HpaII	0.277
SA1 HpaII vs SA3 HpaII	0.010
SA1 HpaII vs SAM HpaII	**0.002**
SA2 HpaII vs SA3 HpaII	0.072
SA2 HpaII vs SAM HpaII	0.019
SA3 HpaII vs SAM HpaII	0.461
Human HpaII vs SA1 HpaII	0.862
Human HpaII vs SA2 HpaII	0.355
Human HpaII vs SA3 HpaII	0.013
Human HpaII vs SAM HpaII	0.003*
Human HpaII vs Bee HpaII	0.009
Bee HpaII vs SA1 HpaII	0.007
Bee HpaII vs SA2 HpaII	0.042
Bee HpaII vs SA3 HpaII	0.613
Bee HpaII vs SAM HpaII	0.873

**p* value = 0.00339 and was thus not significant.

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Method A1. *Homo sapiens* blood DNA extraction

Lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA) (30 ml) was added to 10 ml of human blood in a polypropylene tube, mixed by inversion and placed on ice for a duration of 30 min, during which the contents were mixed every 5 min. The polypropylene tube was then centrifuged at 3 000 rpm for 10 min and the resulting supernatant discarded. Cold phosphate buffered saline (27 mM KCl, 137 mM NaCl, 8 mM Na_2HPO_4 and 1.5 mM KH_2PO_4) (10 ml) was added to and mixed with the pellet, whereafter the contents were centrifuged for a further 10 min. The supernatant was again discarded, the pellet dissolved in 3 ml nuclear lysis buffer (10 mM Tris-HCl, 400 mM NaCl and 2 mM EDTA, pH 8.2), 30 µl Proteinase K (20 mg/ml) and 300 µl 10% (m/v) sodium dodecyl sulfate, and the tube incubated overnight at 56°C. Sodium chloride (6 M) (1 ml) was added and the tube was shaken continuously for 1 min, before centrifuging at 3 000 rpm for 20 min. The supernatant was carefully transferred to a clean polypropylene tube to which 3 volumes ice cold 100% (v/v) ethanol was added. The precipitated DNA was transferred to a clean Eppendorf tube and washed with 70% (v/v) ethanol. The DNA was then collected through centrifugation at 14 000 rpm for 5 min, the ethanol discarded and the pellet resuspended in 200 µl TE buffer.

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Chapter 4

The identification, sequencing and expression analysis of *Diuraphis noxia* DNA methyltransferases and their association to global methylation and hydroxymethylation levels

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4.1 Introduction

Diuraphis noxia (Kurdjumov, Hemiptera: Aphididae – or Russian wheat aphid, RWA) biotypes are morphologically similar, yet display vast differences in their capacity to damage wheat cultivars upon feeding (i.e., their virulence) (Botha 2013). The virulence of the four naturally occurring biotypes in South Africa is as follows in order from least to most virulent, SA1 < SA2 < SA3 < SA4 (Jankielsohn 2014, 2016). There is also a highly virulent, laboratory-reared, South African Mutant (SAM) biotype which developed from SA1 through selection by a prolonged period of feeding on *Dn1* resistant cultivars (Van Zyl and Botha 2008; Swanevelder *et al*. 2010). Despite the emergence of new RWA biotypes in South Africa (Tolmay *et al*. 2007; Jankielsohn 2011, 2016), and other parts of the world, including the United States of America (USA) (Haley *et al*. 2004; Burd *et al*. 2006; Randolph *et al*. 2009) and Argentina (Clua *et al*. 2004), the molecular mechanism underlying the development of new biotypes is currently unknown (Shufran and Payton 2009; Botha *et al*. 2014a). The known genealogy of SA1 and SAM (Van Zyl and Botha 2008; Swanevelder *et al*. 2010), their genetic similarity (Burger and Botha 2017) and their position on either end of the virulence spectrum, renders them particularly useful in the present study, to improve the understanding of the process of biotypification.

Russian wheat aphids release effector/avirulence (avr) proteins into host plants as they feed (Botha *et al*. 2005, 2014a; Walling 2008). In resistant wheat cultivars, avr proteins are recognised by wheat *Dn* (*D. noxia*) gene-encoded resistance (R) proteins, in what is termed an incompatible interaction, resulting in either passive (e.g., tolerant) or active (e.g., antibiotic or antixenotic) plant defence responses (Botha *et al*. 2005, 2006, 2014b; Smith and Clement 2012). However, if the avr protein remains unrecognised (a compatible interaction), as is the case with susceptible cultivars, the aphid is able to damage the host without eliciting the host defence, and is said to be virulent towards the cultivar (Botha *et al*. 2006; Smith and Clement 2012). There is a continuous evolutionary arms race between RWA effector genes and wheat *R* genes, whereby *R* genes evolve to recognise effectors, which in turn are modified to avoid such recognition (Botha 2013). However, despite the important

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function of effectors in either eliciting or avoiding plant defence responses, no RWA effectors have been identified, and none of the genes conferring resistance to RWA (*Dn* genes) have been cloned (Botha *et al*. 2005, 2014b; Smith and Clement 2012).

As the search to identify and clone effector and *Dn* genes continues, some scientists have begun researching different factors that could influence virulence, such as differences in the genomic sequences of *Buchnera aphidicola* housed by different biotypes (Swanevelder *et al*. 2010) or differences in energy production that could influence aphid fitness (De Jager 2014). The possibility of a link between RWA methylation and biotype virulence has also been suggested (Gong *et al*. 2012). In 2012 Gong *et al*. investigated the methylation of four genes encoding salivary gland proteins (putative effector genes) in RWA biotypes US1 and US2, and indeed found these genes to be differentially methylated in the different biotypes. In the initial investigation of South African RWA methylation (Chapter 3), the different biotypes exhibited different banding patterns (after restriction of their DNA with methylation-sensitive enzymes), methylation levels and methylation trends, all of which support a role for methylation in biotypification.

The epigenetic modification of DNA methylation involves the covalent addition of a methyl group to the 5' position of cytosine (Glastad *et al*. 2011; Lyko and Maleszka 2011). In insects, methylation occurs predominantly within genes (Zemach *et al*. 2010; Glastad *et al*. 2011; Lyko and Maleszka 2011), where it is reported to perform two major functions. Firstly, intragenic methylation affects alternative splicing by recruiting or interfering with different DNA binding factors (Hunt *et al*. 2013b; Glastad *et al*. 2014; Yan *et al*. 2015), and secondly, it prevents the initiation of spurious transcription at cryptic binding sites within genes (Hunt *et al*. 2010, 2013a, 2013b). Differences, or changes in methylation level could affect both alternative splicing and spurious transcription, resulting in a different suite of transcripts and ultimately in a different set of proteins being produced. This brings to light the possibility that distinct RWA biotypes could be characterised by different transcript and protein sets, should they have differing methylation levels, and makes clear the need to investigate RWA biotype methylation in greater depth, and at a global, genome-wide

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level. Cloete (2015) found that biotypes SA1 and SAM do indeed have different sets of salivary proteins. However this has not been looked at in conjunction with methylation, and could have arisen by other means.

Here, various aspects of the genes encoding proteins which catalyse methylation, namely the DNA methyltransferases (*DNMTs*) (Goll and Bestor 2005), were assessed, as these could directly influence biotypic methylation levels. The three subfamilies of DNMT proteins perform different functions, with DNMT3 and DNMT1 establishing and maintaining methylation patterns respectively, and DNMT2 methylating both DNA and RNA (Goll and Bestor 2005; Goll *et al*. 2006; Jeltsch *et al*. 2006). Insects have a variety of combinations of the *DNMT* genes, with some lineages having lost one (e.g., *Bombyx mori* and *Triboleum castaneum*) or two (e.g., *Drosophila melanogaster* and *Anopheles gambiae*) subfamilies of *DNMTs*, and others having multiple homologues (e.g., *Apis mellifera*, *Nasonia vitripennis* and *Acyrthosiphon pisum*) within a certain *DNMT* subfamily (Kunert *et al*. 2003; Marhold *et al*. 2004; Xiang *et al*. 2010; Glastad *et al*. 2011; Feliciello *et al*. 2013).

Until recently, there was no knowledge of RWA DNMTs, although, based on the presence of methylation in certain genes (Gong *et al*. 2012), at least one DNMT subfamily was expected to be present in the RWA genome. In 2015 Nicholson *et al*. reported that *D. noxia* has a complete set of methylation-related genes, based on the sequencing of the most virulent US biotype (US2). However, at the commencement of the current research, the RWA *DNMTs* had not yet been reported on. This warranted their identification and sequencing, which in this chapter, were performed for both the hypervirulent SAM biotype, and its parental SA1 biotype. In addition to serving as a comparison for the sequencing results of the Nicholson study, insight into the conservation of *DNMT* sequences during biotypification was also gained (Nicholson *et al*. 2015). The availability of these sequences also enabled the relative quantification of *DNMT* expression. DNA methyltransferase protein activity was also assessed to conclude the investigation of the DNMTs.

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In addition to examining the various aspects of the DNMTs which could influence methylation, the study sought to determine the global levels of methylation of the biotypes. Bisulphite sequencing, the "gold-standard" method for quantifying methylation levels and detecting methylation at single nucleotide resolution, requires a reference genome for comparison to sodium bisulphite-treated DNA (Lister and Ecker 2009; Huang *et al*. 2010; Laird 2010; Krueger *et al*. 2012; Sun *et al*. 2014). The lack of an RWA reference genome at the outset of the current research, meant that bisulphite sequencing was not a viable option for methylation quantification. An antibody specific to 5-methylcytosine (5mC) was instead used to quantify the methylation levels. This antibody, as with bisulphite sequencing, was able to quantify methylation in all sequence contexts, yielding genome-wide methylation levels.

DNA methylation is removed through the process of demethylation, which can occur both passively and actively, with 5-hydroxymethylcytosine (5hmC) being a measurable intermediate of one of the active demethylation pathways (Branco *et al*. 2012; Kohli and Zhang 2013). Hydroxymethylcytosine is formed through the oxidation of 5mC by ten-eleven translocation enzymes (TETs) (Tahiliani *et al*. 2009; Ito *et al*. 2010; Shen *et al*. 2014). The presence of 5hmC has only been reported in a few insects including *A. mellifera*, *T. castaneum*, *N. vitripennis* and *D. melanogaster* (Cingolani *et al*. 2013; Feliciello *et al*. 2013; Wojciechowski *et al*. 2014; Delatte *et al*. 2016; Pegoraro *et al*. 2016; Rasmussen *et al*. 2016). To determine if 5hmC is present in the RWA, and to what extent, an antibody specific to 5hmC was used, providing insight into RWA demethylation for the first time.

The objective of this chapter was thus to characterise the DNA methyltransferases in terms of both expression and sequence, and to relate these observations to the reported virulence levels of the South African RWA biotypes (Jankielsohn 2014, 2016), as well as to the methylation and hydroxymethylation levels of the different biotypes. Four technical objectives were established to achieve the principal objective. These were, firstly, to identify the subfamilies of DNMTs present in the recently sequenced RWA genome (Burger and Botha 2017) through homology searches, as well

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as to clone and sequence the identified *DNMTs* of biotypes SA1 and SAM; secondly, to use these sequences to quantify the baseline expression of the *DNMTs*; thirdly, to quantify the DNMT protein activity through the use of antibodies; and fourthly, to quantify the relative global methylation and hydroxymethylation (indicative of demethylation) levels.

4.2 Methods and materials

4.2.1 Identification, cloning and sequencing of RWA *DNMTs*

4.2.1.1 *In silico* identification of RWA DNMTs

Insect DNA methyltransferases were searched for on the National Center for Biotechnology Information (NCBI – http://www.ncbi.nlm.nih.gov). For the three DNMT subfamilies, the amino acid sequences of results with Refseq accession numbers were saved in both FASTA and GenPept (full) formats. The sequences of related homologues and proteins classified as "methyltransferase-like" were also saved. A sequence-based search of the NCBI was then performed, using the FASTA sequences as queries for a protein BLAST (standard parameters) (Altschul *et al*. 1997). BLAST hits of interest were again those with Refseq accession numbers and "methyltransferase-like" proteins, and the GenPept (full) records of the proteins were saved.

The saved Genpept sequences were used as queries for a BLASTp (standard parameters) (Altschul *et al*. 1997) against the RWA protein build (http://cg-base.org), in Geneious v6.1.6 (http://www.geneious.com, Kearse *et al*. 2012). BLAST results were exported into Microsoft Excel (2010) and sorted based on their % identical sites (ID) and % query coverage (QC). The genes corresponding to the best protein matches were obtained from the RWA genome assembly (GCA_001465515.1) and used for sequencing.

4.2.1.2 Primer design for sequencing of RWA *DNMTs*

Primers (Appendix B, Table B1; Integrated DNA Technologies (IDT), USA) were designed within the coding domain sequences (CDS) of the identified RWA *DNMTs* using Primer3 (http://bioinfo.ut.ee/primer3/, Rozen and Skaletsky 2000). Primer sets were designed such that their

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melting temperatures (T_m) did not differ by more than 2 degrees Celsius, and product sizes ranged between 280 bp and 830 bp. The GC content was between 28% and 45%, and primer dimer T_m, self dimer T_m and hairpin T_m were all below the lowest temperature of the PCR. The primers were then used in a BLASTn analysis (Altschul *et al*. 1997) against the earliest version of the RWA genome (GCA_001465515.1) to ensure they only matched genes of interest, or that only one of the two primers of a set had more than one match, which would result in negligible linear amplification during PCR.

4.2.1.3 Aphid rearing

Parthenogenetic female aphid colonies of the biotypes SA1 and SAM were separately reared in BugDorm cages (MegaView Science Education Services Co. Ltd, Taiwan) in an insectary with continuous artificial lighting from high pressure sodium lamps, a temperature of 22.5°C ± 2.5°C, and a relative humidity of between 35%–40%. Russian wheat aphid SA1 and SAM colonies were maintained on the "Gamtoos S" and "Gamtoos R" wheat cultivars respectively. Seeds were planted in sand-filled pots, and watered daily with a fertiliser that consisted of 77 ml potassium nitrate per 100 l of water, 164 g Sol-u-fert (Kynoch Fertilizers (Pty) Ltd, South Africa) and 2 g Microplex (Ocean Agriculture (Pty) Ltd, South Africa).

4.2.1.4 RNA extraction

For the purpose of making complementary DNA (cDNA), total RNA was extracted from apterous aphids of biotypes SA1 and SAM using the Direct-zol™ RNA MiniPrep kit (Zymogen, USA). Aphids (n=20) were collected into an Eppendorf tube and were immediately flash-frozen in liquid nitrogen, and stored at -80°C until further use. All surfaces and glassware were RNase Away-treated (Thermo Fisher Scientific, USA) prior to extraction. After adding 500 µl TRI Reagent® to the still frozen aphids, the samples were homogenised using a micropestle, and particulates were collected through centrifugation at 12 000 g for 3 min. The supernatant was then carefully transferred into an RNase-free tube. RNA purification, as well as an on-column DNase I (Qiagen, Germany) digestion were carried out as per the manufacturer's guidelines. Following extraction,

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RNA was quantified using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

4.2.1.5 cDNA synthesis

Complementary DNA synthesis was performed using the iScript™ cDNA Synthesis kit (BioRad, USA) in accordance with the provided protocol, and 350 ng of SA1 and SAM total RNA as template. A PCR, using primers for the *murE* gene (Appendix B, Table B1) and varying concentrations of cDNA, was carried out to determine the optimal quantity of cDNA to be used for PCRs. Three reactions were performed containing 2 µl cDNA (SA1 = 1256.4 ng/µl and SAM = 811.8 ng/µl), 2 µl of a 20x dilution of cDNA, and 1 µl of a 20x dilution of cDNA respectively. Other components of the 20 µl reaction included 2.5 U Taq DNA polymerase (Thermo Fisher Scientific, USA), 1x amplification buffer, 0.5 pmoles of both the forward and reverse primers, 0.2 mM dNTPs, and 2.5 mM $MgCl_2$. After an initial 3 min denaturation step at 94°C, 30 cycles at 94°C for 30 sec, 54°C for 30 sec and 72°C for 30 sec were carried out. A final 10 min elongation step was performed at 72°C. PCR products were resolved on an ethidium bromide (2.5 µg/ml) post-stained 3% (m/v) Tris/Acetic acid/Ethylenediaminetetraacetic acid (EDTA) (TAE, 40 mM Tris, 20 mM Acetic acid and 1 mM EDTA, pH 8) agarose gel.

4.2.1.6 PCRs using *DNMT* primers

All PCRs were initially done using the same concentrations of components listed in 4.2.1.5 as well as 2 µl of a 20x dilution of SA1 (1256.4 ng/µl) and SAM (811.8 ng/µl) cDNA. Cycling conditions were also the same as 4.2.1.5 with the exception of the annealing temperatures (T_a) which were chosen based on individual primer T_m. The PCR products were resolved on 3% (m/v) TAE agarose gels stained with ethidium bromide to verify the correct product sizes. PCRs were optimised for primers that amplified non-specifically, by increasing the T_a to a maximum of four degrees above the lowest T_m of the primer pair. If multiple products persisted after PCR optimisation, they were excised from the agarose gel, placed into Eppendorf tubes containing 20 µl dH_2O and stored at -20°C.

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4.2.1.7 Ligation, cloning and sequencing

For primers showing specific amplification (as tested in 4.2.1.6), 4 µl PCR product was ligated into the pTZ57R/T vector (InsTAclone PCR cloning kit, Thermo Fisher Scientific, USA) overnight at 4°C. To obtain DNA from the excised agarose gel fragments, five freeze-thaw cycles (liquid nitrogen/60°C oven) were carried out and the freeze-thawed DNA was quantified through spectrophotometry (NanoDrop 2000 spectrophotometer). Based on these results, differing amounts of freeze-thawed DNA were used, in accordance with the kit's recommendations on the optimal quantity of PCR product for ligation.

Ligation reaction (3 µl) was added to 50 µl thawed DH5α competent cells (Thermo Fisher Scientific, USA) and the mixture was placed on ice for 20 min. Transformation was performed via heat shock at 42°C for 42 sec and 200 µl pre-warmed Luria Broth (LB, 10 g/l Bacto®-tryptone, 10 g/l NaCl and 5 g/l Bacto®-yeast extract) was added to the cells. Transformed cells were then incubated for 1.5 hours at 37°C whilst shaking, before spreading 100 µl onto LB-Agar (1.5% m/v Agar) plates containing 40 µl of both Ampicillin (50 mg/ml) and X-Gal (20 mg/ml). Plates were incubated overnight at 37°C.

White colonies were individually transferred into 6 ml LB containing 1 ml Ampicillin (50 mg/ml), and allowed to grow overnight at 37°C, whereafter 1 µl of inoculum was used for colony PCRs (Güssow and Clackson 1989). The optimised PCR conditions (as in 4.2.1.6) were used to confirm the correct insert size, and the remainder of the inoculum was used for plasmid minipreps following the manufacturer's instructions (Qiagen, Germany). Plasmid minipreps (derived from at least one colony per PCR product) were sent to the Central Analytical Facility (CAF) of Stellenbosch University for bi-directional Sanger sequencing of the insert at the pTZ57R/T multiple cloning site, using the M13 forward and reverse primers (Appendix B, Table B1).

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4.2.1.8 Sequence analysis

Raw sequences were imported into Geneious v6.1.6 and trimmed on either end to remove poor quality or ambiguous base calls. A VecScreen BLAST (http://www.ncbi.nlm.nih.gov/tools/vecscreen/) was then performed using the trimmed sequences to remove any vector DNA. The sequences for both SA1 and SAM biotypes (at least one forward and one reverse per PCR product) were aligned with the respective gene from which primers were designed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/, Sievers *et al*. 2011). The sequence alignments were then analysed for indels and single nucleotide polymorphisms (SNPs). The sequences were also used in a BLASTn analysis on the NCBI (Altschul *et al*. 1997).

4.2.2 *DNMT* expression analysis

4.2.2.1 Primer design for reverse transcription quantitative PCRs (RT-qPCRs)

Primer pairs (Appendix B, Table B2; IDT, USA) were designed from the CDS regions of the sequenced *DNMTs*, as described in 4.2.1.2. The primers, which had a GC content ranging from 30% to 50%, were designed to yield products of between 100 bp and 200 bp in size. Primers were used in a BLASTn analysis (Altschul *et al*. 1997) against the assembled RWA genome (GCA_001465515.1) to ensure they only matched the *DNMT* gene from which they were designed.

4.2.2.2 Aphid rearing

Aphids were reared under the same conditions as in 4.2.1.3. Biotype SA1 was maintained on the "SST 356" wheat cultivar, and both RWA SA2 and SA3 colonies were maintained on "SST 387". The highly virulent SAM biotype was maintained on "SST 398". All SST cultivars were obtained from SENSAKO (Pty) Ltd (South Africa).

4.2.2.3 Collection of aphid heads

Apterous aphids were removed individually from plants using an earbud and placed onto a petri dish under a microscope (Helmut Hund GmbH, Germany) set at its highest magnification. Heads were removed with a liquid nitrogen-cooled scalpel by cutting carefully posterior to the prothorax

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(Appendix B, Figure B1 see A, B), and were shaken off into a liquid nitrogen pre-cooled Eppendorf tube. Three biological replicates (n=3) of 50 aphid heads (n=150) (Appendix B, Figure B1 see C, D) were collected for all four biotypes. Heads were flash-frozen and stored at -80°C until RNA extraction.

4.2.2.4 RNA extraction and cDNA synthesis

All surfaces and glassware were RNase Away-treated prior to RNA extraction, which was performed using the RNeasy Mini kit (Qiagen, Germany) and QIAshredder columns (Qiagen, Germany). A micropestle was used to grind aphid heads to a fine powder, to which 450 µl Buffer RLT was added. The lysate was incubated for 2 min on a heatblock at 56°C, before transfer to a QIAshredder column and centrifugation at 13 000 rpm for 2 min. The rest of the extraction was performed in accordance with the RNeasy Plant Mini kit protocol (Qiagen, Germany) with all further centrifugation steps carried out at 10 000 rpm. On-column DNase I treatment was performed following the manufacturer's guidelines. RNA was eluted using 30 µl RNase-free water and used immediately to synthesise cDNA, as described previously (4.2.1.5). Complementary DNA was quantified at CAF, using the Qubit® 2.0 fluorometer (Thermo Fisher Scientific, USA).

4.2.2.5 RT-qPCR analysis

The relative expression of *DNMT* genes in heads of the RWA biotypes SA1, SA2, SA3 and SAM, was quantified using the Luminaris HiGreen qPCR Master Mix (Thermo Fisher Scientific, USA) and the CFX96 Real-Time System (BioRad, USA). Each 10 µl reaction contained 5 µl Master Mix (2x), 0.25 ng cDNA and between 0.04 and 0.36 pmoles forward and reverse primer. The Taguchi method was used to optimise primer concentrations and T_a (Appendix B, Table B2) for each primer set (Thanakiatkrai and Welch 2012). Each of the three biological replicates per biotype (n=3) was loaded in triplicate (n=9), along with a no template control as a measure of contamination. A five-point two-fold dilution series with a starting concentration of 0.5 ng/µl was also loaded in triplicate, with 2 µl (i.e., 1 ng cDNA for the first standard) cDNA added per reaction. Plates were centrifuged for 5 min at 4 000 rpm to ensure proper mixing of reagents. Cycling commenced at 50°C for 2 min,

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followed by 10 min at 95°C. Forty cycles of 95°C for 15 sec, optimised T_a for 30 sec, and 72°C for 30 sec ensued. A melt curve analysis was performed starting with the T_a and increasing in 0.5°C increments every five sec. The ribosomal genes *L27* and *L32* were used as reference genes as they have previously been shown to be constitutively expressed, in RWA and the pea aphid, respectively (Shakesby *et al*. 2009; Sinha and Smith 2014). Biotype SA1 samples were used as the control samples against which expression was measured using Pfaffl's methodology (Pfaffl 2001).

