Diuraphis noxia biotype recognition by the wheat integrated domain nucleotide-binding leucine-rich repeat, *TaAdnr1*

by

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Table of contents

I. Declaration i

II. Acknowledgements ii

III. List of Tables iii

IV. List of Figures iii

V. General abbreviations v

Chapter 1 1

Introduction 1

1.1 Introduction 2

1.2 References 4

Chapter 2 7

Literature review 7

2.1 Wheat 8

2.2 The Russian wheat aphid 9

2.2.1 The *D. noxia* biotypification 9

2.2.2 Symptoms of *D. noxia* infestation 11

2.2.3 Feeding by *D. noxia* 11

2.2.4 The aphid salivary effectors 12

2.3 Plant innate immunity 14

2.3.1 PTI 15

2.3.2 ETI 15

2.4 Plant wound responses 24

2.4.1 The reactive oxygen species 24

2.4.2 Sieve tube occlusion 25

2.4.3 Protein plugging 26

2.4.4 Systemic acquired resistance 26

2.5 Existing wheat R-genes against *D. noxia* 27

2.6 The wheat-*D. noxia* interaction in South Africa 28

2.7 Virus-induced gene silencing 29

2.8 References 34

2.9 Conclusion 52

Chapter 3 53

Materials and Methods 53
3.1 Analysis of the full-length *TaAdnr1* sequence in *Triticum aestivum* L. 54
3.1.1 Plant Material 54
3.1.2 RWA biotype maintenance and infestation 54
3.1.3 Analysis of the full-length *TaAdnr1* sequence 54
3.1.4 RNA extraction and cDNA synthesis 55
3.1.5 Determination of the full length of *TaAdnr1* 56
3.2 RT-qPCR expression analysis of *TaAdnr1* 60
3.3 