4.2.2.6 Statistical analysis

Microsoft Excel (2010)/XLSTAT Premium (Addinsoft Inc., USA) were used for the statistical analysis, and SigmPlot (2001) was used to plot graphs showing the average readings and standard deviation. An ANOVA was performed to test for significant differences between the sample means, with the level of significance set at $p \leq 0.05$. The model assumptions of ANOVA (i.e., homoscedasticity and normality of the residuals), were tested for using Levene's test and the Shapiro-Wilk test respectively (significance set at $p \leq 0.05$ for both tests). If the ANOVA null hypothesis – that the means of the treatment groups are equal – was rejected, a Fisher's LSD test was then performed.

4.2.3 DNMT protein activity quantification

4.2.3.1 Aphid rearing

Conditions were the same as in 4.2.1.3 and colonies of all biotypes were maintained on the "SST 356" wheat cultivar.

4.2.3.2 Protein extraction

Three replicates (n=3) of 150 apterous aphids (n=450) of biotypes SA1, SA2, SA3 and SAM were collected, flash-frozen and stored at -80°C until use. A micropestle was used to grind aphids into a fine powder, to which 100 µl phosphate buffered saline (50 mM NaH_2PO_4, 50 mM Na_2HPO_4 and 150 mM NaCl, pH 7.5), 10 µl phenylmethylsulphonyl fluoride (1 mM) and 10 µl dithiothreitol

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(1 mM) were added. Homogenised mixtures were centrifuged at 15 000 rpm (4°C) for 10 min to pellet the cell debris and the resulting supernatant was transferred to a clean Eppendorf tube.

4.2.3.3 Protein quantification

Proteins were quantified using the Bradford protein assay (Bradford 1976). Each reaction contained 150 µl dH_2O, 40 µl Quick Start™ Bradford 1x dye reagent (BioRad, USA) and 10 µl sample or Bovine Serum Albumin standard at concentrations of 2 mg/ml, 1.5 mg/ml, 1 mg/ml, 0.75 mg/ml, 0.5 mg/ml, 0.25 mg/ml and 0.125 mg/ml (BioRad, USA). Absorbance was measured at 600 nm using the Glomax®-Multi Detection System (Promega, USA). SigmaPlot was used to calculate the R^2 of the standard curve, and protein concentration (expressed as mg protein/ml) was calculated using the following formula.

$$[\text{Protein}] = \frac{(\text{Sample absorbance - blank absorbance})}{(\text{Standard absorbance - blank absorbance})} \text{ x [Standard] x dilution factor}$$

4.2.3.4 Antibody-mediated quantification of DNMT protein activity

DNA methyltransferase protein activity was quantified following the guidelines provided with Abcam's colourimetric DNMT Activity Quantification kit (Abcam, UK), and using the maximum recommended amount of protein extract, 5 µl (ranging from 7.69 to 10.96 µg, standardised using the formula below) of each of the three biological replicates per biotype (n=3). Absorbance was measured using the Glomax®-Multi Detection System, and DNA methyltransferase activity in OD/h/µg (optical density/hour/microgram) was calculated using the formula below. The statistical analysis was performed as in section 4.2.2.6.

$$\text{Protein activity} = \frac{(\text{Sample OD - Blank OD})}{(\text{Protein amount (µg) x hour})} \text{ x } 1000$$

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4.2.4 Global methylation quantification

4.2.4.1 DNA extraction

Genomic DNA was extracted from apterous aphids of the biotypes SA1, SA2, SA3 and SAM (as reared in section 4.2.2.2) using DNAzol® Reagent (Thermo Fisher Scientific, USA) following a modified protocol. Aphids (n=50) were collected using a soft-bristled brush for each of the three biological repeats (n=3) per biotype (n=150). Homogenised mixtures were centrifuged at 10 000 rpm for 15 min to pellet the cell debris. The resulting supernatant was transferred to a clean 1.5 ml Eppendorf tube, whereafter 500 µl ice cold 100% (v/v) ethanol was added and the tubes were left overnight at -20°C to precipitate the DNA. Precipitated DNA was transferred to a new Eppendorf tube and washed using 75% (v/v) ethanol. The DNA was then collected through centrifugation at 10 000 rpm for 5 min. An additional wash and centrifugation step were carried out and the resulting pellets were air-dried. DNA was resuspended in 50 µl low Tris-EDTA (TE, 10 mM Tris-HCl and 0.1 mM EDTA, pH 8) buffer and quantified using the Qubit® 2.0 fluorometer at CAF. DNA quality was assessed through gel electrophoresis on a 3% (m/v) TAE agarose gel, stained with ethidium bromide as previously described.

4.2.4.2 Antibody-mediated quantification of methylated DNA

Global levels of methylation were determined utilising Abcam's colourimetric Methylated DNA Quantification kit (Abcam, UK) using 150 ng DNA of the three biological repeats per biotype (n=3). A slight modification of the protocol was followed in the 'methylation capture' section (11.2.2 of the protocol), whereby incubation of DNA and diluted capture antibody was performed for 15 hours at room temperature in the dark to allow for optimal antibody binding, as opposed to one hour at room temperature. The final plate incubation, after addition of the developer solution, was carried out for the maximum recommended time of 10 min. Absorbance at 450 nm was read in triplicate (n=9) within five min of adding the stop solution, using the Glomax®-Multi Detection System.

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Relative methylation levels were calculated for each sample using the following formula:

$$\text{Relative 5mC \%} = \frac{(\text{Sample OD - Negative control OD})/S}{(\text{Positive control OD - Negative control OD}) \times 2/P} \times 100$$

where 5mC is 5-methylcytosine, OD is optical density, S is the amount of sample DNA in ng and P is the amount of positive control in ng. The statistical analysis was carried out as in section 4.2.2.6.

4.2.5 Global hydroxymethylation quantification

4.2.5.1 Antibody-mediated quantification of hydroxymethylated DNA

Global hydroxymethylation levels were quantified using Abcam's colourimetric Hydroxymethylated DNA Quantification kit (Abcam, UK), in accordance with the provided protocol. Differing amounts of a freshly extracted DNA sample from each biotype were loaded in triplicate (n=3), and standardised using the formula below (refer to S, the amount of sample DNA). The final plate incubation was carried out for 10 min, whereafter absorbance at 450 nm was read using the Glomax®-Multi Detection System. Relative hydroxymethylation levels were calculated for each sample using the following formula:

$$\text{Relative 5hmC \%} = \frac{(\text{Sample OD - Negative control II OD})/S}{(\text{Positive control OD - Negative control II OD}) \times 5/P} \times 100$$

where 5hmC is 5-hydroxymethylcytosine, OD is optical density, S is the amount of sample DNA in ng and P is the amount of positive control in ng. For methods relating to the statistical analysis, see section 4.2.2.6.

4.3 Results

4.3.1 Identification and sequencing of RWA *DNMTs*

4.3.1.1 *In silico* identification of RWA DNMTs

The BLASTp analysis performed using the insect DNMTs against the RWA proteins, revealed that for the three DNMT subfamilies, the best match to the RWA protein build was the respective *A. pisum* DNMT proteins. For DNMT1, the best match was identified as g9062.t1, a RWA protein

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which aligned to *A. pisum* XP_001942632.1 with an 80.6% ID and a 70.94% QC. A second RWA protein, g16165.t1 also aligned to XP_001942632.1 with a 91.1% ID and a 19.07% QC. An alignment of g9062.t1 and g16165.t1 with DNMT1 proteins of other insects, revealed that these two proteins are likely encoded by a single gene, because the 3' region of g9062.t1 and the 5' region of g16165.t1 overlap/align perfectly (Appendix B, Figure B2).

g2520.t1, a putative DNMT2 RWA protein, matched strongly to *A. pisum* XP_001949338 with an 89.9% ID and a 100% QC. A putative RWA DNMT3A (g20164.t1) and DNMT3B (g24379.t1) protein were also identified which matched *A. pisum* XP_008178776 and XP_003240668 respectively. The %ID and %QC values were 71 and 35.38 (g20164.t1), and 82.1 and 64.45 (g24379.t1) respectively.

4.3.1.2 Sequencing of RWA *DNMTs*

Based on a PCR using primers for the *murE* gene, testing various cDNA concentrations, 2 µl of a 20x dilution of SA1 and SAM cDNA was used for the PCRs using the *DNMT* primers (4.2.1.6) (Appendix B, Figure B3). After trimming the raw sequences, 99.8% of the bases of all the sequences had a Q score of at least 20, 98.8% had a Q score of at least 30, and 94.5% had a Q score of at least 40. Alignments of the sequenced SA1 and SAM *DNMT* genes, along with the genes from which the sequencing primers were designed, revealed 14 SNPs (Figures 4.1–4.5). Thirteen of these SNPs were found within a single biotype (i.e., in either SA1 or SAM) and the remaining SNP in g9062.t1 was present between the SA1 and SAM biotypes. When the sequences were used in a BLASTn analysis on the NCBI, the best alignments were to the RWA methyltransferases from bioproject PRJNA310344, and matched with at least a 99% ID and 99% QC (Appendix B, Figures B4–B8). PRJNA310344, the RWA US2 RefSeq genome assembly, was derived from the GenBank assembly (bioproject PRJNA233413), which itself was based on the whole genome shotgun project JOTR00000000 (the topic of Nicholson *et al*.'s 2015 article).

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g9062.t1      AAGCATTAATAGGTATAAACACTGAATATGCAGATTATTTATTACTTGAACCACACCCTA 540
SA1_9062_1F   AAGCATTAATAGGTATAAACACTGAATATGCAGATTATTTATTACTTGAACCACACCCTA 288
SA1_9062_1R   AAGCATTAATAGGTATAAACACTGAATATGCAGATTATTTATTACTTGAACCACACCCTA 118
SA1_9062_2F   AAGCATTAATAGGTATAAACACTGAATATGCAGATTATTTATTACTTGAACCACACCCTA 288
SA1_9062_2R   AAGCATTAATAGGTATAAACACTGAATATGCAGATTATTTATTACTTGAACCACACCCTA 115
SAM_9062_1F   AAGCATTAATAGGTATAAACACTGAATATGCAGATTATTTATTACTTGAACCACACCCTA 288
SAM_9062_1R   ---------------------------ATGCAGATTATTTATTACTTGAACCACACCCTA 33
SAM_9062_2F   AAGCATTAATAGGTAAAAACACTGAATATACAGATTATTTATTACTTGAACCACACCCTA 284
SAM_9062_2R   AAGCATTAATAGGTAAAAACACTGAATATACAGATTATTTATTACTTGAACCACACCCTA 110
                                         ** ******************************

g9062.t1      ATTATAAAAAATATATGACATCTGTAATTGAAAAGATAAATCTGAGCAAAATAGTAATTG 600
SA1_9062_1F   ATTATAAAAAATATATGACATCTGTAATTGAAAAGATAAATCTGAGCAAAATAGTAATTG 348
SA1_9062_1R   ATTATAAAAAATATATGACATCTGTAATTGAAAAGATAAATCTGAGCAAAATAGTAATTG 178
SA1_9062_2F   ATTATAAAAAATATATGACATCTGTAATTGAAAAGATAAATCTGAGCAAAATAGTAATTG 348
SA1_9062_2R   ATTATAAAAAATATATGACATCTGTAATTGAAAAGATAAATCTGAGCAAAATAGTAATTG 175
SAM_9062_1F   ATTATAAAAAATATATGACATCTGTAATTGAAAAGATAAATCTGAGCAAAATAGTAATTG 348
SAM_9062_1R   ATTATAAAAAATATATGACATCTGTAATTGAAAAGATAAATCTGAGCAAAATAGTAATTG 93
SAM_9062_2F   ATTATAAAAAATATATGACATCTGTAATTGAAAAGATAAATCTGAGCAAAATAGTAATTG 344
SAM_9062_2R   ATTATAAAAAATATATGACATCTGTAATTGAAAAGATAAATCTGAGCAAAATAGTAATTG 170
              ************************************************************

g9062.t1      AAAAAATGTTAGACAATCATGAATCTGATGATTCAACTTATGAAGATATTTTAAATTATG 660
SA1_9062_1F   AAAAAATGTTAGACAATCATGAATCTGATGATTCAACTTATGAAGATATTTTAAATTATG 408
SA1_9062_1R   AAAAAATGTTAGACAATCATGAATCTGATGATTCAACTTATGAAGATATTTTAAATTATG 238
SA1_9062_2F   AAAAAATGTTAGACAATCATGAATCTGATGATTCAACTTATGAAGATATTTTAAATTATG 408
SA1_9062_2R   AAAAAATGTTAGACAATCATGAATCTGATGATTCAACTTATGAAGATATTTTAAATTATG 235
SAM_9062_1F   AAAAAATGTTAGACAATCATGAATCTGATGATTCAACTTATGAAGATATTTTAAATTATG 408
SAM_9062_1R   AAAAAATGTTAGACAATCATGAATCTGATGATTCAACTTATGAAGATATTTTAAATTATG 153
SAM_9062_2F   AAAAAATGTTAGACAATCATGAATCTGATGATTCAACTTATGAAGATATTTTAAATTATG 404
SAM_9062_2R   AAAAAATGTTAGACAATCATGAATCTGATGATTCAACTTATGAAGATATTTTAAATTATG 230
              ************************************************************

g9062.t1      TTGTAACATCTATAAACCCAGCAACAGGGAATACATTTTCAGAAGAAGATCTTATTACTC 720
SA1_9062_1F   TTATAACATCTATAAACCCAGCAACAGGGAATACATTTTCAGAAGAAGATCTTATTACTC 468
SA1_9062_1R   TTATAACATCTATAAACCCAGCAACAGGGAATACATTTTCAGAAGAAGATCTTATTACTC 298
SA1_9062_2F   TTATAACATCTATAAACCCAGCAACAGGGAATACATTTTCAGAAGAAGATCTTATTACTC 468
SA1_9062_2R   TTATAACATCTATAAACCCAGCAACAGGGAATACATTTTCAGAAGAAGATCTTATTACTC 295
SAM_9062_1F   TTGTAACATCTATAAACCCAGCAACAGGGAATACATTTTCAGAAGAAGATCTTATTACTC 468
SAM_9062_1R   TTGTAACATCTATAAACCCAGCAACAGGGAATACATTTTCAGAAGAAGATCTTATTACTC 213
SAM_9062_2F   TTGTAACATCTATAAACCCAGCAACAGGGAATACATTTTCAGAAGAAGATCTTATTACTC 464
SAM_9062_2R   TTGTAACATCTATAAACCCAGCAACAGGGAATACATTTTCAGAAGAAGATCTTATTACTC 290
              ** *********************************************************

g9062.t1      ATGCACAGTTTGTACTCGATCAAGTAACTGACTACGACTCAATTGGCATGTATGATTTTC 780
SA1_9062_1F   ATGCACAGTTTGTACTCGATCAAGTAACTGACTACGACTCAATTGGCATGTATGATTTTC 528
SA1_9062_1R   ATGCACAGTTTGTACTCGATCAAGTAACTGACTACGACTCAATTGGCATGTATGATTTTC 358
SA1_9062_2F   ATGCACAGTTTGTACTCGATCAAGTAACTGACTACGACTCAATTGGCATGTATGATTTTC 528
SA1_9062_2R   ATGCACAGTTTGTACTCGATCAAGTAACTGACTACGACTCAATTGGCATGTATGATTTTC 355
SAM_9062_1F   ATGCACAGTTTGTACTCGATCAAGTAACTGACTACGACTCAATTGGCATGTATGATTTTC 528
SAM_9062_1R   ATGCACAGTTTGTACTCGATCAAGTAACTGACTACGACTCAATTGGCATGTATGATTTTC 273
SAM_9062_2F   ATGCACAGTTTGTACTCGATCAAGTAACTGACTACGACTCAATTGGCATGTATGATTTTC 524
SAM_9062_2R   ATGCACAGTTTGTACTCGATCAAGTAACTGACTACGACTCAATTGGCATGTATGATTTTC 350
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g9062.t1       CATTATCTGAAACTCAATGTGTTGAAACTTTAACCCAACTTTCAGGTGCCACAAAATCTG 840
SA1_9062_1F    CATTATCTGAAACTCAATGTGTTGAAACTTTA---------------------------- 560
SA1_9062_1R    CATTATCTGAAACTCAATGTGTTGAAACTTTAACCCAACTTTCAGGTGCCACAAAATCTG 418
SA1_9062_2F    CATTATCTGAAACTCAATGTG------------------------------------- 549
SA1_9062_2R    CATTATCTGAAACTCAATGTGTTGAAACTTTAACCCAACTTTCAGGTGCCACAAAATCTG 415
SAM_9062_1F    CATTATCTGAAACTCAATGTGTTGAAACTTTAACCCAACTTTC----------------- 571
SAM_9062_1R    CATTATCTGAAACTCAATGTGTTGAAACTTTAACCCAACTTTCAGGTGCCACAAAATCTG 333
SAM_9062_2F    CGTTATCTGAAACTCTATGTGTTGAAACTTTAACCCAACTTTCA---------------- 568
SAM_9062_2R    CGTTATCTGAAACTCTATGTGTTGAAACTTTAACCCAACTTTCAGGTGCCACAAAATCTG 410
               * ************* *****
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Figure 4.1. SA1 and SAM sequences aligned to g9062.t1, a putative *DNMT1* RWA gene, from which the primers were designed. Asterisks indicate a perfect alignment of a base between all sequences. Highlighted regions are SNPs between (turquoise), and within (yellow) biotypes.

```
g16165.t1      GTGTCATTCAAAAACAGCATGATCCTTAAATTAACTCTTAGATGTATTACACGTATGGGC 180
SA1_16165_1F   GTGTCATTCAAAAACAGCATGATCCTTAAATTAACTCTTAGATGTATTACACGTATGGGC 139
SA1_16165_1R   -------------------------------------TTAGATGTATTACACGTATGGGC 23
SAM_16165_1F   GTGTCATTCAAAAACAGCATGATCCTTAAATTAACTCTTAGATGTATTACACGTATGGGC 142
SAM_16165_1R   GTGTCATTCAAAAACAGCATGATCCTTAAATTAACTCTTAGATGTATTACACGTATGGGC 60
SAM_16165_2F   GTGTCATTCAAAAACAGCATGATCCTTAAATTAACTCTTAGATGTATTACACGTATGGGC 142
SAM_16165_2R   ----CATTCAAAAACAGCATGATCCTTAAATTAACTCTTAGATGTATTACACGTATGGGC 56
                                                 ***********************

g16165.t1      TACCAGTGTACCTTTGGTATCTTACAAGCTGGTAACTTTGGTGTACCTCAAACTAGAAGG 240
SA1_16165_1F   TACCAGTGTACCTTTGGTATCTTACAAGCTGGTAACTTTGGTGTACCTCAAACTAGAAGG 199
SA1_16165_1R   TACCAGTGTACCTTTGGTATCTTACAAGCTGGTAACTTTGGTGTACCTCAAACTAGAAGG 83
SAM_16165_1F   TACCAGTGTACCTTTGGTATCTTACAAGCTGGTAACTTTGGTGTACCTCAAACTAGAAGG 202
SAM_16165_1R   TACCAGTGTACCTTTGGTATCTTACAAGCTGGTAACTTTGGTGTACCTCAAACTAGAAGG 120
SAM_16165_2F   TACCAGTGTACCTTTGGTATCTTACAAGCTGGTAACTTTGGTGTACCTCAAACTAGAAGG 202
SAM_16165_2R   TACCAGTGTACCTTTGGTATCTTACAAGCTGGTAACTTTGGTGTACCTCAAACTAGAAGG 116
               ************************************************************

g16165.t1      AGGTTAATTATTATGGCTGCAGCTCCTGGTGAAAATTTGCCTTTTTATCCTGAGCCTATA 300
SA1_16165_1F   AGGTTAATTATTATGGCTGCAGCTCCTGGTGAAAATTTGCCTTTTTATCCTGAGCCTATA 259
SA1_16165_1R   AGGTTAATTATTATGGCTGCAGCTCCTGGTGAAAATTTGCCTTTTTATCCTGAGCCTATA 143
SAM_16165_1F   AGGTTAATTATTATGGCTGCAGCTCCTGGTGAAAATTTGCCTTTTTATCCTGAGCCTATA 262
SAM_16165_1R   AGGTTAATTATTATGGCTGCAGCTCCTGGTGAAAATTTGCCTTTTTATCCTGAGCCTATA 180
SAM_16165_2F   AGGTTAATTATTATGGCTGCAGCTCCTGGTGAAAATTTGCCTTTTTATCCTGAGCCTATA 262
SAM_16165_2R   AGGTTAATTATTATGGCTGCAGCTCCTGGTGAAAATTTGCCTTTTTATCCTGAGCCTATA 176
               ************************************************************

g16165.t1      AACGTCTTTAATAGAAAAAGTTCAAGTCTAAGTGTTCAGGTTGGTGATAAAA-AATTCAA 359
SA1_16165_1F   AACGTCTTTAATAGAAAAAGTTCAAGTCTAAGTGTTCAGGTTGGTGATAAAA-AATTCAA 318
SA1_16165_1R   AACGTCTTTAATAGAAAAAGTTCAAGTCTAAGTGTTCAGGTTGGTGATAAAA-AATTCAA 202
SAM_16165_1F   AACGTCTTTAATAGAAAAAGTTCAAGTCTAAGTGTTCAGGTTGGTGATAAAAAAATTCAA 322
SAM_16165_1R   AACGTCTTTAATAGAAAAAGTTCAAGTCTAAGTGTTCAGGTTGGTGATAAAAAAATTCAA 240
SAM_16165_2F   AACGTCTTTAATAGAAAAAGTTCAAGTCTAAGTGTTCAGGTTGGTGATAAAA-AATTCAA 321
SAM_16165_2R   AACGTCTTTAATAGAAAAAGTTCAAGTCTAAGTGTTCAGGTTGGTGATAAAA-AATTCAA 235
               ************************************************** *******

g16165.t1      AACTAATTGCATCTATAATGATTCTGCTCCTTTGAGAACCCTCACAGTATATGATGCTTG 419
SA1_16165_1F   AACTAATTGCATCTATAATGATTCTGCTCCTTTGAGAACCCTCACAGTATATGATGCTTG 378
SA1_16165_1R   AACTAATTGCATCTATAATGATTCTGCTCCTTTGAGAACCCTCACAGTATATGATGCTTG 262
SAM_16165_1F   AACTAATTGCATCTATAATGATTCTGCTCCTTTGAGAACCCTCACAGTATATGATGCTTG 382
SAM_16165_1R   AACTAATTGCATCTATAATGATTCTGCTCCTTTGAGAACCCTCACAGTATATGATGCTTG 300
SAM_16165_2F   AACTAATTGCATCTATAATGATTCTGCTCCTTTGAGAACCCTCACAGTATATGATGCTTG 381
SAM_16165_2R   AACTAATTGCATCTATAATGATTCTGCTCCTTTGAGAACCCTCACAGTATATGATGCTTG 295
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g16165.t1      GTCTGATTTACCTGAAATATCAAATGGAGCTTTTCAAGAAGAAATTCCATACTGTTCTAC 479
SA1_16165_1F   GTCTGATTTACCTGAAATATCAAATGGAGCTTTTCAAGAAGAAATTCCATACTGTTCTAC 438
SA1_16165_1R   GTCTGATTTACCTGAAATATCAAATGGAGCTTTTCAAGAAGAAATTCCATACTGTTCTAC 322
SAM_16165_1F   GTCTGATTTACCTGAAATATCAAATGGAGCTTTTCAAGAAGAAATTCCATACTGTTCTAC 442
SAM_16165_1R   GTCTGATTTACCTGAAATATCAAATGGAGCTTTTCAAGAAGAAATTCCATACTGTTCTAC 360
SAM_16165_2F   GTCTGATTTACCTGAAATATCAAATGGAGCTTTTCAAGAAGAAATTCCATACTGTTCTAC 441
SAM_16165_2R   GTCTGATTTACCTGAAATATCAAATGGAGCTTTTCAAGAAGAAATTCCATACTGTTCTAC 355
               ************************************************************

g16165.t1      TCCCATTACACATTTACAGAAATTATTAAGATATCCAGACAATCGATATGCAGAATCTAT 539
SA1_16165_1F   TCCCATTACACATTTACAGAAATTATTAAGATATCCAGACAATCGATATGCAGAATCTAT 498
SA1_16165_1R   TCCCATTACACATTTACAGAAATTATTAAGATATCCAGACAATCGATATGCAGAATCTAT 382
SAM_16165_1F   TCCCATTACACATTTACAGAAATTATTAAGATATCCAGACAATCGATATGCAGAATCTAT 502
SAM_16165_1R   TCCCATTACACATTTACAGAAATTATTAAGATATCCAGACAATCGATATGCAGAATCTAT 420
SAM_16165_2F   TCCCATTACACATTTACAGAAATTATTAAGATATCCAGACAATCGATATGCAGAATCTAT 501
SAM_16165_2R   TCCCATTACACATTTACAGAAATTATTAAGATATCCAGACAATCGATATGCAGAATCTAT 415
               ************************************************************

g16165.t1      ACTGAGTGATCATATATGCAAGGAAATGTCATCTTTGGTTCAAGCTAGAATAGCACTAAT 599
SA1_16165_1F   ACTGAGTGATCATATATGCAAGGAAATGTCATCTTTGGTTCAAGCTAGAATAGCACTAAT 558
SA1_16165_1R   ACTGAGTGATCATATATGCAAGGAAATGTCATCTTTGGTTCAAGCTAGAATAGCACTAAT 442
SAM_16165_1F   ACTGAGTGATCATATATGCAAGGAAATGTCATCTTTGGTTCAAGCTAGAATAGCACTAAT 562
SAM_16165_1R   ACTGAGTGATCATATATGCAAGGAAATGTCATCTTTGGTTCAAGCTAGAATAGCACTAAT 480
SAM_16165_2F   ACTGAGTGATCATATATGCAAGGAAATGTCATCTTTGGTTCAAGCTAGAATAGCACTAAT 561
SAM_16165_2R   ACTGAGTGATCATATATGCAAGGAAATGTCATCTTTGGTTCAAGCTAGAATAGCACTAAT 475
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g16165.t1      ACCAGTAGGTGAAGGAAGTGACTGGAGAGACCTACCCAACATTATAGTACAATTACCTGA 659
SA1_16165_1F   ACCAGT------------------------------------------------------ 564
SA1_16165_1R   ACCAGTAGGTGAAGGAAGTGACTGGAGAGACCTACCCAACATTATAGTACAATTACCTGA 502
SAM_16165_1F   ACCAGTAGGTGAAGGAAGTGACTGGAGAGACCTACCCAACATTATAGTACAATT------ 616
SAM_16165_1R   ACCAGTAGGTGAAGGAAGTGACTGGAGAGACCTACCCAACATTATAGTACAATTACCTGA 540
SAM_16165_2F   ACCAGTAGGTGAAGGAAGTGACTGGAGAGACCTACCCAACATTATAGTACAATTACCTG- 620
SAM_16165_2R   ACCAGTAGGTGAAGGAAGTGACTGGAGAGACCTACCCAACATTATAGTACAATTACCTGA 535
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Figure 4.2. SA1 and SAM sequences aligned to g16165.t1, a putative *DNMT1* RWA gene, from which the primers were designed. Asterisks indicate a perfect alignment of a base between all sequences. Highlighted regions are possible sequencing errors (grey).

```
g2520.t1       GGATTTGAAAGTTCATTAGCTAGAGACAAATTAGTTACCGCTCTGAGTCAGTCGGGTTTT 360
SA1_2520_1F    GGATTTGAAAGTTCATTAGCTAGAGACAAATTAGTTACCGCTCTGAGTCAGTCGGGTTTT 378
SA1_2520_1R    GGATTTGAAAGTTCATTAGCTAGAGACAAATTAGTTACCGCTCTGAGTCAGTCGGGTTTT 132
SA1_2520_2F    GGATTTGAAAGTTCATTAGCTAGAGACAAATTAGTTACCGCTCTGAGTCAGTCGGGTTTT 383
SA1_2520_2R    GGATTTGAAAGTTCATTAGCTAGAGACAAATTAGTTACCGCTCTGAGTCAGTCGGGTTTT 103
SA1_2520_3F    GGATTTGAAAGTTCATTAGCTAGAGACAAATTAGTTACCGCTCTGAGTCAGTCGGGTTTT 379
SA1_2520_3R    -------AAAGTTCATTAGCTAGAGACAAATTAGTTACCGCTCTGAGTCAGTCGGGTTTT 53
SAM_2520_1F    GGATTTGAAAGTTCATTAGCTAGAGACAAATTAGTTACCGCTCTGAGTCAGTCGGGTTTT 381
SAM_2520_1R    GGATTTGAAAGTTCATTAGCTAGAGACAAATTAGTTACCGCTCTGAGTCAGTCGGGTTTT 92
SAM_2520_2F    GGATTTGAAAGTTCATTAGCTAGAGACAAATTAGTTACCGCTCTGAGTCAGTCGGGTTTT 381
SAM_2520_2R    GGATTTGAAAGTTCATTAGCTAGAGACAAATTAGTTACCGCTCTGAGTCAGTCGGGTTTT 93
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g2520.t1       ACATATAGAGAATTTCTTCTTAGTCCTGTACACTTTGGAATTTGCAATTCAAGATTGAGG 420
SA1_2520_1F    ACATATAGAGAATTTCTTCTTAGTCCTGTACACTTTGGAATTTGCAATTCAAGATTGAGG 438
SA1_2520_1R    ACATATAGAGAATTTCTTCTTAGTCCTGTACACTTTGGAATTTGCAATTCAAGATTGAGG 192
SA1_2520_2F    ACATATAGAGAATTTCTTCTTAGTCCTGTACACTTTGGAATTTGCAATTCAAGATTGAGG 443
SA1_2520_2R    ACATATAGAGAATTTCTTCTTAGTCCTGTACACTTTGGAATTTGCAATTCAAGATTGAGG 163
SA1_2520_3F    ACATATAGAGAATTTCTTCTTAGTCCTGTACACTTTGGAATTTGCAATTCAAGATTGAGG 439
SA1_2520_3R    ACATATAGAGAATTTCTTCTTAGTCCTGTACACTTTGGAATTTGCAATTCAAGATTGAGG 113
SAM_2520_1F    ACATATAGAGAATTTCTTCTTAGTCCTGTACACTTTGGAATTTGCAATTCAAGATTGAGG 441
SAM_2520_1R    ACATATAGAGAATTTCTTCTTAGTCCTGTACACTTTGGAATTTGCAATTCAAGATTGAGG 152
SAM_2520_2F    ACATATAGAGAATTTCTTCTTAGTCCTGTACACTTTGGAATTTGCAATTCAAGATTGAGG 441
SAM_2520_2R    ACATATAGAGAATTTCTTCTTAGTCCTGTACACTTTGGAATTTGCAATTCAAGATTGAGG 153
               ************************************************************

g2520.t1       TATTATTTATTAGCCAAGAAGAAACCATTAGATTTTGCAATATCCTTACAAAATGATATC 480
SA1_2520_1F    TATTATTTATTAGCCAAGAAGAAACCATTAGATTTTGCAATATCCTTACAAAATGATATC 498
SA1_2520_1R    TATTATTTATTAGCCAAGAAGAAACCATTAGATTTTGCAATATCCTTACAAAATGATATC 252
SA1_2520_2F    TATTATTTATTAGCCAAGAAGAAACCATTAGATTTTGCAATATCCTTACAAAATGATATC 503
SA1_2520_2R    TATTATTTATTAGCCAAGAAGAAACCATTAGATTTTGCAATATCCTTACAAAATGATATC 223
SA1_2520_3F    TATTATTTATTAGCCAAGAAGAAACCATTAGATTTTGCAATATCCTTACAAAATGATATC 499
SA1_2520_3R    TATTATTTATTAGCCAAGAAGAAACCATTAGATTTTGCAATATCCTTACAAAATGATATC 173
SAM_2520_1F    TATTATTTATTAGCCAAGAAGAAACCATTAGATTTTGCAATATCCTTACAAAATGATATC 501
SAM_2520_1R    TATTATTTATTAGCCAAGAAGAAACCATTAGATTTTGCAATATCCTTACAAAATGATATC 212
SAM_2520_2F    TATTATTTATTAGCCAAGAAGAAACCATTAGATTTTGCAATATCCTTACAAAATGATATC 501
SAM_2520_2R    TATTATTTATTAGCCAAGAAGAAACCATTAGATTTTGCAATATCCTTACAAAATGATATC 213
               ************************************************************

g2520.t1       ATAACTGAAAATAATTGGGACGATAAATTGTGCAGCCGTGTGAAACAAGTTTCAGATGTA 540
SA1_2520_1F    ATAACTGAAAATAATTGGGACGATAAATTGTGCAGCCGTGTGAAACAAGTTTCAGATGTA 558
SA1_2520_1R    ATAACTGAAAATAATTGGGACGATAAATTGTGCAGCCGTGTGAAACAAGTTTCAGATGTA 312
SA1_2520_2F    ATAGCTGAAAATAATTGGGACGATAAATTGTGCAGCCGTGTGAAACAAGTTTCAGATGTA 563
SA1_2520_2R    ATAGCTGAAAATAATTGGGACGATAAATTGTGCAGCCGTGTGAAACAAGTTTCAGATGTA 283
SA1_2520_3F    ATAGCTGAAAATAATTGGGACGATAAATTGTGCAGCCGTGTGAAACAAGTTTC------- 552
SA1_2520_3R    ATAGCTGAAAATAATTGGGACGATAAATTGTGCAGCCGTGTGAAACAAGTTTCAGATGTA 233
SAM_2520_1F    ATAACTGAGAATAATTGGGACGATAAATTGTGCAGCCGTGTGAAACAAGTTTCAGATGTA 561
SAM_2520_1R    ATAACTGAGAATAATTGGGACGATAAATTGTGCAGCCGTGTGAAACAAGTTTCAGATGTA 272
SAM_2520_2F    ATAACTGAAAATAATTGGGACGATAAATTGTGCAGCCGTGTGAAACAAGTTTCAGATGTA 561
SAM_2520_2R    ATAACTGAAAATAATTGGGACGATAAATTGTGCAGCCGTGTGAAACAAGTTTCAGATGTA 273
               *** **** *******************************************
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Figure 4.3. SA1 and SAM sequences aligned to g2520.t1, a putative *DNMT2* RWA gene, from which the primers were designed. Asterisks indicate a perfect alignment of a base between all sequences. Highlighted regions are SNPs within a biotype (yellow).

```
g20164.t1      TTTAAATGATATTGTTGATGATTGCAATGAGAATATTGCCAAGTTGGTTGTTCAATATTA 120
SA1_20164_1F   ----------------------------------------AGTTGGTTGTTCAATATTA 19
SA1_20164_1R   ----------TTTGTTGATGATTGCAATGAGAATATTGCCAAGTTGGTTGTTCAATATTA 50
SA1_20164_2F   ----------------------------------------AGTTGGTTGTTCAATATTA 19
SA1_20164_2R   ----------TTTGTTGATGATTGCAATGAGAATATTGCCAAGTTGGTTGTTCAATATTA 50
SAM_20164_1F   ---------------------------------TATTGCCAAGTTGGTTGTTCAATATTA 27
SAM_20164_1R   ----------TTTGTTGATGATTGCAATGAGAATATTGCCAAGTTGGTTGTTCAATATTA 50
SAM_20164_2F   ---------------------------TGAGAATATTGCCAAGTTGGTTGTTCAATATTA 33
SAM_20164_2R   ----------TTTGTTGATGATTGCAATGAGAATATTGCCAAGTTGGTTGTTCAATATTA 50
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g20164.t1      TCCAAACAAGGAGGATTATAACAAAGATAAATTTTTTGATCAAAATGATTGCATTAAAAA 180
SA1_20164_1F   TCCAAACAAGGAGGATTATAACAAAGATAAATTTTTTGATCAAAATGATTGCATTAAAAA 79
SA1_20164_1R   TCCAAACAAGGAGGATTATAACAAAGATAAATTTTTTGATCAAAATGATTGCATTAAAAA 110
SA1_20164_2F   TCCAAACAAGGAGGATTATAACAAAGATAAATTTTTTGATCAAAATGATTGCATTAAAAA 79
SA1_20164_2R   TCCAAACAAGGAGGATTATAACAAAGATAAATTTTTTGATCAAAATGATTGCATTAAAAA 110
SAM_20164_1F   TCCAAACAAGGAGGATTATAACAAAGATAAATTTTTTGATCAAAATGATTGCATTAAATA 87
SAM_20164_1R   TCCAAACAAGGAGGATTATAACAAAGATAAATTTTTTGATCAAAATGATTGCATTAAATA 110
SAM_20164_2F   TCCAAACAAGGAGGATTATAACAAAGATAAATTTTTTGATCAAAATGATTGCATTAAAAA 93
SAM_20164_2R   TCCAAACAAGGAGGATTATAACAAAGATAAATTTTTTGATCAAAATGATTGCATTAAAAA 110
               ********************************************************* *

g20164.t1      ATTTTTCTCCTTTTTACAAATAAAAAACAAAATGGATGCATTGCAGAAGGATGGTAAGCC 240
SA1_20164_1F   ATTTTTCTCCTTTTTACAAATAAAAAACAAAATGGATGCATTGCTGAAGGATGGTAAGCC 139
SA1_20164_1R   ATTTTTCTCCTTTTTACAAATAAAAAACAAAATGGATGCATTGCTGAAGGATGGTAAGCC 170
SA1_20164_2F   ATTTTTCTCCTTTTTACAAATAAAAAACAAAATGGATGCATTGCAGAAGGATGGTAAGCC 139
SA1_20164_2R   ATTTTTCTCCTTTTTACAAATAAAAAACAAAATGGATGCATTGCAGAAGGATGGTAAGCC 170
SAM_20164_1F   ATCTTTCTCCTTTTTACAAATAAAAAACAAAATGGATGCATTGCAGAAGGATGGTAAGCC 147
SAM_20164_1R   ATCTTTCTCCTTTTTACAAATAAAAAACAAAATGGATGCATTGCAGAAGGATGGTAAGCC 170
SAM_20164_2F   ATTTTTCTCCTTTTTACAAATAAAAAACAAAATGGATGCATTGCAGAAGGATGGTAAGCC 153
SAM_20164_2R   ATTTTTCTCCTTTTTACAAATAAAAAACAAAATGGATGCATTGCAGAAGGATGGTAAGCC 170
               ** ***************************************** ***************

g20164.t1      TATTTTTTGGGCTTTTGAAACAAGTGCTGCAATTAAAATAGGAGAACAAAAAACAATTTC 300
SA1_20164_1F   TATTTTTTGGGCTTTTGAAACAAGTGCTGCAATTAAAATAGGAGAACAAAAAACAATTTC 199
SA1_20164_1R   TATTTTTTGGGCTTTTGAAACAAGTGCTGCAATTAAAATAGGAGAACAAAAAACAATTTC 230
SA1_20164_2F   TATTTTTTGGGCTTTTGAAACAAGTGCTGCAATTAAAATAGGAGAACAAAAAACAATTTC 199
SA1_20164_2R   TATTTTTTGGGCTTTTGAAACAAGTGCTGCAATTAAAATAGGAGAACAAAAAACAATTTC 230
SAM_20164_1F   TATTTTTTGGGCTTTTGAAACAAGTGCTGCAATTAAAATAGGAGAACAAAAAACAATTTC 207
SAM_20164_1R   TATTTTTTGGGCTTTTGAAACAAGTGCTGCAATTAAAATAGGAGAACAAAAAACAATTTC 230
SAM_20164_2F   TATTTTTTGGGCTTTTGAAACAAGTGCTGCAATTAAAATAGGAGAACAAAAAACAATTTC 213
SAM_20164_2R   TATTTTTTGGGCTTTTGAAACAAGTGCTGCAATTAAAATAGGAGAACAAAAAACAATTTC 230
               ************************************************************

g20164.t1      GAGATTTCTGAACACTCAACCAATAATCATCGGTATGGAGACAAATGATGTCCAACAAAG 360
SA1_20164_1F   GAGATTTCTGAACACTCAACCAATAATCATCGGTATGGAGACAAATGATGTCCAACAAAG 259
SA1_20164_1R   GAGATTTCTGAACACTCAACCAATAATCATCGGTATGGAGACAAATGATGTCCAACAAAG 290
SA1_20164_2F   GAGATTTCTGAACACTCAACCAATAATCATCGGTATGGAGACAAATGATGTCCAACAAAG 259
SA1_20164_2R   GAGATTTCTGAACACTCAACCAATAATCATCGGTATGGAGACAAATGATGTCCAACAAAG 290
SAM_20164_1F   GAGATTTCTGAACACTCATCCAATAATCATCGGTATGGAGACAAATGATGTCCAACAAAG 267
SAM_20164_1R   GAGATTTCTGAACACTCATCCAATAATCATCGGTATGGAGACAAATGATGTCCAACAAAG 290
SAM_20164_2F   GAGATTTCTGAACACTCAACCAATAATCATCGGTATGGAGACAAATGATGTCCAACAAAG 273
SAM_20164_2R   GAGATTTCTGAACACTCAACCAATAATCATCGGTATGGAGACAAATGATGTCCAACAAAG 290
               ****************** ****************************************

g20164.t1      AAGCCGGTTTATTTGGACTAACATCACTATACTCAAAGAAAACATCAAACAATTAACAAC 420
SA1_20164_1F   AAGCCGGTTTATTTGGACTAACATCACTATACTCAAGGAAAACATCAAACAATTAACAAC 319
SA1_20164_1R   AAGCCGGTTTATTTGGACTAACATCACTATACTCAAGGAAAACATCAAACAATTAACAAC 350
SA1_20164_2F   AAGCCGGTTTATTTGGACTAACATCACTATACTCAAAGAAAACATCAAACAATTAACAAC 319
SA1_20164_2R   AAGCCGGTTTATTTGGACTAACATCACTATACTCAAAGAAAACATCAAACAATTAACAAC 350
SAM_20164_1F   AAGCCGGTTTATTTGGACTAACATCACTATACTCAAAGAAAACATCAAACAATTAACAAC 327
SAM_20164_1R   AAGCCGGTTTATTTGGACTAACATCACTATACTCAAAGAAAACATCAAACAATTAACAAC 350
SAM_20164_2F   AAGCCGGTTTATTTGGACTAACATCACTATACTCAAAGAAAACATCAAACAATTAACAAC 333
SAM_20164_2R   AAGCCGGTTTATTTGGACTAACATCACTATACTCAAAGAAAACATCAAACAATTAACAAC 350
               ************************************ **********************
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```
g20164.t1      TCAGAATATTTATCTTCATAAAATGCCAAAATCTATTGGAAGAAGATCAAAATTTTATAA 480
SA1_20164_1F   TC------------------------------------------------------------ 321
SA1_20164_1R   TC------------------------------------------------------------ 352
SA1_20164_2F   TC------------------------------------------------------------ 321
SA1_20164_2R   TC------------------------------------------------------------ 352
SAM_20164_1F   TC------------------------------------------------------------ 329
SAM_20164_1R   TC------------------------------------------------------------ 352
SAM_20164_2F   TC------------------------------------------------------------ 335
SAM_20164_2R   TC------------------------------------------------------------ 352
               **
```

Figure 4.4. SA1 and SAM sequences aligned to g20164.t1, a putative *DNMT3A* RWA gene, from which the primers were designed. Asterisks indicate a perfect alignment of a base between all sequences. Highlighted regions are SNPs within a biotype (yellow).

```
g24379.t1      TGGAACTATCTTAGTGTAAAAGCTGCTAGAGAAAATACACCTTTTTATTGGTTATTTGAA 420
SA1_24379_1F   ------TATCTTAGTGTAAAAGCTGCTAGAGAAAATACACCTTTTTATTGGTTATTTGAA 54
SA1_24379_1R   TGGAACTATCTTAGTGTAAAAGCTGCTAGAGAAAATACACCTTTTTATTGGTTATTTGAA 72
SA1_24379_2F   -----CTATCTTAGTGTAAAAGCTGCTAGAGAAAATACACCTTTTTATTGGTTATTTGAA 55
SA1_24379_2R   TGGAACTATCTTAGTGTAAAAGCTGCTAGAGAAAATACACCTTTTTATTGGTTATTTGAA 72
SAM_24379_1F   TGGAACTATCTTAGTGTAAAAGCTGCTAGAGAAAATACACCTTTTTATTGGTTATTTGAA 62
SAM_24379_1R   TGGAACTATCTTAGTGTAAAAGCTGCTAGAGAAAATACACCTTTTTATTGGTTATTTGAA 72
SAM_24379_2F   TGGAACTATCTTAGTGTAAAAGCTGCTAGAGAAAATACACCTTTTTATTGGTTATTTGAA 77
SAM_24379_2R   TGGAACTATCTTAGTGTAAAAGCTGCTAGAGAAAATACACCTTTTTATTGGTTATTTGAA 72
                     ****************************************************

g24379.t1      AATGTGGCAAGCATGGAAATCAAAAATAGAGATACCATTTCAAAGTTTTTTGAATATCAA 480
SA1_24379_1F   AATGTGGCAAGTATGGAAATCAAAAATAGAGATACCATTTCAAAGTTTTTTGAATATCAA 114
SA1_24379_1R   AATGTGGCAAGTATGGAAATCAAAAATAGAGATACCATTTCAAAGTTTTTTGAATATCAA 132
SA1_24379_2F   AATGTGGCAAGTATGGAAATCAAAAATAGAGATACCACTTCAAAGTTTTTTGAATATCAA 115
SA1_24379_2R   AATGTGGCAAGTATGGAAATCAAAAATAGAGATACCACTTCAAAGTTTTTTGAATATCAA 132
SAM_24379_1F   AATGTGGCAAGCATGGAAATCAAAAATAGAGATACCATTTCAAAGTTTTTTGAATATCAA 122
SAM_24379_1R   AATGTGGCAAGCATGGAAATCAAAAATAGAGATACCATTTCAAAGTTTTTTGAATATCAA 132
SAM_24379_2F   AATGTGGCAAGTATGGAAATCAAAAATAGAGATACCATTTCAAAGTTTTTTGAATATCAA 137
SAM_24379_2R   AATGTGGCAAGTATGGAAATCAAAAATAGAGATACCATTTCAAAGTTTTTTGAATATCAA 132
               *********** ************************* *********************

g24379.t1      CCAATTATTTTTGATTCATTACACTTCAGCCCACAGCGACGTAGACGATATTTTTGGTCT 540
SA1_24379_1F   CCAATTATTTTTGATTCATTACACTTCAGCCCACAGCGACGTAGACGATATTTTTGGTCT 174
SA1_24379_1R   CCAATTATTTTTGATTCATTACACTTCAGCCCACAGCGACGTAGACGATATTTTTGGTCT 192
SA1_24379_2F   CCAATTATTTTTGATTCATTACACTTCAGCCCACAGCGACGTAGACGATATTTTTGGTCT 175
SA1_24379_2R   CCAATTATTTTTGATTCATTACACTTCAGCCCACAGCGACGTAGACGATATTTTTGGTCT 192
SAM_24379_1F   CCAATTATTTTTGATTCATTACACTTCAGCCCACAGCGACGTAGACGATATTTTTGGTCT 182
SAM_24379_1R   CCAATTATTTTTGATTCATTACACTTCAGCCCACAGCGACGTAGACGATATTTTTGGTCT 192
SAM_24379_2F   CCAATTATTTTTGATTCATTACACTTCAGCCCACAGCGACGTAGACGATATTTTTGGTCT 197
SAM_24379_2R   CCAATTATTTTTGATTCATTACACTTCAGCCCACAGCGACGTAGACGATATTTTTGGTCT 192
               ************************************************************

g24379.t1      AATTTCCCGGGTATTAAACAGTTGGCTTATTCTCGGGAGATGGATGACTATGAAATGATT 600
SA1_24379_1F   AATTTCCCGGGTATTAAACAGTTGGCTTATTCTCGGGAGATGGATGACTATGAAATGATT 234
SA1_24379_1R   AATTTCCCGGGTATTAAACAGTTGGCTTATTCTCGGGAGATGGATGACTATGAAATGATT 252
SA1_24379_2F   AATTTCCCGGGTATTAAACAGTTGGCTTATTCTCGGGAGATGGATGACTATGAAATGATT 235
SA1_24379_2R   AATTTCCCGGGTATTAAACAGTTGGCTTATTCTCGGGAGATGGATGACTATGAAATGATT 252
SAM_24379_1F   AATTTCCCGGGTATTAAACAGTTGGCTTATTCTCGGGAGATGGATGACTATGAAATGATT 242
SAM_24379_1R   AATTTCCCGGGTATTAAACAGTTGGCTTATTCTCGGGAGATGGATGACTATGAAATGATT 252
SAM_24379_2F   AATTTCCCGGGTATTAAACAGTTGGCTTATTCTCGGGAGATGGATGACTATGAAATGATT 257
SAM_24379_2R   AATTTCCCGGGTATTAAACAGTTGGCTTATTCTCGGGAGATGGATGACTATGAAATGATT 252
               ************************************************************
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g24379.t1     GATTCAAAACTTGAAGATTATTTAGAGAAGAATTTAGACCGACAGGCTAATGTAGTAAAA 660
SA1_24379_1F  GATTCAAAACTTGAAGATTATTTAGAGAAGAATTTAGACCGACAGGCTAATGTAGTAAAA 294
SA1_24379_1R  GATTCAAAACTTGAAGATTATTTAGAGAAGAATTTAGACCGACAGGCTAATGTAGTAAAA 312
SA1_24379_2F  GATTCAAAACTTGAAGATTATTTAGAGAAGAATTTAGACCGACAGGCTAATGTAGTAAAA 295
SA1_24379_2R  GATTCAAAACTTGAAGATTATTTAGAGAAGAATTTAGACCGACAGGCTAATGTAGTAAAA 312
SAM_24379_1F  GATTCAAAACTTGAAGATTATTTAGAGAAGAATTTAGACCGACAGGCTAACGTAGTAAAA 302
SAM_24379_1R  GATTCAAAACTTGAAGATTATTTAGAGAAGAATTTAGACCGACAGGCTAACGTAGTAAAA 312
SAM_24379_2F  GATTCAAAACTTGAAGATTATTTAGAGAAGAATTTAGACCGACAGGCTAATGTAGTAAAA 317
SAM_24379_2R  GATTCAAAACTTGAAGATTATTTAGAGAAGAATTTAGACCGACAGGCTAATGTAGTAAAA 312
              ************************************************* *********

g24379.t1      ATTGGTACCATCACTTCAAAAAGAAGTTGCTTACAAGATA 700
SA1_24379_1F   ATTGGTACCATCACTTCAAAA------------------- 315
SA1_24379_1R   ATTGGTACCATCACTTCAAAA------------------- 333
SA1_24379_2F   ATTGGTACCATCACTTCAAAA------------------- 316
SA1_24379_2R   ATTGGTACCATCACTTCAAAA------------------- 333
SAM_24379_1F   ATTGGTACCATCACTTCAAAA------------------- 323
SAM_24379_1R   ATTGGTACCATCACTTCAAAA------------------- 333
SAM_24379_2F   ATTGGTACCATCACTTCAAAA------------------- 338
SAM_24379_2R   ATTGGTACCATCACTTCAAAA------------------- 333
               *********************
```

Figure 4.5. SA1 and SAM sequences aligned to g24379.t1, a putative *DNMT3B* RWA gene, from which the primers were designed. Asterisks indicate a perfect alignment of a base between all sequences. Highlighted regions are SNPs within a biotype (yellow).

4.3.2 *DNMT* expression analysis

The expression of aphid head *DNMTs* among the biotypes was next investigated (see Appendix B for RT-qPCR standard curves, efficiencies (E) and R^2 values (Appendix B, Figures B9–B13), as well as the melt curves of the genes examined (Appendix B, Figures B14–B18)). As seen in Figure 4.6, the *DNMT1* expression of biotypes SA2 and SA3 was down-regulated in comparison to biotype SA1. Biotype SAM's *DNMT1* expression was, however, up-regulated when compared to that of biotype SA1. As the *p* value of the ANOVA was 0.416 for *L27* and 0.362 for *L32* (Appendix B, Table B3), the null hypothesis failed to be rejected, and no significant differences between the mean *DNMT1* expression levels were detected between the biotypes.

The *DNMT2* gene was the most stably expressed of the three *DNMT* genes tested, and as with *DNMT1*, no significant differences in expression were found between any of the biotypes (Figure 4.7; Appendix B, Table B4). Biotype SA1 exhibited the highest *DNMT2* expression level, with the exception of biotype SA3's *DNMT2* expression when normalised using *L27* (SA1 = 1 vs SA3 = 1.01).

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The expression of *DNMT3* showed the most inter-biotype variation of the three *DNMT* subfamilies (Figure 4.8). Biotype SA2's *DNMT3* expression was up-regulated when compared to biotype SA1. The expression of the two more virulent biotypes, SA3 and SAM, was down-regulated in comparison to that of SA1. The null hypothesis of the ANOVA was rejected (Appendix B, see Table B5 for *p* values), and Fisher's LSD test revealed that the *DNMT3* expression levels of SA3 and SAM, when normalised using *L27* and *L32*, were significantly lower than that of SA2 (Appendix B, Table B5). When the stringency of the Fisher's LSD test was reduced to a *p* value of ≤ 0.1 (Appendix B, Table B5), a significant difference between SA1 and SAM's *DNMT3* expression became apparent (when normalised using both reference genes), with SAM's expression being significantly lower than SA1's. At the same level of significance, SA3's *DNMT3* expression, normalised using *L32*, was also significantly lower than that of SA1.

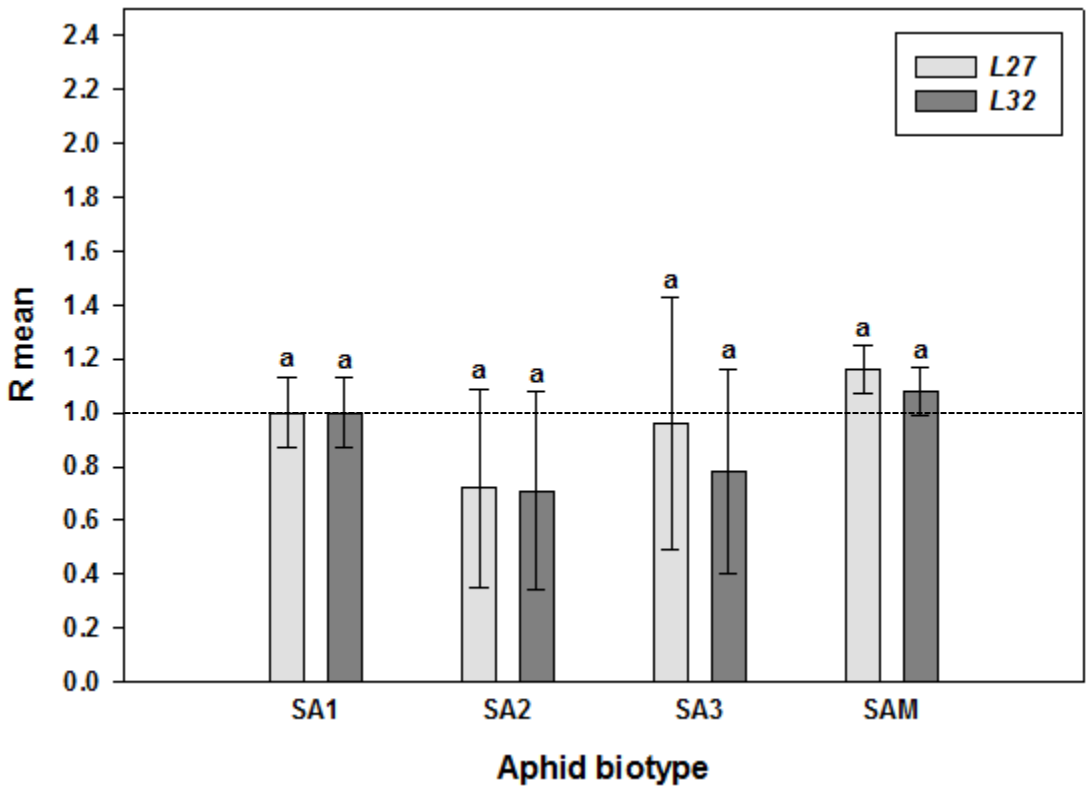

Figure 4.6. A comparison of the average relative expression (R mean) of *DNMT1* of South African RWA biotype heads. Fold changes in expression are shown relative to the SA1 samples, the expression of which was set at 1, as indicated by the dotted line. The light and dark grey bars represent the expression when normalised against the reference genes *L27* and *L32* respectively, and the error bars indicate standard deviation. Different alphabetic letters indicate significant differences ($p \leq 0.05$) (none in this case).

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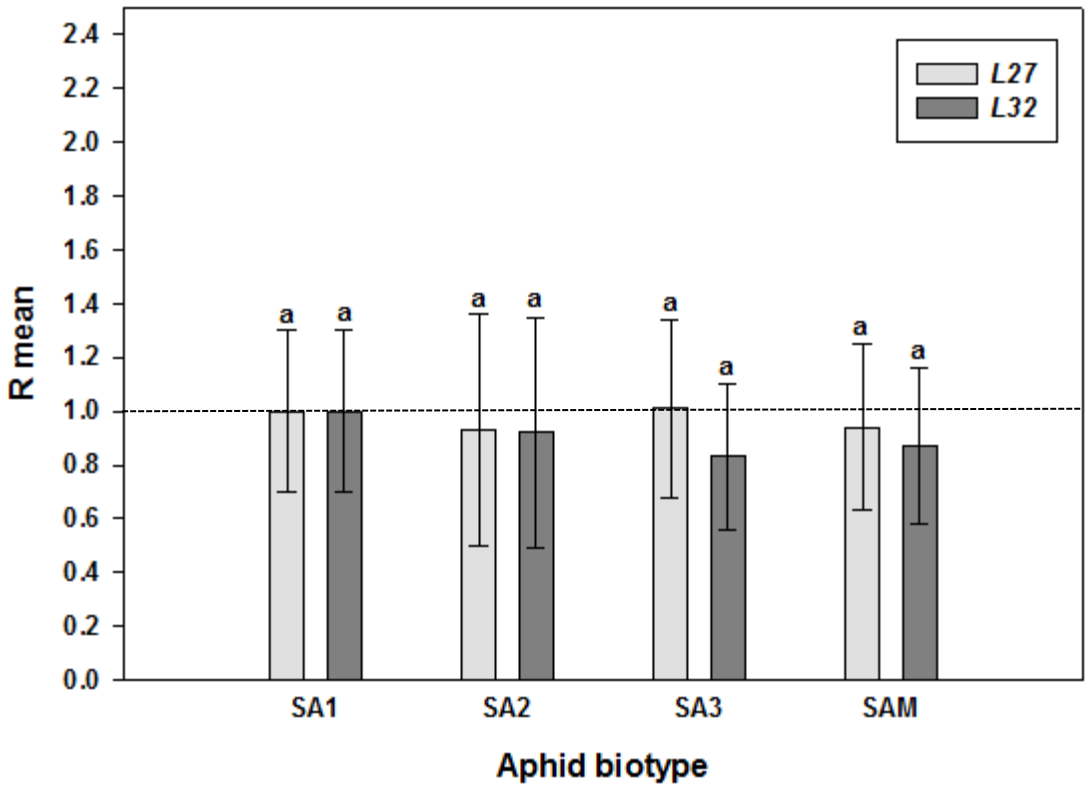

Figure 4.7. A comparison of the average relative expression (R mean) of *DNMT2* of South African RWA biotype heads. Fold changes in expression are shown relative to the SA1 samples, the expression of which was set at 1, as indicated by the dotted line. The light and dark grey bars represent the expression when normalised against the reference genes *L27* and *L32* respectively, and the error bars indicate standard deviation. Different alphabetic letters indicate significant differences ($p \leq 0.05$) (none in this case).

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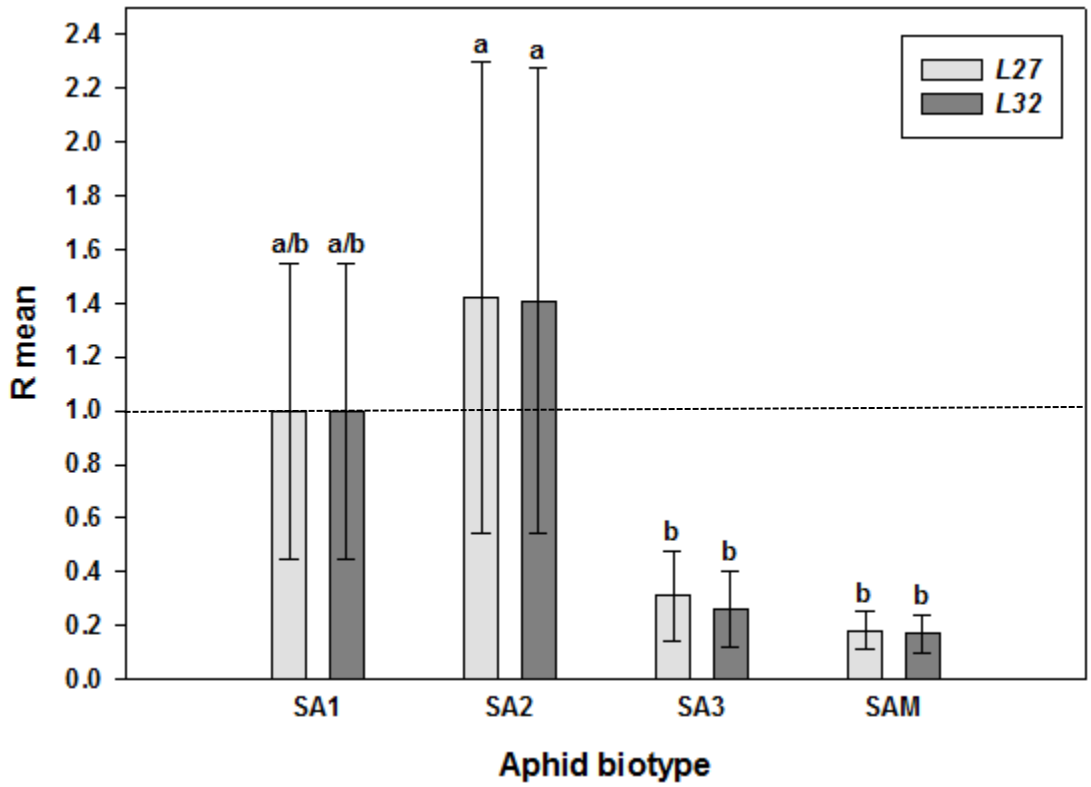

Figure 4.8. A comparison of the average relative expression (R mean) of *DNMT3* of South African RWA biotype heads. Fold changes in expression are shown relative to the SA1 samples, the expression of which was set at 1, as indicated by the dotted line. The light and dark grey bars represent the expression when normalised against the reference genes *L27* and *L32* respectively, and the error bars indicate standard deviation. Different alphabetic letters indicate significant differences ($p \leq 0.05$).

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4.3.3 DNMT protein activity quantification

The DNMT protein activity (OD/h/µg) ranged from 44.80 to 53.54, with biotype SAM exhibiting the lowest DNMT protein activity of the four biotypes (Figure 4.9). The DNMT protein activity levels did not differ significantly between the biotypes (Appendix B, Table B6).

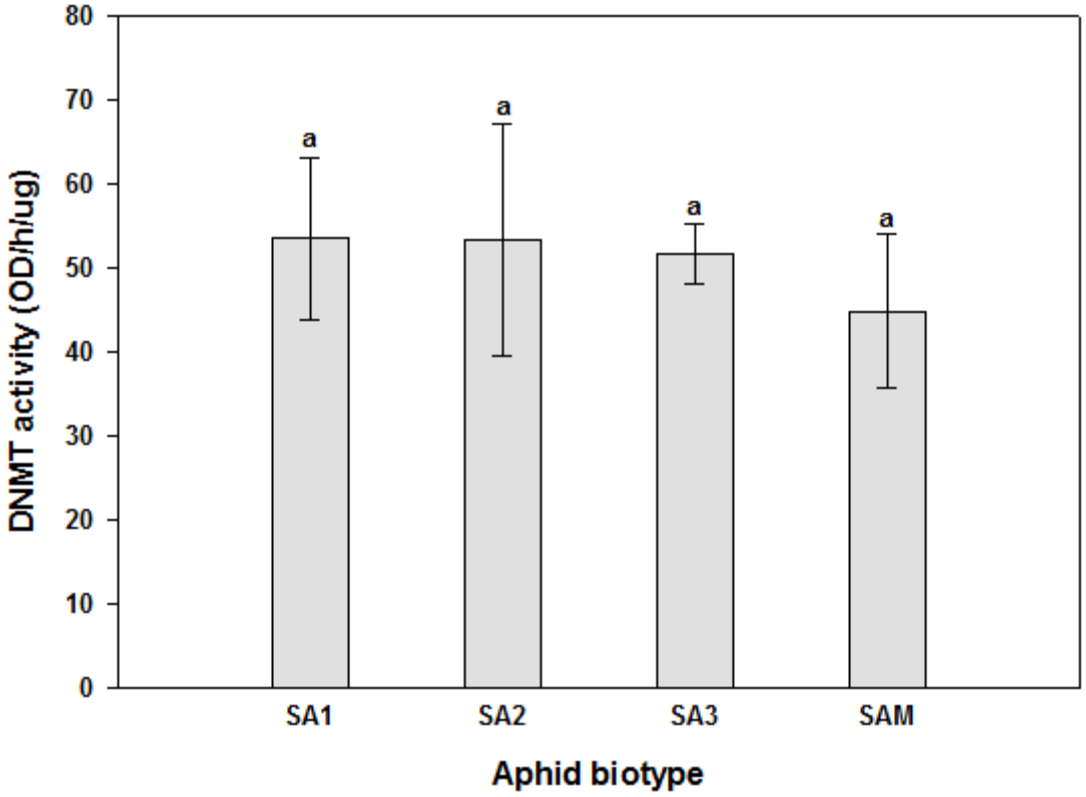

Figure 4.9. DNMT protein activity (OD/h/µg) of South African RWA biotypes SA1, SA2, SA3 and SAM, with error bars indicating the standard deviation. Different alphabetic letters indicate significant differences ($p \leq 0.05$) (none in this case).

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4.3.4 Global methylation and hydroxymethylation quantification

Antibodies specific to 5mC and 5hmC were used to gain insight into the methylation and demethylation occurring in the South African RWA biotypes at the global level. The use of the 5mC antibody revealed similar levels of global methylation between the four biotypes tested (Appendix B, Table B7), which ranged from 0.1% to 0.16% (Figure 4.10).

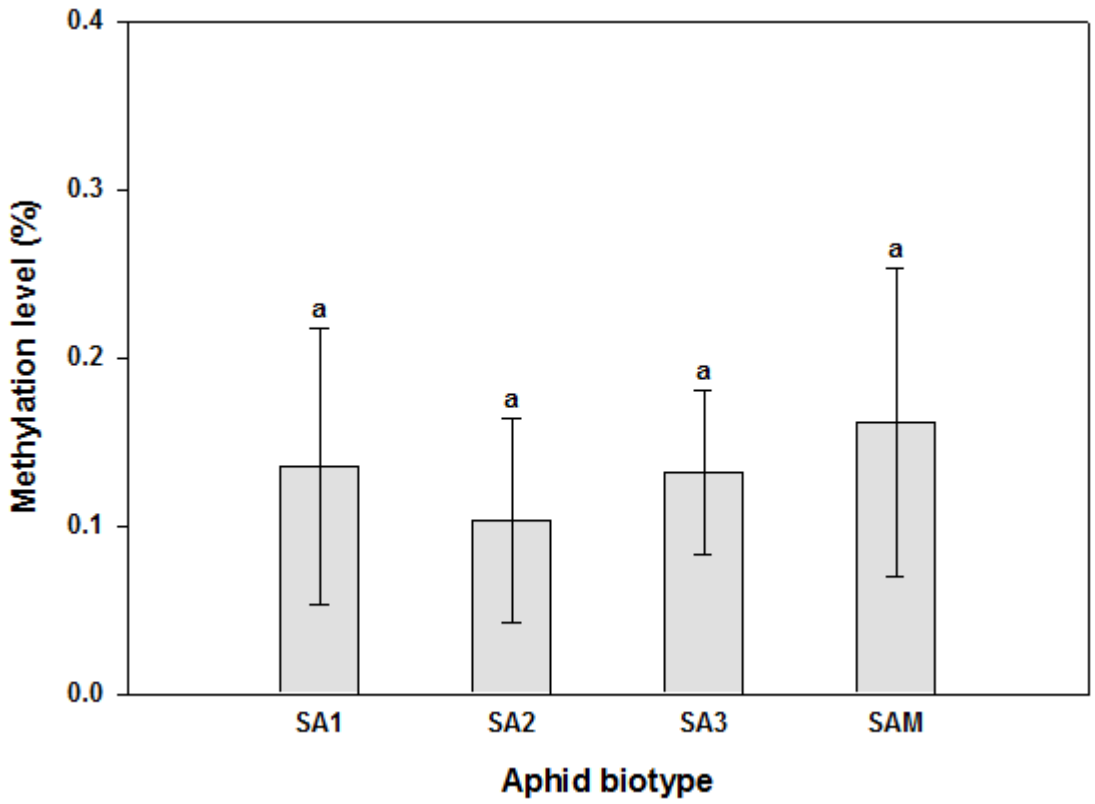

Figure 4.10. Global methylation levels (%) of South African RWA biotypes SA1, SA2, SA3 and SAM, with error bars indicating the standard deviation. Different alphabetic letters indicate significant differences ($p \leq 0.05$) (none in this case).

The hydroxymethylation levels ranged from 0.02% to 0.46%, with biotype SA2 displaying the lowest, and biotype SAM displaying the highest 5hmC levels respectively (Figure 4.11). The ANOVA null hypothesis was rejected ($p < 0.0001$), with Fisher's LSD test revealing that biotype SA2's global 5hmC level was significantly lower than that of the other three biotypes, whilst biotype SAM's level was significantly higher than that of the other three biotypes (Appendix B, Table B8). The global 5hmC levels of biotypes SA1 and SA3 did not differ significantly from each other (p=0.233).

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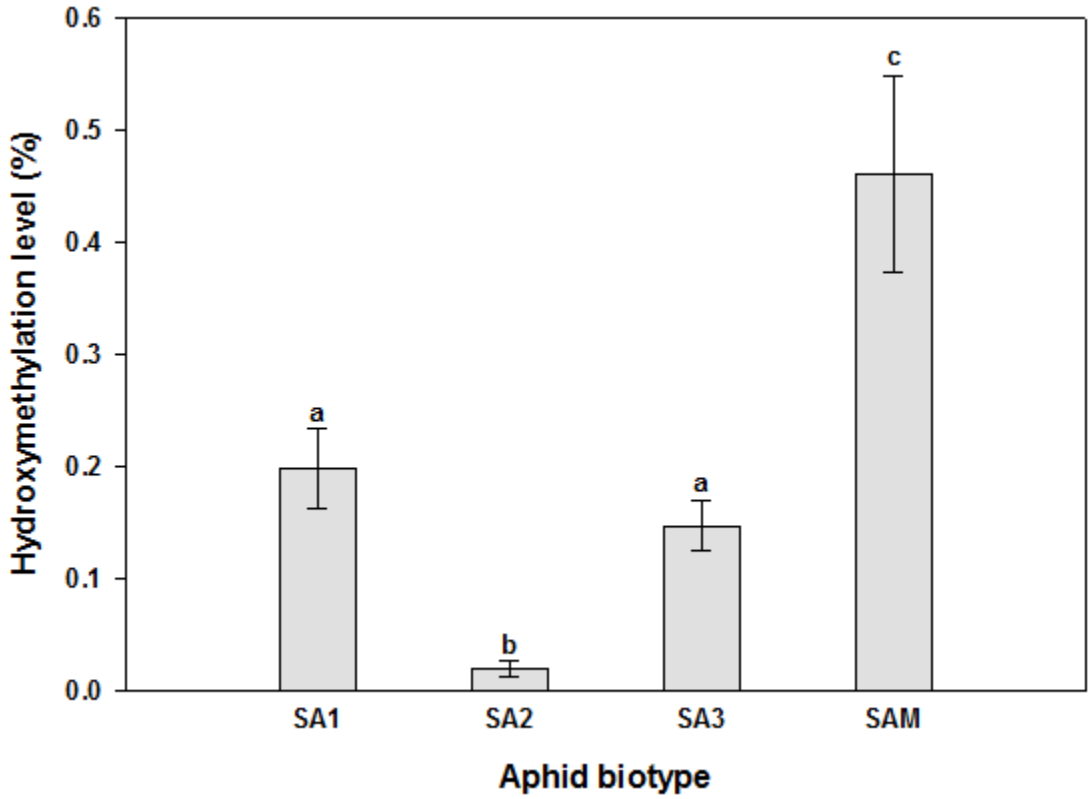

Figure 4.11. Global hydroxymethylation levels (%) of South African RWA biotypes SA1, SA2, SA3 and SAM, with error bars indicating the standard deviation. Different alphabetic letters indicate significant differences ($p \leq 0.05$).

4.4 Discussion

Integrated pest management programmes depend heavily on the breeding of wheat cultivars which provide resistance against RWA (Tolmay *et al*. 1997; Smith and Clement 2012; Botha 2013; Sinha and Smith 2014). The effectiveness of these cultivars, however, is often short-lived as aphids overcome the resistance they impart (Botha *et al*. 2005, 2010; Tagu *et al*. 2008; Sinha and Smith 2014). Understanding how new aphid biotypes develop, as well as the mechanisms they employ to exert their virulence, enabling the breakdown of plant resistance, are of utmost importance if resistant cultivars are to be used to their full potential (Botha *et al*. 2014a). The availability of the highly virulent mutant RWA biotype (SAM) (Van Zyl and Botha 2008; Swanevelder *et al*. 2010), alongside South Africa's naturally occurring biotypes (SA1, SA2, SA3 and SA4) (Walters *et al*. 1980; Tolmay *et al*. 2007; Jankielsohn 2011, 2014, 2016) presents a unique opportunity for the study of biotypification. Despite having developed from SA1, which only renders *dn3*-containing

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cultivars susceptible (Jankielsohn 2011), SAM has the remarkable ability to overcome the resistance of all the *Dn* genes that have been introduced and/or documented (Botha 2013; Botha *et al*. 2014a). SAM thus serves as a model to resolve aphid biotypification.

In the present study, the *DNMT* genes (of SA1 and SAM) were identified, sequenced and compared. The *DNMT* expression and DNMT protein activity of the various biotypes were also quantified. Additionally, global methylation and hydroxymethylation levels were quantified, with the goal of understanding if methylation (and the enzymes which catalyse its addition) and demethylation are related to the reported RWA virulence levels (Jankielsohn 2014, 2016).

4.4.1 Identification and sequencing of RWA *DNMTs*

The initial homology-based search for the RWA DNMTs was performed at the protein level as opposed to the DNA level, as this circumvented problems related to the degeneracy of the genetic code (Lagerkvist 1978), such as the possibility of two similar DNMT proteins being encoded by quite different nucleotide sequences, which might not have been detected as the best BLASTn match, and may have been overlooked. Of the five RWA proteins identified as putative DNMTs, the strongest match to the DNMT proteins of the closely related *A. pisum* was g2520.t1, the putative DNMT2 protein. This result was expected, as the DNMT2 subfamily is the most conserved of the three DNMT subfamilies (Goll and Bestor 2005; Schaefer and Lyko 2007, 2010).

The primers used for sequencing the *DNMTs* were designed based on the coding domain sequences of the genes corresponding to the best protein matches, so as to look for conservation/variation only in the exonic regions of the genes. The motivation for this is that introns, and the variation therein (e.g., SNPs or indels) would be spliced out during mRNA processing (Tilgner *et al*. 2012). Ward and Cooper (2010) do, however, caution that mutations in introns can lead to intron retention, the activation of cryptic splice sites or exon skipping, should they occur at splice sites, and these would affect the sequence of the mRNA and thus cDNA that was synthesised.

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As with other Hemiptera investigated thus far, including *A. pisum* and *Nilaparvata lugens*, at least one *DNMT* gene from each *DNMT* subfamily was identified in RWA (Walsh *et al*. 2010; Zhang *et al*. 2015). *Acyrthosiphon pisum* has two *DNMT1* and *DNMT3* genes and one *DNMT2* gene (Walsh *et al*. 2010), whilst *N. lugens* has one gene of each *DNMT* subfamily (Zhang *et al*. 2015). Here one putative *DNMT1* and *DNMT2* gene, as well as two putative *DNMT3* genes (*DNMT3A* and *DNMT3B*), were reported on.

The SA1 and SAM *DNMTs* of the three subfamilies have highly conserved nucleotide sequences, with only one SNP present between the two biotypes (in the *DNMT1* gene), which could have a functional protein effect (Figure 4.1). This high level of sequence conservation makes clear two things. Firstly, the biotypification of SA1 to SAM had little effect on the *DNMT* sequences, and secondly, there were no mutations in intronic splice sites between SA1 and SAM that resulted in major sequence differences. The 13 intrabiotypic SNPs that occurred could possibly be ascribed to heterozygosity (Figures 4.1, 4.3–4.5). The similarity of the *DNMT* sequences implies that SA1 and SAM have a similar potential for methylating their genomes. The BLASTn analysis on the NCBI using these sequences revealed that the high level of sequence conservation between SA1 and SAM extended to RWA biotype US2 (Appendix B, Figures B4–B8), indicating that the RWA *DNMT* subfamilies appear to be highly conserved, even in geographically distinct RWA populations.

4.4.2 *DNMT* expression analysis

The absence of available literature on RWA DNMTs prompted an investigation into the baseline *DNMT* expression (i.e., expression of aphids not challenged with resistance) of South African RWA. The expression results were analysed with the different functions of the DNMTs in mind. It should be noted that except where otherwise stated, the functions of the DNMTs have been characterised in mammals. However, it is widely assumed that the insect DNMTs have the same functions as their mammalian orthologues (Wang *et al*. 2006; Glastad *et al*. 2014), and conclusions were drawn accordingly.

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The expression analysis was performed using cDNA transcribed from aphid head RNA. The decision to use aphid heads as opposed to whole aphids, was based on the fact that effector proteins (which, if detected by plant resistance proteins, trigger plant defences) are produced in the salivary glands, located in aphid heads (Botha *et al*. 2005, 2006). The *DNMT* expression within aphid heads could directly affect the methylation of effector genes. This in turn, based on the two main functions of intragenic methylation, could alter the splice variants produced by the effector gene, and/or the regulation of spurious transcription at cryptic binding sites or intragenic promoters, thereby influencing aphid virulence. Knowledge of baseline aphid head *DNMT* expression, and ultimately of how *DNMT* expression changes when aphids are challenged with resistance, will prove useful in understanding aphid virulence.

It is possible to excise the salivary glands from the aphid head (Cloete 2015). However, the time-consuming nature of this complex process could result in changes in gene expression occurring during excision. By using a liquid nitrogen-cooled scalpel to cut off the aphid head, and by placing the still frozen head directly into a pre-cooled Eppendorf tube, the expression of the head tissue is more likely to remain unaltered.

4.4.2.1 *DNMT1* expression

DNA methyltransferase 1 is a maintenance methyltransferase, the function of which is to accurately copy the DNA methylation pattern from the parent strand of DNA to the daughter strand during replication (Goll and Bestor 2005). As DNA replication is a process which occurs throughout the life cycle of the aphid, and requires the constant copying of methylation patterns, the aphid biotype with the highest global methylation level would be expected to have the highest *DNMT1* expression level, and the opposite would be expected for the biotype with the lowest global methylation level. The results presented in Figures 4.6 (*DNMT1* expression) and 4.10 (global methylation) are in agreement with this, and follow a very similar trend. The global methylation levels of biotypes SA2 and SA3 are lower than that of SA1 (albeit only slightly), and this is consistent with the down-regulation of *DNMT1* expression of these biotypes in comparison to SA1's *DNMT1* expression.

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SAM is the only biotype which has a global methylation level higher than SA1. Similarly, SAM is the only biotype which has an up-regulated *DNMT1* expression when compared to SA1. The *DNMT1* expression levels are thus clearly related to/explained by the amount of methylation present within the biotypes.

4.4.2.2 *DNMT2* expression

DNA methyltransferase 2 is able to methylate both DNA and transfer RNA (tRNA), and has a much higher enzymatic activity towards the latter, where it specifically targets cytosine 38 of $tRNA^{Asp}_{GUC}$, $tRNA^{Gly}_{GCC}$ and $tRNA^{Val}_{AAC}$ (Goll *et al*. 2006; Jeltsch *et al*. 2006, 2016; Schaefer and Lyko 2007, 2010). The similar *DNMT2* expression levels across the four biotypes are likely due to a number of important biological processes involving DNMT2-mediated tRNA methylation. For example, methylation of cytosine 38 of $tRNA^{Asp}_{GUC}$ in mice increases translational fidelity by allowing time for the proper discrimination between $tRNA^{Asp}_{GUC}$ and the near-cognate codon $tRNA^{Glu}_{UUC}$ (Jeltsch *et al*. 2016). As $tRNA^{Asp}_{GUC}$ is also methylated by DNMT2 in *Drosophila* (Schaefer *et al*. 2010), its methylation could play a similar role in insect translation.

Transfer RNA methylation also promotes tRNA stability in *Drosophila* by protecting the tRNA molecules from stress-induced cleavage (Schaefer *et al*. 2010; Durdevic *et al*. 2013b; Jeltsch *et al*. 2016). This is an important function of DNMT2 tRNA methylation, as tRNA fragments can compete with double-stranded RNA (dsRNA) as a substrate for Dicer-2, thus affecting the efficiency of the small-interfering RNA (siRNA) pathway (Durdevic *et al*. 2013b). Transfer RNA fragments also repress translation (in both mammals and *Drosophila*) and thus inhibit protein synthesis (Yamasaki *et al*. 2009; Durdevic *et al*. 2013b; Jeltsch *et al*. 2016). The numerous regulatory roles of DNMT2-mediated tRNA methylation underlie the need for similar *DNMT2* expression across biotypes.

The use of *DNMT2* mutant *Drosophila* has revealed that DNMT2 is important for both innate immunity against RNA viruses (Durdevic *et al*. 2013a), and in the response to both oxidative and heat stress (Schaefer *et al*. 2010). DNA methyltransferase 2 mutant *Drosophila* display an increased

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sensitivity to oxidative treatments (paraquat and H_2O_2), and heat-stressed *DNMT2* mutant *Drosophila* exhibit a reduced lifespan (Schaefer *et al*. 2010).

The slightly higher *DNMT2* expression level exhibited by SA1, when compared to SAM, might be explained by the different responses these biotypes invoke upon feeding on resistant wheat cultivars. When SA1 feeds on wheat with antibiotic (e.g., "Tugela *Dn1*") or antixenotic (e.g., "Tugela *Dn5*") modes of resistance, an oxidative burst (elevated H_2O_2) occurs at the feeding sites (Botha *et al*. 2014b; Burger *et al*. 2017). This is accompanied by higher levels of peroxidase activity (Botha *et al*. 2014b). Peroxidases are reactive oxygen species (ROS) enzymes which regulate the levels of H_2O_2, and have a role in both the production of H_2O_2 (Ślesak *et al*. 2007; Almagro *et al*. 2009), and its breakdown to water (Giorgio *et al*. 2007; Ślesak *et al*. 2007; Sharma *et al*. 2012). Biotype SAM feeding, however, is not associated with an oxidative burst or increased peroxidase activity levels, because SAM avoids detection by wheat hosts (Botha *et al*. 2014a). The aphids used in this study were fed on susceptible cultivars, and thus were not faced with oxidative stress as a means of plant defence. The slightly higher baseline expression of *DNMT2* in biotype SA1 when compared to SAM, could indicate that SA1 is poised to increase its *DNMT2* (which is involved in protecting against oxidative stress) expression should it be challenged by an oxidative burst.

4.4.2.3 *DNMT3* expression

DNA methyltransferase 3 has long been known as a *de novo* methyltransferase (Okano *et al*. 1999; Goll and Bestor 2005), which establishes new methylation patterns by methylating previously unmethylated sites (Kunert *et al*. 2003; Schaefer and Lyko 2007). The *DNMT3* expression of the two more virulent biotypes used in this study, SA3 and SAM, is down-regulated in comparison to the two less virulent biotypes, SA1 and SA2, and this decrease in expression could therefore be advantageous from a virulence perspective. Given that *de novo* methylation is known to occur in response to environmental stimuli (Zhang *et al*. 2015; Standage *et al*. 2016), a plausible explanation for the lower *DNMT3* expression is that the more virulent biotypes are aware that they are not being

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challenged by environmental stressors (i.e., resistance), and thus expend less energy on *DNMT3* transcription.

A role for DNMT3A in the facilitation of transcription has also been identified, with DNMT3A-dependent methylation of gene bodies promoting transcription by antagonising Polycomb repression (Wu *et al*. 2010). Although the aphid effector genes are yet to be identified (Botha *et al*. 2005, Smith and Clement 2012), it is possible that they contain DNMT3A binding sites within their gene bodies, and that their transcription could be facilitated by DNMT3A binding and subsequent methylation. In the current study, SA1 and SA2's *DNMT3A* expression, and therefore DNMT3A protein production, is up-regulated in comparison to the more virulent biotypes. The presence of more DNMT3A protein could result in increased transcription of the effector genes and a concomitant increase in effector protein production.

Since it is the recognition of aphid effector proteins by plant resistance proteins that initiates plant defence responses (in wheat cultivars resistant to aphids) (Botha *et al*. 2005, 2006), the increase in the amount of effector proteins produced by SA1 and SA2 would allow for the easier detection of these biotypes by plants. Indeed, there are more *Dn* resistance genes (and cultivars into which these have been bred) which provide resistance against biotypes SA1 and SA2, than there are which provide resistance against SA3, SA4 and SAM (Botha 2013; Botha *et al*. 2014a; Jankielsohn 2014, 2016). The *DNMT3A* expression tested here was that of aphids reared on susceptible cultivars which did not contain any *Dn* genes. The fact that SA1 and SA2 have higher *DNMT3A* expression (and perhaps greater effector protein production) under such conditions, provides insight into why they are the least virulent biotypes. Quantifying the *DNMT3A* expression of aphids challenged by resistance will yield valuable information on DNMT3A's possible involvement in effector transcription.

Other functions of DNMT3 are a role in the removal of 5mC and 5hmC (Chen *et al*. 2012, 2013) and a proposed involvement in the maintenance of methylation, by being able to "methylate sites missed by DNMT1 activity" (Jones and Liang 2009). However, as the DNMT3-mediated removal

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of 5mC and 5hmC is dependent on certain redox conditions (Chen *et al*. 2012, 2013), and has only been shown to occur *in vitro* (Chen *et al*. 2012, 2013), it is difficult to draw conclusions regarding the *DNMT3* expression and its potential demethylating and dehydroxymethylating activities in RWA. DNA methyltransferase 3 is assumed to help maintain methylation in densely methylated areas of the mammalian genome (Jones and Liang 2009). Although the global methylation levels of RWA have now been determined (Figure 4.10), the distribution of methylation within the genome will need to be assessed through bisulphite sequencing, before conclusions relating to the density of methylation and the possible maintenance role of DNMT3 can be drawn.

4.4.3 DNMT protein activity

The similarity of the DNMT protein activity between the biotypes is expected based on the fact that the global methylation levels (Figure 4.10) are so similar, with a range of only 0.06%. It is difficult to partition the activity of the different DNMT proteins, as the kit measured the total DNMT activity, that of DNMT1, DNMT3A and DNMT3B. This, unfortunately, makes it impossible to compare the expression of the individual *DNMTs* to their protein activity. However, biotype SAM's *DNMT1* expression was slightly higher than that of biotype SA1 (Figure 4.6), whilst its *DNMT3* expression was signigicantly lower than that of SA1, at a significance level of $p \leq 0.1$ (Figure 4.8). The combination of the two is reflected in the total measured DNMT activity being slightly lower than that of SA1.

4.4.4 Global methylation

The study by Gong *et al*. (2012) remains the only report on RWA methylation (apart from Chapter 3). The methylation levels reported here provide the first information on the global, genome-wide methylation of RWA. The global methylation levels did not differ significantly between the biotypes and were thus unable to account for the reported difference in virulence levels of the South African RWA biotypes (Jankielsohn 2014, 2016).

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The global methylation levels (0.1%–0.16%) are in line with other reports of insect methylation. For example, the global methylation levels of *A. mellifera* (Lyko *et al*. 2010), *B. mori* (Xiang *et al*. 2010), the ants *Camponotus floridanus* and *Harpegnathos saltator* (Bonasio *et al*. 2012) and *N. vitripennis* (Beeler *et al*. 2014) are all between 0.1% and 0.2%. These levels were, however, determined using bisulphite sequencing, and caution should be taken when directly comparing global methylation levels determined using different techniques (Ye *et al*. 2013).

The current study used the same antibody as that of Panikar *et al*. (2015), who investigated adult *D. melanogaster* methylation, to measure global methylation, and thus allows a more direct comparison. Although other authors have reported lower levels of adult *D. melanogaster* methylation using bisulphite sequencing (0% – Lyko *et al*. 2000), liquid chromatography tandem mass spectrometry (0.034% – Capuano *et al*. 2014) and thin layer chromatography (0.05%–0.1% – Gowher *et al*. 2000), Panikar *et al*. (2015) found the adult *D. melanogaster* genome to be approximately 0.5% methylated. Russian wheat aphids thus have low, but detectable levels of methylation which are approximately 0.2 to 0.4 fold of that of the model organism *D. melanogaster*, as measured using the same technique.

4.4.5 Global demethylation

The similarity between the RWA biotypes with regards to methylation levels, *DNMT1* expression levels (responsible for maintaining the methylation levels), and the summed protein activity of the maintenance and *de novo* methyltransferases, prompted an investigation into the RWA hydroxymethylation levels. This was conducted to see if the removal of methylation may be related to the reported differences in virulence of the RWA biotypes (Jankielsohn 2014, 2016).

The hydroxylation of methylated cytosines by TET enzymes, resulting in the formation of 5hmC, is one of various active demethylation mechanisms (Tahiliani *et al*. 2009; Ito *et al*. 2010; Branco *et al*. 2012; Shen *et al*. 2014). The initial functional characterisation of TETs was performed in mammals, which have three TET enzymes, namely TET1, TET2 and TET3 (Iyer *et al*. 2009; Tahiliani *et al*. 2009). In contrast to this, invertebrates possess only a single TET orthologue (Pastor *et al*. 2013;

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Wojciechowski *et al*. 2014), which has been identified in insects containing hydroxymethylation, including *A. mellifera* (Cingolani *et al*. 2013; Wojciechowski *et al*. 2014), *T. castaneum* (Feliciello *et al*. 2013), *N. vitripennis* (Pegoraro *et al*. 2016) and *D. melanogaster* (Dunwell *et al*. 2013). In 2014, Wojciechowski *et al*. functionally characterised the *A. mellifera* TET orthologue, AmTET, and concluded that, like the mammalian TETs, AmTET is capable of hydroxylating 5mC to form 5hmC. The presence of measurable amounts of 5hmC in the four RWA biotypes tested, suggests that at least one active demethylation pathway (i.e., hydroxylation of 5mC by TET) is present in RWA. Thus, although a homology search for a RWA TET gene/protein was not performed, the RWA genome likely contains a TET orthologue which orchestrates active demethylation.

The results obtained (Figure 4.11) suggest that RWA biotype SAM has a significantly greater capacity to actively demethylate DNA than any of the other South African biotypes. The high level of hydroxymethylation exhibited by SAM was not, however, totally unexpected, because SAM was also found to exhibit the highest level of hemimethylation (at the external cytosine) when its methylation was investigated using the methylation-sensitive amplification polymorphism (MSAP) technique (Chapter 3).

Hemimethylated DNA arises during DNA replication, as the newly synthesised daughter strand contains unmodified cytosines (Jeltsch 2002; Goll and Bestor 2005). It can also arise during both passive (replication-dependent) and active demethylation (Ehrlich and Lacey 2013). An example of the latter is the TET-mediated hydroxylation of a 5mC base on one of the two DNA strands. The hydroxymethylated base can then be directly converted back to cytosine through the dehydroxymethylase activity of DNMT3A and DNMT3B (Chen *et al*. 2012). Alternatively, it can be deaminated to 5-hydroxymethyluracil (5hmU) by the activation-induced cytidine deaminase (AID)/apolipoprotein B mRNA editing enzyme, catalytic polypeptide (APOBEC) family of cytidine deaminases. Hydroxymethyluracil is subsequently recognised and removed by glycosylases, and replaced with an unmodified cytosine via base excision repair mechanisms (Cortellino *et al*. 2011; Guo *et al*. 2011; Branco *et al*. 2012; Hashimoto *et al*. 2012). The hydroxymethylated base can also

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be converted to 5-formylcytosine (5fC) and then 5-carboxylcytosine (5caC) (both of which are removed via glycosylases and BER mechanisms) through the oxidative action of TET enzymes (He *et al*. 2011; Maiti and Drohat 2011; Branco *et al*. 2012). Regardless of the mechanism of 5hmC removal/conversion, the resulting DNA is hemimethylated. The higher level of hydroxymethylation present in SAM, may thus underlie the increased hemimethylation level seen in this biotype.

Thus, after scrutinising the data using multiple methodologies (i.e., the 5hmC antibody and the MSAP technique), it can be concluded with fair confidence that SAM undergoes more demethylation than its parent biotype SA1, as well as biotypes SA2 and SA3. This demethylation is likely to occur at specific sets of genes depending on the environmental cue/stress SAM is faced with, as opposed to occurring globally (although global, genome-wide demethylation was measured). As gene bodies are the predominant site of methylation in insects (Zemach *et al*. 2010; Glastad *et al*. 2011; Lyko and Maleszka 2011), it is likely that it is this methylation that will be removed. The removal of intragenic methylation of certain genes could alter the transcripts that are produced, by exposing cryptic binding sites or intragenic promoters (Maunakea *et al*. 2010; Hunt *et al*. 2013a) and/or affect the splice variants that are produced, through methylation's involvement in alternative splicing (Lyko and Maleszka 2011; Shukla *et al*. 2011; Bonasio *et al*. 2012; Maunakea *et al*. 2013; Glastad *et al*. 2014; Yan *et al*. 2015). As demethylation can occur in a matter of hours (Glastad *et al*. 2011), the greater capability of SAM to demethylate its genome, provides SAM with more flexibility to adapt to changing environments, and may underlie SAM's ability to overcome plant resistance.

4.5 Conclusion

The global methylation levels of the RWA biotypes, measured using an antibody specific to 5mC, were comparable to that of adult *D. melanogaster*, as measured using the same antibody. The RWA global methylation levels were also comparable to the global methylation levels of various other insects, measured using bisulphite sequencing. Although the methylation levels were found to differ slightly between the RWA biotypes, it appears to be biotype SAM's ability to demethylate its

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genome (or parts thereof) that gives SAM an advantage in terms of adapting to environmental stressors, and overcoming the resistance imparted by resistant wheat cultivars.

Many of the aspects of methylation that were investigated here were similar between the different biotypes, as is to be expected given the similarity of the methylation levels detected. The similarity of the DNMTs was evident at the nucleotide level (between SA1 and SAM), the level of transcripts produced (for *DNMT1* and *DNMT2*), and the protein activity level. Interestingly, the expression of *DNMT3*, which methylates DNA in a *de novo* fashion in response to environmental stimuli (Zhang *et al*. 2015; Standage *et al*. 2016), was lower (and in some cases significantly so) in the more virulent biotypes, SA3 and SAM. Two explanations were offered for this, the first being that the more virulent biotypes were aware that they were not being challenged by a resistant food source, and conserved energy by producing less *DNMT3* transcripts. The possibility of DNMT3A binding sites being present on SA1 and SA2 effector genes was also raised. The implication thereof, given DNMT3A's role in the facilitation of transcription, is that the increased *DNMT3A* expression of these two biotypes would result in elevated effector transcript and protein levels, thus leading to their easier detection by plant resistance proteins.

4.6 References

4.6.1 Journal articles

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4.6.2 Websites

Cereal Genomics Base	http://cg-base.org
Clustal Omega	http://www.ebi.ac.uk/Tools/msa/clustalo/
Geneious v6.1.6	http://www.geneious.com
NCBI	http://www.ncbi.nlm.nih.gov
Primer3	http://bioinfo.ut.ee/primer3/
VecScreen	http://www.ncbi.nlm.nih.gov/tools/vecscreen/

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4.7 Appendix B

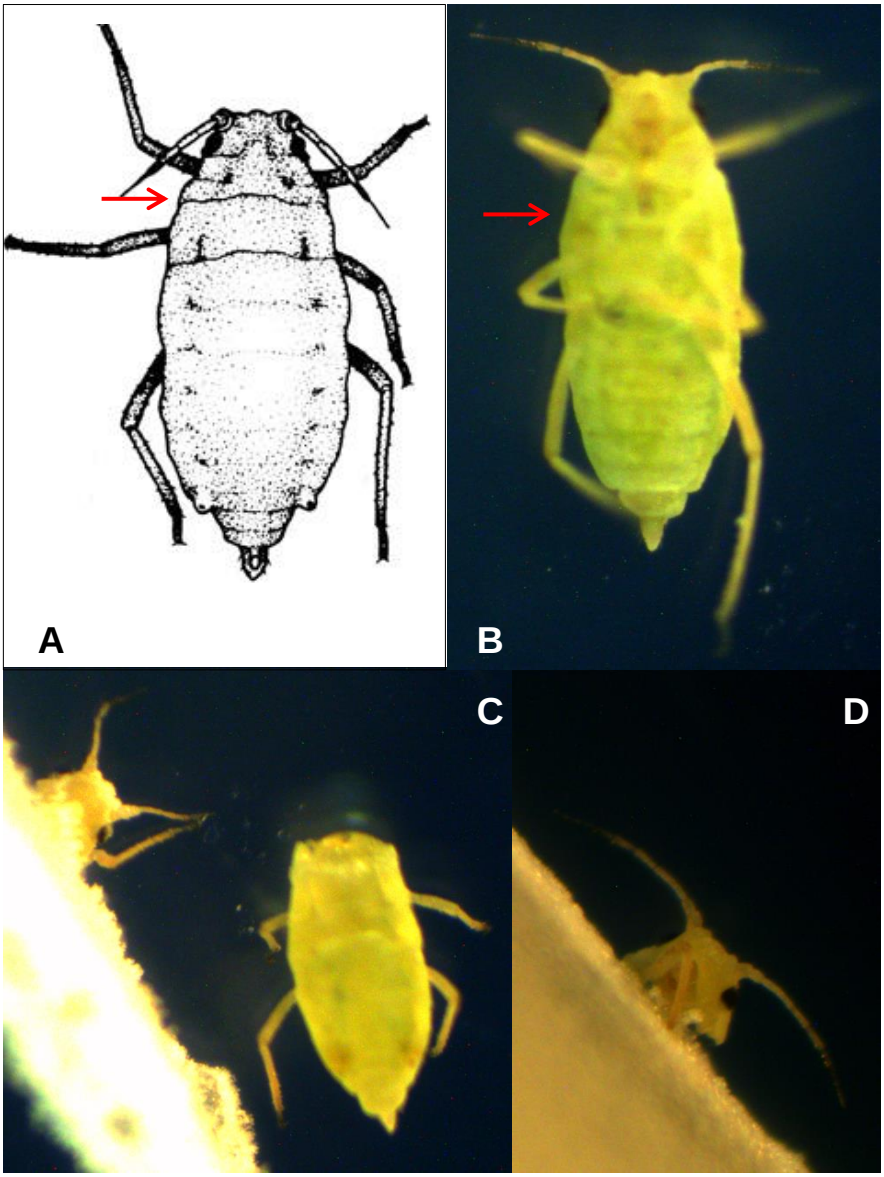

Figure B1. Aphid head excision. (A) Drawing of the dorsal side of a RWA. Sourced from http://pnwsteep.wsu.edu/tillagehandbook/chapter8/images/081390-1.gif. (B) Photograph of the ventral side of a RWA. The arrows in A and B indicate the posterior side of the prothorax, the position at which the heads were cut off. (C) An image showing the separated aphid head and body, with the head frozen to the scalpel. (D) A higher magnification of the severed aphid head. Photographs B, C and D were taken with a DCM510 microscope CMOS camera.

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```
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g16165.t1       ------------------------------------------------------------ 0
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g16165.t1       ---------------------------------------------MNRFNSGQYSLFKNSLI 17
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                                                          *****   ** ****

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g9062.t1        ------------------------------------------------------------ 829
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```

Figure B2. Alignment (Clustal Omega) of insect DNMT1 proteins shows the overlap (highlighted area) of the 3' region of *D. noxia* g9062.t1 and the 5' region of *D. noxia* g16165.t1. EFN76367 = DNA (cytosine-5)-methyltransferase 1 [Harpegnathos saltator]; EHJ76342 = DNA cytosine-5 methyltransferase [Danaus plexippus]; NP_001036980 = DNA cytosine-5 methyltransferase [Bombyx mori]; NP_001164521 = DNA methyltransferase 1a [Nasonia vitripennis]; NP_001164522 = DNA methyltransferase 1a [Apis mellifera]; XP_001942632.1 = PREDICTED: similar to DNA (cytosine-5-)-methyltransferase [Acyrthosiphon pisum]; XP_001942687.1 = PREDICTED: similar to DNA (cytosine-5-)-methyltransferase 1 [Acyrthosiphon pisum]; XP_003398214 = PREDICTED: DNA (cytosine-5)-methyltransferase PliMCI-like [Bombus terrestris]; XP_003493144 = PREDICTED: DNA (cytosine-5)-methyltransferase 1-like [Bombus impatiens]; XP_003702004 = PREDICTED: DNA (cytosine-5)-methyltransferase PliMCI-like

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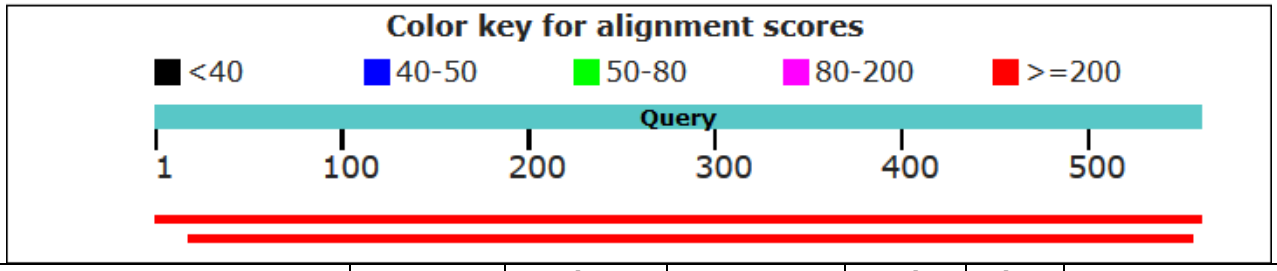
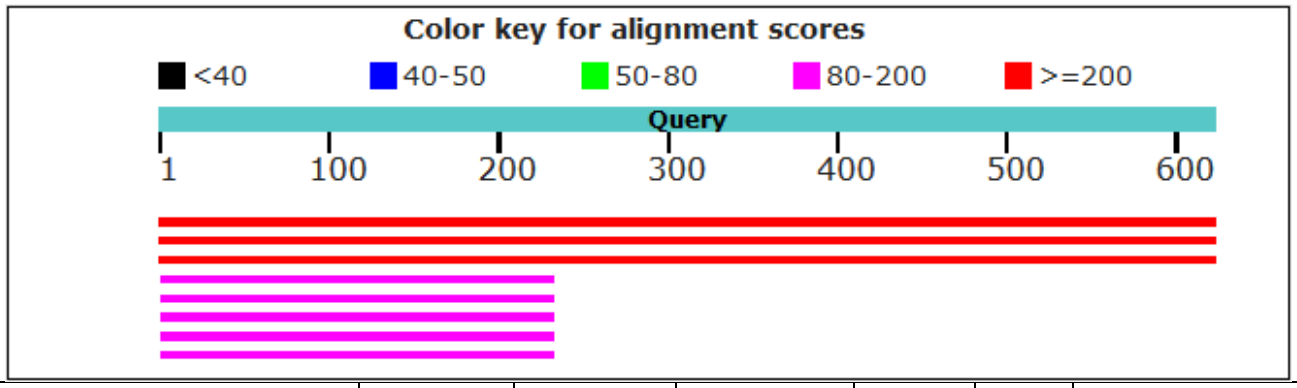


Description	Max score	Total score	Query cover	E value	Ident	Accession
PREDICTED: Diuraphis noxia DNA (cytosine-5)-methyltransferase PliMCI-like (LOC107173344), mRNA	1031	1031	100%	0.0	99%	XM_015523853.1
PREDICTED: Diuraphis noxia DNA (cytosine-5)-methyltransferase PliMCI-like (LOC107173325), mRNA	407	407	96%	2e-109	81%	XM_015523831.1

Figure B4. NCBI BLASTn using the sequenced g9062.t1 CDS as query.



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PREDICTED: Diuraphis noxia DNA (cytosine-5)-methyltransferase 1-like (LOC107172625), partial mRNA	689	689	100%	0	87%	XM_015522916.1
PREDICTED: Acyrthosiphon pisum DNA (cytosine-5)-methyltransferase PliMCI (LOC103308886), mRNA	675	675	100%	0	86%	XM_008183068.2
PREDICTED: Bombyx mori DNA cytosine-5 methyltransferase (Dnmt1),	95.3	95.3	37%	1e-15	74%	XM_012695406.1

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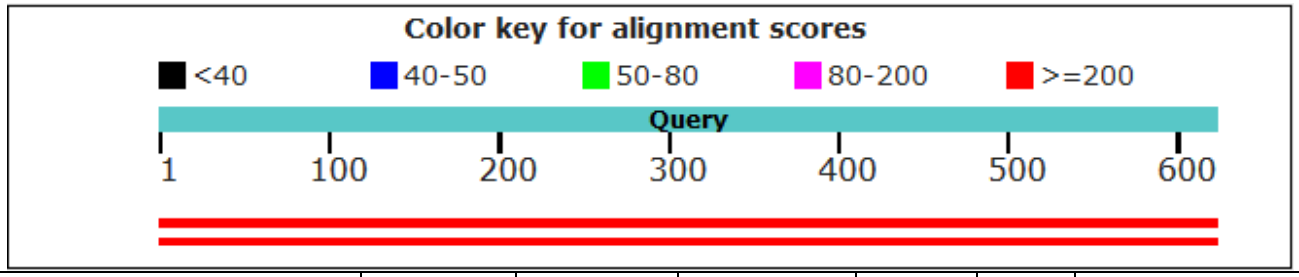
transcript variant X1, mRNA						
Bombyx mori DNA cytosine-5 methyltransferase (Dnmt1), mRNA	95.3	95.3	37%	1e-15	74%	NM_001043515.2
Cloning vector pENTRL21H8STREPTEV DNA, complete sequence	95.3	95.3	37%	1e-15	74%	LC010239.1
Bombyx mori mRNA for DNA-C5-methyltransferase-1, complete cds	95.3	95.3	37%	1e-15	74%	LC010238.1
Bombyx mori BmDnmt1 mRNA for DNA cytosine-5 methyltransferase, complete cds	95.3	95.3	37%	1e-15	74%	AB194008.1

Figure B5. NCBI BLASTn using the sequenced g16165.t1 CDS as query.



Description	Max score	Total score	Query cover	E value	Ident	Accession
PREDICTED: Diuraphis noxia tRNA (cytosine(38)-C(5))-methyltransferase (LOC107164163), mRNA	1151	1151	100%	0.0	100%	XM_015511881.1
PREDICTED: Acyrthosiphon pisum tRNA (cytosine(38)-C(5))-methyltransferase (LOC100167127), mRNA	918	918	100%	0.0	93%	XM_001949303.4

Figure B6. NCBI BLASTn using the sequenced g2520.t1 CDS as query.

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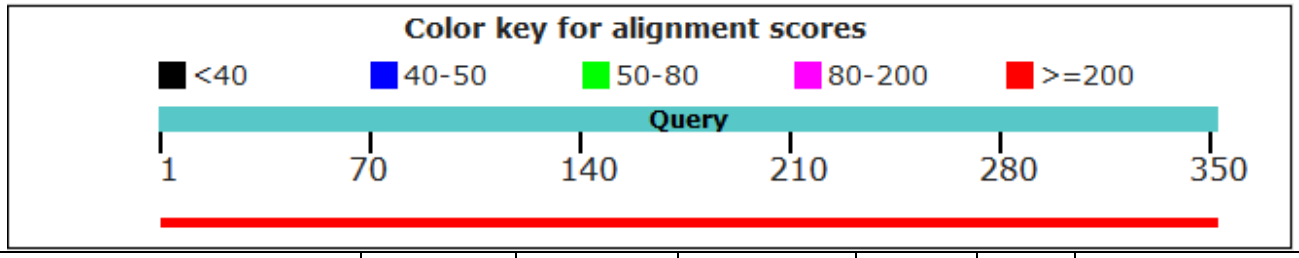

Description	Max score	Total score	Query cover	E value	Ident	Accession
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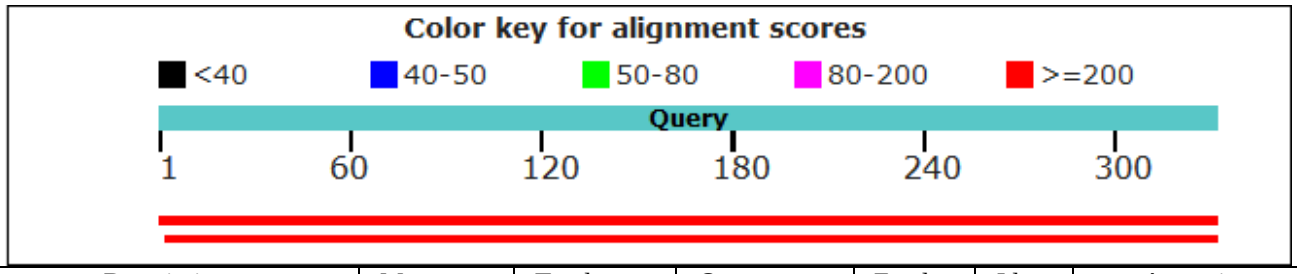
Figure B7. NCBI BLASTn using the sequenced g20164.t1 CDS as query.



Description	Max score	Total score	Query cover	E value	Ident	Accession
PREDICTED: Diuraphis noxia DNA (cytosine-5)-methyltransferase 3B-like (LOC107165102), mRNA	612	612	100%	2e-171	99%	XM_015513182.1
PREDICTED: Acyrthosiphon pisum DNA (cytosine-5)-methyltransferase 3B-like (LOC100572675), mRNA	309	309	99%	2e-80	84%	XM_016807077.1

Figure B8. NCBI BLASTn using the sequenced g24379.t1 CDS as query.

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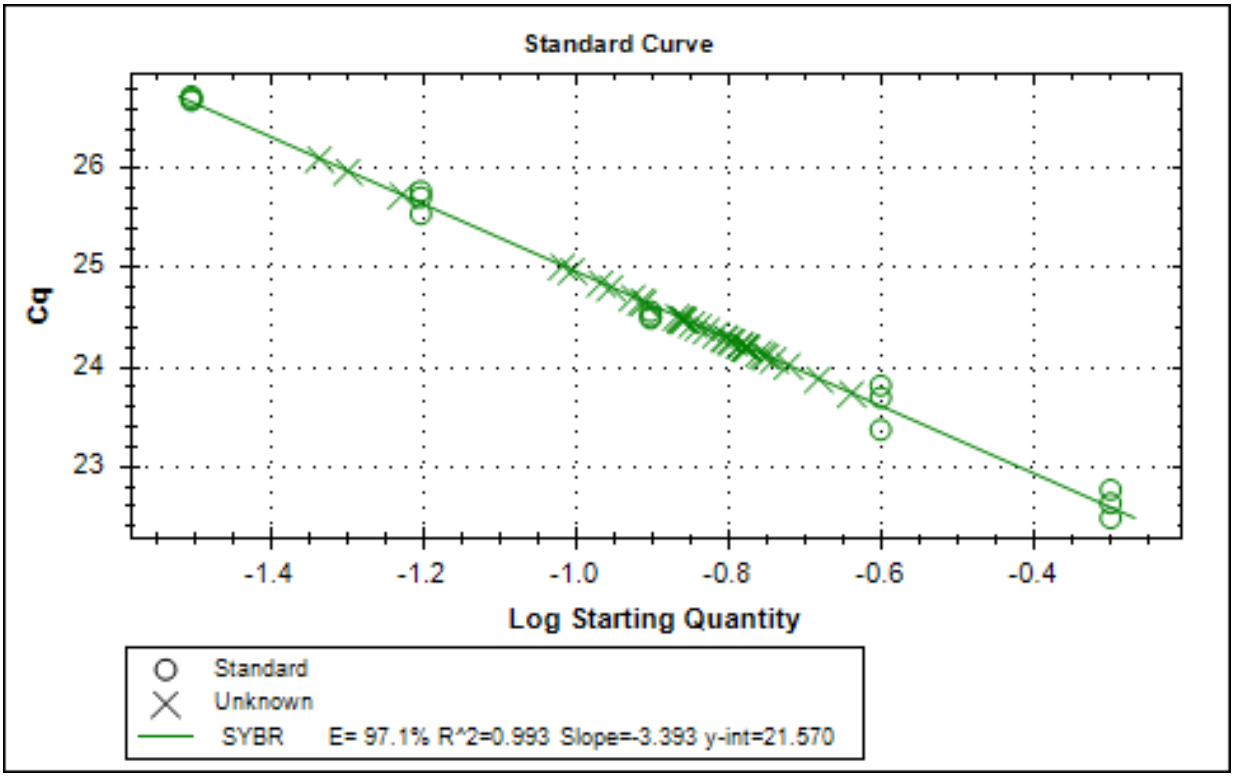

Figure B9. Standard curve for the *L27* gene.

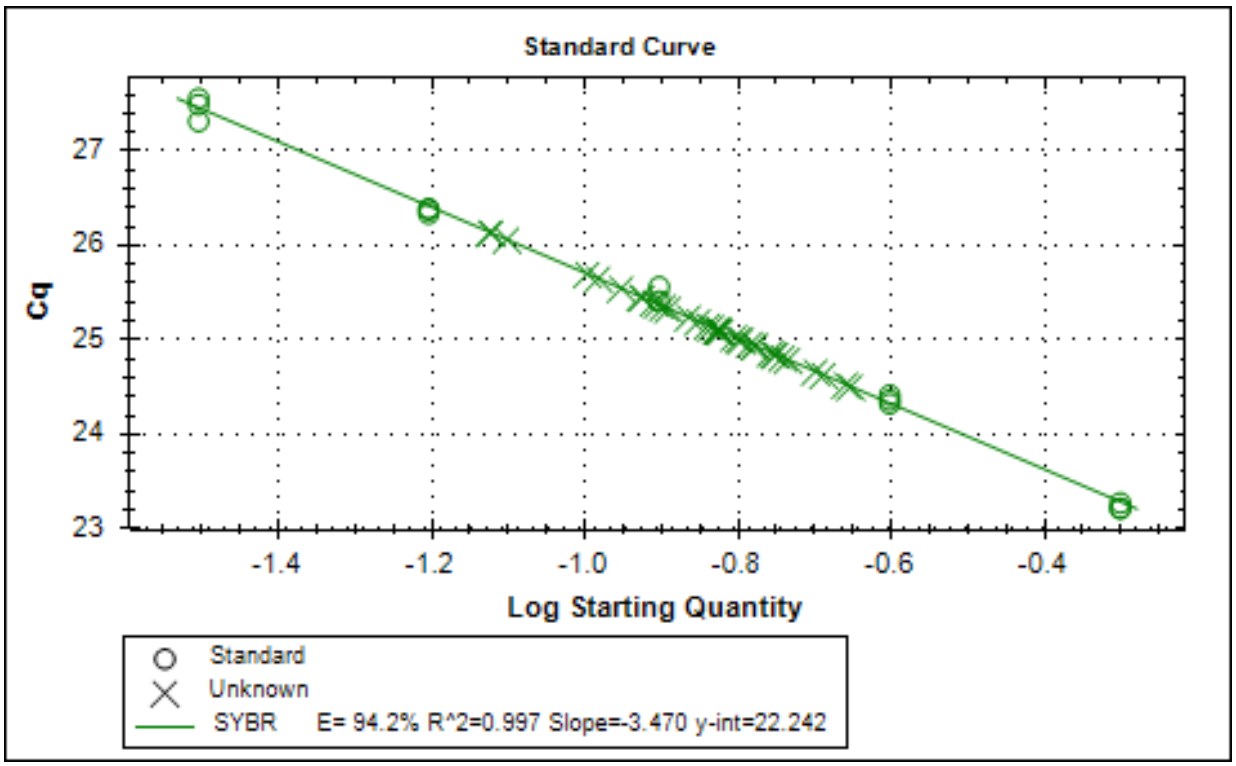

Figure B10. Standard curve for the *L32* gene.

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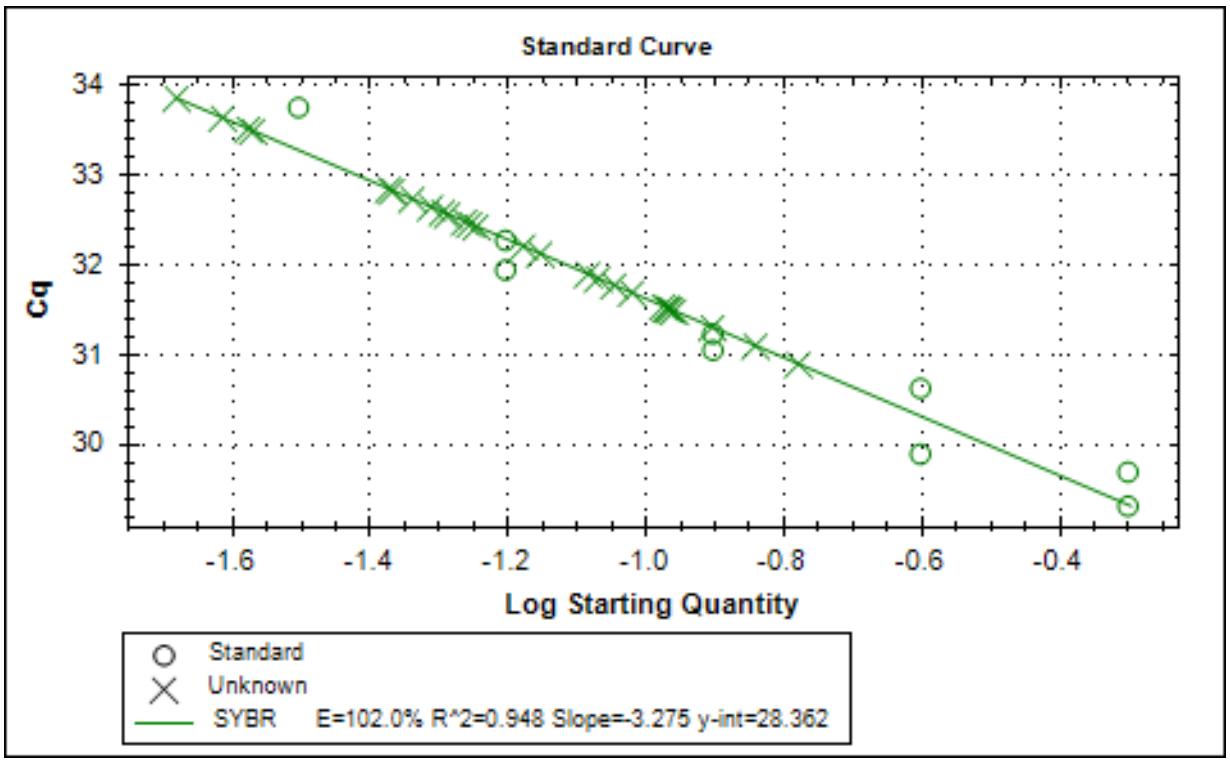

Figure B11. Standard curve for the *DNMT1* (g16165.t1) gene.

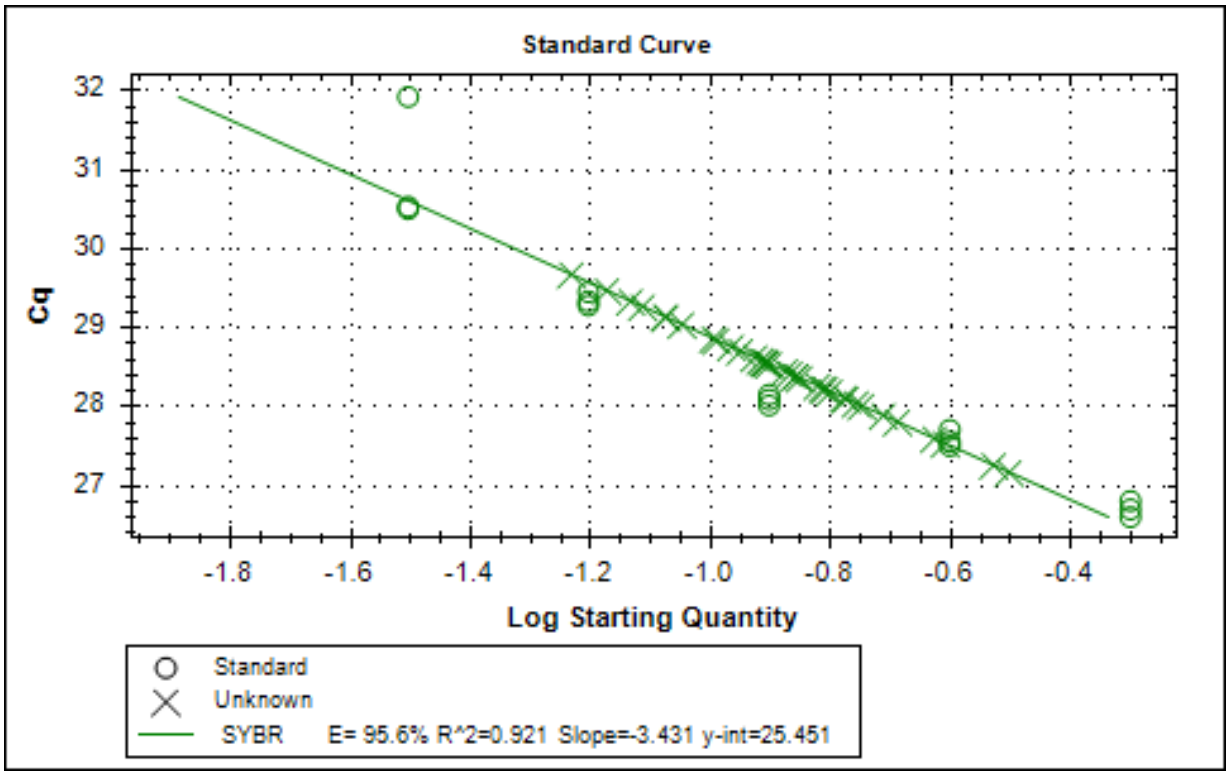

Figure B12. Standard curve for the *DNMT2* (g2520.t1) gene.

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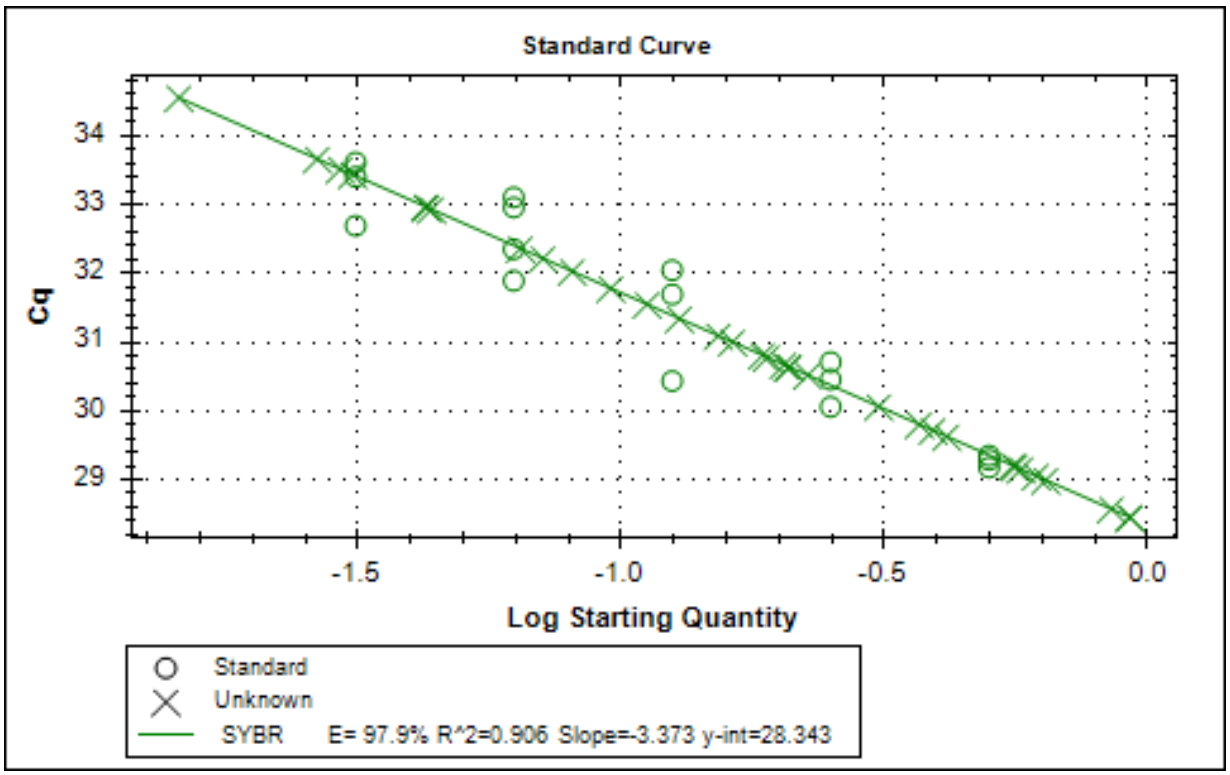

Figure B13. Standard curve for the *DNMT3A* (g20164.t1) gene.

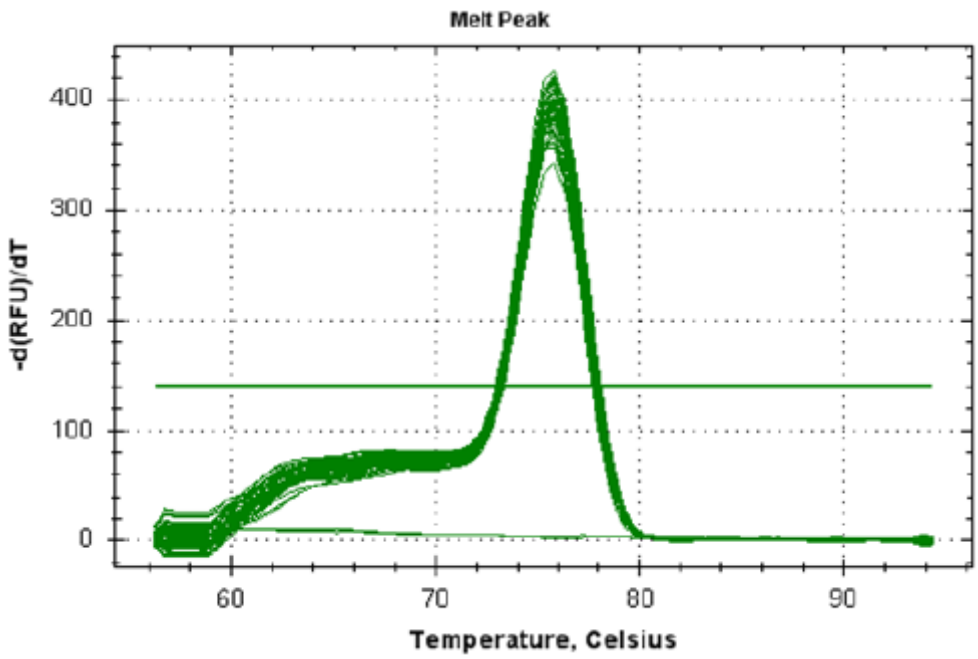

Figure B14. Melt curve of the *L27* primer set.

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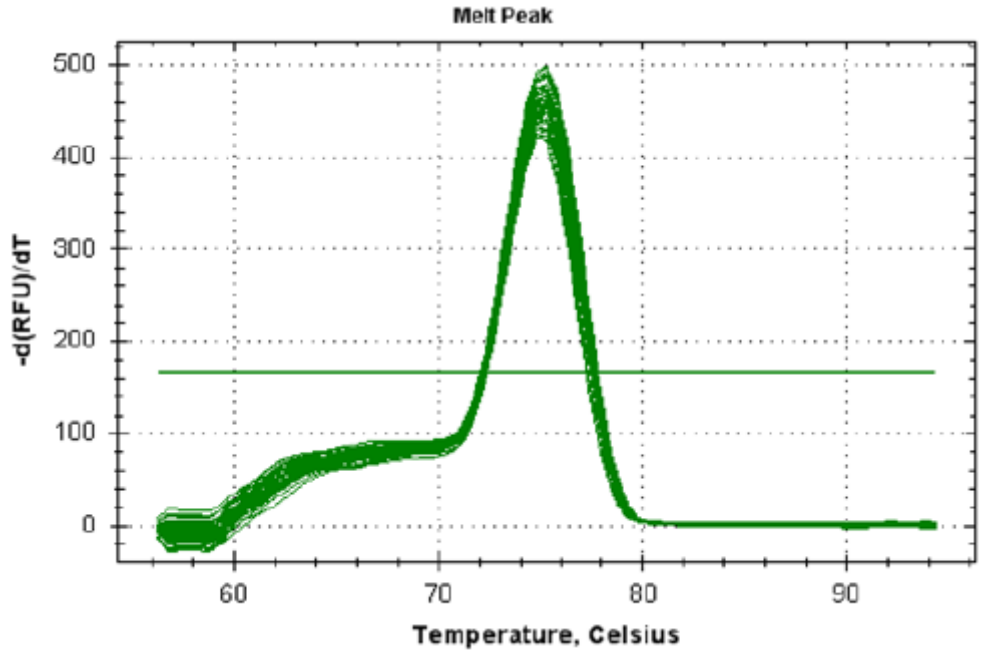

Figure B15. Melt curve of the *L32* primer set.

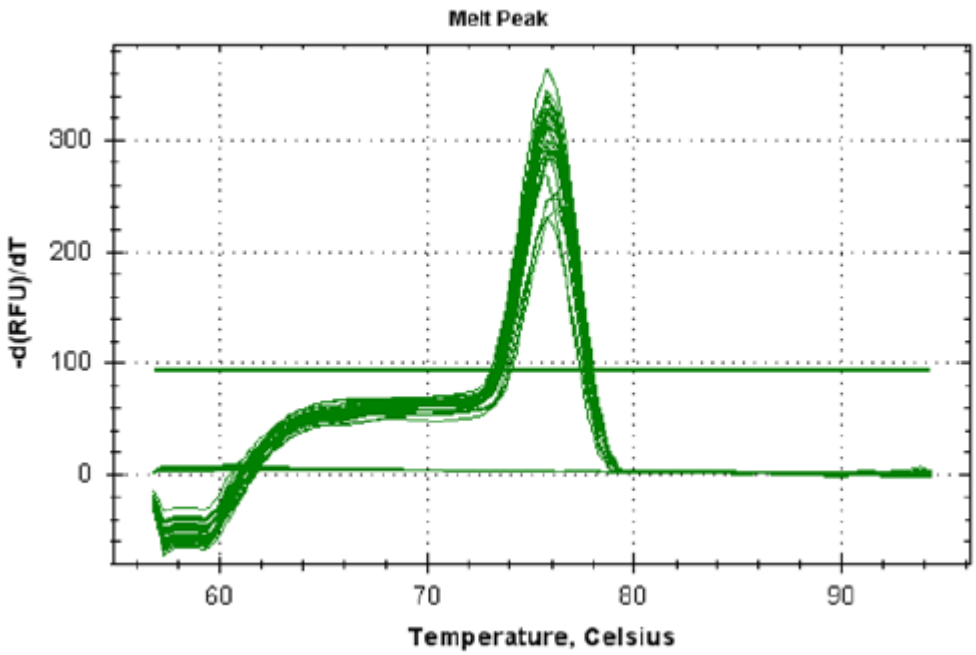

Figure B16. Melt curve of the *DNMT1* (g16165.t1) gene.

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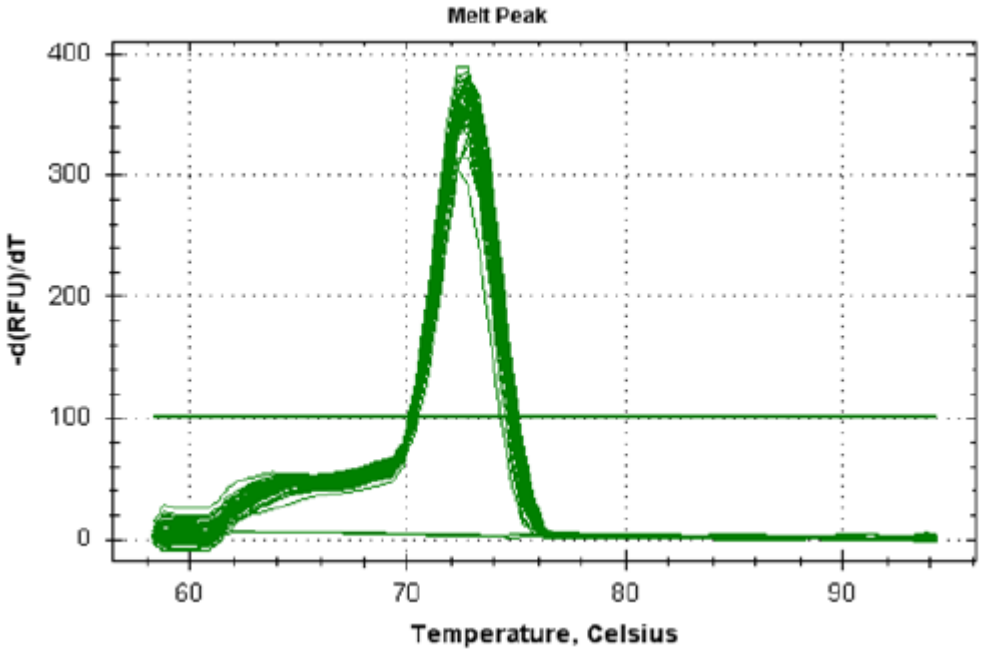

Figure B17. Melt curve of the *DNMT2* (g2520.t1) gene.

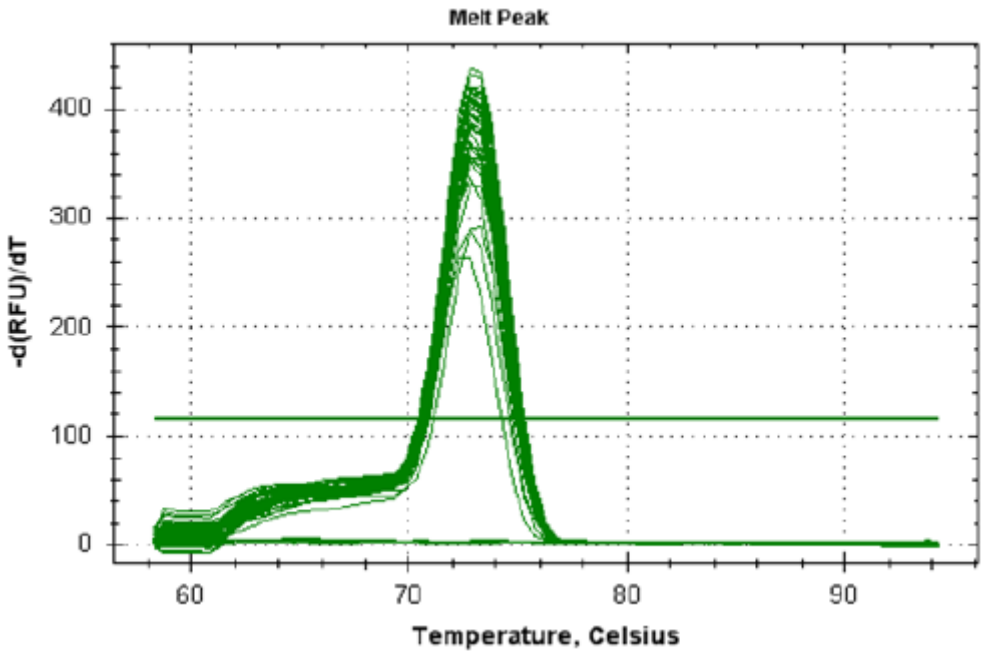

Figure B18. Melt curve of the *DNMT3A* (g20164.t1) gene.

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Table B1. Sequences of primers designed to amplify regions of *DNMT* (subfamily 1 through 3) coding domain sequences. The *murE* primer sequences, used to test for an appropriate cDNA concentration, and the M13 primers (used for sequencing) matching regions of the pTZ57R/T vector, are also given.

***DNMT* subfamily**	***D. noxia* gene**	**Forward primer sequence (5'–3')**	**Reverse primer sequence (5'–3')**
1	g16165.t1	TCATGGTGGGTATACAAAAGT	TTCTGGCCAATATTCATTGTTT
1	g9062.t1	TTCATTGTTTCTTCACTTTCT	AGGTTTTGGCCAATATTTTCA
2	g2520.t1	TCAGTGGTATTGGTGGAATG	TTTTGCTGACTGGAAAGTCT
3A	g20164.t1	TGTTGATGATTGCAATGAGAA	TCTGAGTTGTTAATTGTTTGATGT
3B	g24379.t1	TGACTTTTACCGTGTTTGGA	CTTTTTGAAGTGATGGTACCAA
	murE	ATCTTCTTAGCCAAATTTGTC CACA	ACTGCAGGAATAGCACCAGC
M13		GTAAAACGACGGCCAGT	CAGGAAACAGCTATGAC

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Table B2. Sequences of primers designed for RT-qPCR analysis of a single *D. noxia* gene from each *DNMT* subfamily. Reference gene sequences and the literature citing them are also noted. Optimal forward and reverse primer concentrations, and annealing temperature for each primer pair are also given.

***DNMT* subfamily**	***D. noxia* gene**	**Forward (F) primer sequence (5'–3')**	**Reverse (R) primer sequence (5'–3')**	**F primer [] (pmoles)**	**R primer [] (pmoles)**	**T_a (°C)**	**Reference**
1	g16165.t1	AGATGTATTACACGTATGGGC	AGACGTTTATAGGCTCAGGA	0.16	0.36	56.8	Current study
2	g2520.t1	GCTCTGAGTCAGTCGGGTTT	CACACGGCTGCACAATTTAT	0.36	0.04	58.3	Current study
3A	g20164.t1	GGCTTTTGAAACAAGTGCTGC	AACCGGCTTCTTTGTTGGAC	0.16	0.36	58.3	Current study
	L27	ACCAGCACGATTTTACCAGATTTC	CGTAGCCTGCCCTCGTGTA	0.16	0.36	56.3	Sinha and Smith 2014
	L32	CGTCTTCGGACTCTGTTGTCAA	CAAAGTGATCGTTATGACAAACTCAA	0.36	0.36	56.3	Shakesby *et al*. 2009

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Table B3. Probability values obtained from the ANOVA, Shapiro-Wilk and Levene's test (level of significance of $p \leq 0.05$ for these three tests) using the *DNMT1* RT-qPCR results.

***DNMT1* (*L27*)**		***DNMT1* (*L32*)**	
Test	***p* value**	**Test**	***p* value**
ANOVA	0.416	**ANOVA**	0.362
Shapiro-Wilk	0.719	**Shapiro-Wilk**	0.701
Levene	0.210	**Levene**	0.205

Table B4. Probability values obtained from the ANOVA, Shapiro-Wilk and Levene's test (level of significance of $p \leq 0.05$ for these three tests) using the *DNMT2* RT-qPCR results. Probability values below the significance threshold are in boldface.

***DNMT2* (*L27*)**		***DNMT2* (*L32*)**	
Test	***p* value**	**Test**	***p* value**
ANOVA	0.988	**ANOVA**	0.926
Shapiro-Wilk	**0.039***	**Shapiro-Wilk**	0.058
Levene	0.766	**Levene**	0.652

*The ANOVA is robust against deviations from normality (McDonald 2008; Schmider *et al*. 2010) and was thus performed despite a Shapiro-Wilk *p* value of less than 0.05.

Table B5. Probability values obtained from the ANOVA, Shapiro-Wilk, Levene and Fisher's LSD test (level of significance of $p \leq 0.05$ for these four tests) using the *DNMT3* RT-qPCR results. Probability values below the significance threshold are in boldface.

***DNMT3* (*L27*)**		***DNMT3* (*L32*)**	
Test	***p* value**	**Test**	***p* value**
ANOVA	**0.045**	**ANOVA**	**0.038**
Shapiro-Wilk	0.147	**Shapiro-Wilk**	0.123
Levene	**0.012***	**Levene**	**0.010***
Fisher's LSD comparison		**Fisher's LSD comparison**	
SA1 vs SA2	0.331	SA1 vs SA2	0.341
SA1 vs SA3	0.123	SA1 vs SA3	0.096**
SA1 vs SAM	0.072**	SA1 vs SAM	0.067**
SA2 vs SA3	**0.025**	SA2 vs SA3	**0.020**
SA2 vs SAM	**0.015**	SA2 vs SAM	**0.014**
SA3 vs SAM	0.738	SA3 vs SAM	0.821

* Although the Levene's test *p* value was less than 0.05, the ANOVA was still performed because "parametric tests are not particularly sensitive to violations" of the assumption of homoscedasticity (McDonald 2008).

** Significant at a level of $p \leq 0.1$.

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Table B6. Probability values obtained from the ANOVA, Shapiro-Wilk and Levene's test (level of significance of $p \leq 0.05$ for these three tests) using the DNMT protein activity results.

Test	*p* value
ANOVA	0.670
Shapiro-Wilk	0.869
Levene	0.204

Table B7. Probability values obtained from the ANOVA, Shapiro-Wilk and Levene's test (level of significance of $p \leq 0.05$ for these three tests) using the relative global methylation level (%5mC) results. Probability values below the significance threshold are in boldface.

Test	*p* value
ANOVA	0.298
Shapiro-Wilk	**0.038***
Levene	**0.011****

* The ANOVA is robust against deviations from normality (McDonald 2008; Schmider *et al*. 2010) and was thus performed despite a Shapiro-Wilk *p* value of less than 0.05.

** Although the Levene's test *p* value was less than 0.05, the ANOVA was still performed because "parametric tests are not particularly sensitive to violations" of the assumption of homoscedasticity (McDonald 2008).

Table B8. Probability values obtained from the ANOVA, Shapiro-Wilk, Levene and Fisher's LSD test (level of significance of $p \leq 0.05$ for these four tests) using the relative global hydroxymethylation level (%5hmC) results. Probability values below the significance threshold are in boldface.

Test	*p* value
ANOVA	**< 0.0001**
Shapiro-Wilk	0.371
Levene	**0.015***
Fisher's LSD comparison	
SA1 vs SA2	**0.002**
SA1 vs SA3	0.233
SA1 vs SAM	**< 0.0001**
SA2 vs SA3	**0.010**
SA2 vs SAM	**< 0.0001**
SA3 vs SAM	**< 0.0001**

* Although the Levene's test *p* value was less than 0.05, the ANOVA was still performed because "parametric tests are not particularly sensitive to violations" of the assumption of homoscedasticity (McDonald 2008).

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Chapter 5

Summary

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5.1 Summary

Wheat production is severely afflicted by the economically important agricultural pest, *Diuraphis noxia* (Kurdjumov, Hemiptera: Aphididae – or Russian wheat aphid, RWA), owing to its induction of leaf-rolling and chloroplast damage, symptoms which reduce yield and can lead to plant death (Fouché *et al*. 1984; Burd and Burton 1992; Burd and Elliott 1996; Heng-Moss *et al*. 2003; Botha *et al*. 2006). The recent spread of the RWA to Australia serves as a reminder of the importance of being able to effectively control RWA infestation (International Plant Protection Convention – https://www.ippc.int/en/countries/australia/pestreports/). Currently, the most effective method of control is the use of resistant wheat cultivars (Porter *et al*. 2009). However, the continuous development of aphid biotypes which are able to overcome these resistant sources lessens their period of efficacy (Botha *et al*. 2005, 2010; Tagu *et al*. 2008; Sinha and Smith 2014). There is thus a dire need to understand, on the molecular level, how biotypes develop, and what causes certain aphid populations to become more virulent (Shufran and Payton 2009; Botha *et al*. 2014a).

Various factors that could influence biotypification have been explored using biotypes expressing different levels of virulence. These include differences in the composition of the genome of the sole endosymbiont (*Buchnera aphidicola*) in different biotypes (Swanevelder *et al*. 2010; Burger *et al*. 2017), differences in the mitochondrial genes between biotypes (De Jager 2014), differences in saliva composition (Cloete 2015) and the genomes of aphid biotypes (Burger and Botha 2017). In 2012, Gong *et al*. alluded to a link between methylation and RWA virulence. However, the contribution of this molecular mechanism to RWA biotypification has remained for the most part unexplored. The value of its exploration lies largely in the fact that methylation does not involve changes of the genome itself, but rather leads to alterations of gene expression (Roberts and Gavery 2012; Mukherjee *et al*. 2015), through the addition (Jeltsch 2002) and removal (Branco *et al*. 2012) of methyl groups at cytosine residues. This is especially relevant given the findings of Burger and Botha's 2017 study, wherein the closely related aphid biotypes SA1 and SAM (which differ greatly in virulence level), were found to be extremely similar at the genetic level.

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The current study thus sought to determine the possible role of methylation in biotypification and the associated increase in virulence. In order to investigate and compare the methylation of different biotypes, a reliable method of methylation detection and quantification was necessary. To this end, two methods, namely methylation-sensitive amplification polymorphism (MSAP) (Reyna-López *et al*. 1997) and restriction site-specific fluorescent labelling (RSSFL), were tested for their ability to detect and quantify RWA methylation.

The MSAP technique was successful in detecting methylation in the CG and CC dinucleotide contexts. The use of HpaII and MspI in the MSAP methodology reveals polymorphisms that arise as a result of differences in the methylation state of certain loci. This proved especially useful when investigating the methylation of the least (SA1) and most (SAM) virulent South African RWA biotypes. Because SAM developed from SA1 (Van Zyl and Botha 2008; Swanevelder *et al*. 2010), changes in methylation which occurred during SAM's evolution, could be tracked through the identification of polymorphic loci between SA1 and SAM. The MSAP banding patterns, and specifically the number of unique MspI and unique HpaII bands, could also be used to quantify overall, internal and external methylation levels (Kronforst *et al*. 2008).

Twenty-two polymorphic loci were identified between biotypes SA1 and SAM, 18 of which were as a result of a gain in methylation during SAM's development. Although the identity of these fragments remains unknown, their gain in methylation is clearly beneficial to SAM, from a virulence perspective. As a gain of methylation in the genes which encode RWA effectors would lead to their tighter transcriptional regulation, and differences in the splicing variants that are produced, both of which could aid SAM in its avoidance of plant detection (Botha *et al*. 2014a), it was proposed that the newly methylated loci might reside on effector genes. The next step to be performed in future experiments, would be to excise and sequence the polymorphic fragments, and to then use homology searches to ascertain the identity and function of these putative genes. Although the RWA effector genes are still to be cloned (Botha *et al*. 2005; Smith and Clement 2012), there are genes which have been identified as putative effectors (Cloete 2015, Visser 2016).

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Furthermore, as effector proteins are found within aphid saliva, the genes encoding them are expected to contain a secretion signal (Carolan *et al*. 2011), a feature which would be useful in determining if the newly methylated loci are indeed found in genes encoding effectors.

The overall methylation levels of SA1 and SAM indicated that SAM's methylation level increased as it evolved from SA1. It is important to note that the overall methylation level is based on the number of unique MspI and HpaII bands (of the total 637 loci) within the individual biotype, and not on polymorphisms between the biotypes. Two things became apparent when the overall methylation level was dissected into internal and external methylation levels. Firstly, SA1 and SAM exhibit more methylation in the CC than the CG dinucleotide context, which is unusual for insects (Lyko and Maleszka 2011), and secondly, the largest increase in methylation during SAM's development, occurred at the external cytosine of 5' CCGG 3' sites. It was thus suggested that an increase in external cytosine hemimethylation may contribute to increased aphid virulence in certain biotypes.

Despite the ability of MSAP to both detect and quantify RWA methylation, this technique is not without its shortcomings. The first is common to all methodologies that make use of the isoschizomers HpaII and MspI (Laird 2010), and is the fact that the use of these enzymes only allows the detection/quantification of methylation in the CG and CC dinucleotide context. Although this captures the methylation present in the most common dinucleotide context for methylation in insects, CG (Lyko and Maleszka 2011), it fails to assay other dinucleotide contexts. This is problematic, given the increasing number of reports on insects, including RWA (Gong *et al*. 2012), the genomes of which contain methylation in contexts other than CG (Krauss *et al*. 2009; Walsh *et al*. 2010; Smith *et al*. 2012; Feliciello *et al*. 2013). The use of only a limited number of primer sets (in this case 7), and the fact that these primers contain selective nucleotides, decreases the area of the genome which is assayed in methylation (Meudt and Clarke 2007). For example, only methylation sites upstream of an A or T were surveyed in the current study. When making use of MSAP, one thus needs to be certain of the outcome one wants to achieve, because as Weiner *et al*.

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(2013) mention MSAP serves to provide a "preliminary survey of DNA methylation patterns". The use of MSAP in the current study, however, greatly enriched the field of knowledge of RWA methylation.

The second method assessed for its ability to detect and quantify RWA methylation was RSSFL. This novel technique makes use of a fluorescently labelled adaptor which binds to the sticky ends produced after HpaII and MspI restriction, with the fluorescence intensity providing an indication of the relative amount of restriction that occurs using each enzyme. The RSSFL technique was unable to detect (and thus quantify) the presence of methylation because HpaII and MspI are capable of restricting DNA with more than one methylation state (i.e., both enzymes restrict unmethylated DNA, as well as other methylated states). This characteristic of the isoschizomers made it impossible to partition the fluorescence readings into those based on the restriction of sites that were methylated, and those that were unmethylated. The RSSFL technique did, however, prove useful in detecting methylation trends of the RWA biotypes, when it was used in conjunction with appropriate controls. Such controls need to be organisms whose methylation has previously been quantified, and the current study made use of *Homo sapiens* and *Apis mellifera capensis* DNA. The methylation trends that emerged suggested that biotype SAM has a lower methylation level than the other biotypes (SA1, SA2 and SA3), the fluorescence levels of which were similar to the more highly methylated human sample. This was in contrast to the MSAP results where SAM was found to have the highest methylation level. This discrepancy could however be explained by the fact that MSAP does not survey all 5' CCGG 3' sites.

The investigation of the RSSFL and MSAP techniques provided insight into the methylation trends, patterns and levels of the different biotypes, but an accurate quantification of the global, genome-wide methylation level was yet to be performed. This was achieved through the use of an antibody specific to 5-methylcytosine (5mC), which was able to detect methylation in all sequence contexts. The resulting global methylation levels ranged between 0.1% and 0.16%, did not differ significantly, and were similar to that of *Drosophila melanogaster*, the methylation of which was

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quantified using the same antibody (Panikar *et al*. 2015). The results obtained using the antibody were much lower than those obtained using MSAP. This was not, however, unexpected, as the MSAP technique is known to over-estimate methylation levels (Smith *et al*. 2012; Yan *et al*. 2015).

With a greater understanding of the methylation patterns and levels, the study next sought to investigate factors which could influence the methylation of the different biotypes, with a focus on the characterisation of the DNA methyltransferases (DNMTs) which catalyse methylation (Goll and Bestor 2005). The DNMTs of the related SA1 and SAM biotypes were first identified (revealing one putative *DNMT1* and *DNMT2* gene, as well as two putative *DNMT3* genes (*DNMT3A* and *DNMT3B*)), cloned and sequenced. The sequences of these genes in SA1 and SAM, as well as RWA US2, were highly conserved, indicating that these biotypes have a similar capacity for methylating their genomes.

Quantifying the baseline expression levels of *DNMT1*, *DNMT2* and *DNMT3A* formed an important part of the current research, because all future studies which involve *DNMT* expression of the South African RWA, will make use of this information as a point of reference. These studies could include quantifying *DNMT* expression of aphids fed on different sources of resistance, or of aphids in which a *DNMT* gene has been silenced. The silencing of *DNMT1* and *DNMT3* in *Nilaparvata lugens* led to a decrease in fecundity, and has sparked interest in the possibility of regulating DNA methylation as a means of pest management (Zhang *et al*. 2015). If the transferability of this idea is to be tested for RWA, the efficacy of double-stranded RNA-mediated interference (RNAi) would require the level of *DNMT* expression to be quantified, and compared to the baseline expression.

The baseline expression of both *DNMT1* and *DNMT2* did not differ significantly between the RWA biotypes. The *DNMT1* expression levels displayed a similar trend to the methylation levels (quantified using the antibody specific to 5mC) of the biotypes. As DNMT1 is responsible for the maintenance of methylation (Goll and Bestor 2005), it follows that the biotype with the lowest methylation level should also have the lowest *DNMT1* expression, and *vice versa*, as was shown here.

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Transfer RNA (tRNA) methylation is mediated by DNMT2 (Jeltsch *et al*. 2006; Schaefer and Lyko 2010), and the similar levels of *DNMT2* expression across the biotypes are probably as a result of the regulatory roles performed by methylated tRNA in a variety of important biological processes. These include the prevention of stress-induced tRNA cleavage (Schaefer *et al*. 2010; Durdevic *et al*. 2013; Jeltsch *et al*. 2016), the product of which (tRNA fragments) negatively affects protein synthesis (Yamasaki *et al*. 2009; Durdevic *et al*. 2013; Jeltsch *et al*. 2016) and the efficiency of the small-interfering RNA (siRNA) pathway (Durdevic *et al*. 2013). Transfer RNA methylation also aids in the discrimination of near-cognate codons, thereby increasing translational fidelity (Jeltsch *et al*. 2016).

A study using *DNMT2* mutant *Drosophila*, uncovered a role of DNMT2 in protection against oxidative stress (Schaefer *et al*. 2010). This function of DNMT2, coupled with the fact that SA1, but not SAM, induces an oxidative burst upon feeding on resistant cultivars (Botha *et al*. 2014a, 2014b; Burger *et al*. 2017), could account for the slightly higher *DNMT2* expression level of SA1 in comparison to SAM. Although fed on susceptible cultivars in this experiment, biotype SA1 appears to be poised to increase its *DNMT2* expression, should it feed on resistant plants, where it would be challenged by an oxidative burst.

The *DNMT3* expression of the less virulent biotypes (SA1 and SA2) was found to be higher than that of the more virulent biotypes (SA3 and SAM), with a significant difference observed between the expression of SA2 and the more virulent biotypes. Two explanations for the higher *DNMT3* expression were put forward, one of which was based on the ability of DNMT3A to facilitate transcription (Wu *et al*. 2010), as well as the possibility that genes encoding effectors could contain DNMT3A binding sites. Although the effector-encoding genes still need to be identified (Botha *et al*. 2005; Smith and Clement 2012), and sequenced before DNMT3A binding sites can be searched for, the presence of such sites would result in the facilitation of the transcription of these genes, and a rise in the amount of effector protein. Unlike biotype SAM, less virulent aphid biotypes are not able to avoid plant detection (Botha *et al*. 2014a, 2014b; Burger *et al*. 2017), and it is the presence

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of effector proteins in their saliva which makes their infestation known to the plant (Walling 2008; Botha *et al*. 2014a). The higher *DNMT3A* expression levels could thus partially explain the low virulence levels of SA1 and SA2. The second explanation was based on the fact that DNMT3 methylation is environmentally responsive (Zhang *et al*. 2015; Standage *et al*. 2016). It is possible that the more virulent biotypes are cognisant of the fact that they are not being faced with resistance, and produce less DNMT3 transcripts (and proteins) as a means of energy conservation.

Further characterisation of the DNMTs involved the quantification of their level of protein activity. The resulting levels did not differ significantly, a finding that was expected, based on the similar global methylation levels of the biotypes, but which was also interesting, seeing that the expression of *DNMT3* was found to differ significantly between some of the biotypes. As the kit used measured the combined activity of DNMT1 and DNMT3, it was not possible to partition the activity levels into that of DNMT1 and DNMT3, to allow a comparison between the activity and expression levels of these two DNMTs.

The quantification of the hydroxymethylation (5hmC) levels of the different biotypes proved extremely useful, revealing a number of important findings. Firstly, as 5hmC is formed via ten-eleven translocation (TET)-mediated hydroxylation of 5mC (Tahiliani *et al*. 2009; Ito *et al*. 2010; Shen *et al*. 2014), and 5hmC was detected in all the biotypes, at least one active pathway of demethylation is present in RWA. By implication, there should also be at least a single TET homologue present in the RWA genome.

Secondly, the highly virulent SAM biotype has a much greater ability to demethylate its genome, as revealed by SAM's significantly higher 5hmC level. SAM's enhanced demethylation capability could also underlie the high level of external hemimethylation seen in this biotype when analysed using MSAP, as hemimethylation arises not only during replication (Jeltsch 2002; Goll and Bestor 2005), but also during demethylation (Ehrlich and Lacey 2013). Demethylation has important implications for gene regulation, which include changes in the splice variants that will be produced, on account of methylation's role in the regulation of alternative splicing (Lyko and Maleszka 2011;

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Shukla *et al*. 2011; Bonasio *et al*. 2012; Maunakea *et al*. 2013; Glastad *et al*. 2014; Yan *et al*. 2015). An increase in spurious transcription arising from the exposure of intragenic promoters or cryptic binding sites could also occur (Mandrioli 2007; Hunt *et al*. 2010, 2013a, 2013b; Maunakea *et al*. 2010). As future studies reveal more information regarding which specific genes are demethylated, the intricacies of RWA demethylation will become clearer. What is clear from the present results is that SAM, sometime during its evolution from SA1, has gained an increased ability to demethylate its genome, which has afforded this biotype greater flexibility/plasticity to adapt to changing environments, including the deployment of resistant wheat cultivars.

Thirdly, it is possible that the high level of 5hmC observed in SAM is becoming fixed as a *bona fide* epigenetic characteristic. The use of MSAP and the antibody specific to 5hmC both provide evidence of SAM's enhanced demethylation capability, and the likelihood of the existence of a TET homologue. There are three avenues of 5hmC removal, one of them being the direct conversion of 5hmC to unmethylated cytosine by DNMT3A and DNMT3B (Chen *et al*. 2012). Biotype SAM's *DNMT3A* expression was the lowest of the four biotypes. Thus, if DNMT3A conversion of 5hmC is the favoured mechanism of 5hmC removal/conversion in RWA (this is yet to be determined), the relatively low *DNMT3A* expression would result in less 5hmC being removed. One study has already shown that 5hmC has the potential of being a *bona fide* epigenetic characteristic because certain proteins bind specifically to 5mC and others to 5hmC (Spruijt *et al*. 2013). Furthermore, intronic 5hmC of *A. mellifera* has been shown to be involved in alternative splicing (Cingolani *et al*. 2013). This presents many new avenues for exploration of demethylation and 5hmC functions in RWA.

In conclusion, the RSSFL technique is useful for detecting methylation trends, whilst MSAP enables both the detection and quantification of methylation in the CG and CC dinucleotide contexts, at a subset of anonymous 5' CCGG 3' sites. The use of the antibody specific to 5mC provides an accurate quantification of global methylation in all sequence contexts. The four South African biotypes have similar global methylation levels. The DNMTs have highly conserved

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nucleotide sequences, as well as similar levels of *DNMT1* and *DNMT2* expression, and protein activity. The differences in *DNMT3* expression appear to be related to the virulence of the respective biotypes. The biotypes exhibit varying levels of hydroxymethylation, with SAM's level being significantly higher than the other biotypes. Thus whilst the methylation levels, and most of the aspects of the enzymes which catalyse methylation are similar between the biotypes, it is the ability to demethylate its genome, which affords SAM (and perhaps other biotypes) a greater level of plasticity/flexibility, thereby enabling a higher level of virulence.

5.2 References

5.2.1 Journal articles

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5.2.2 Websites

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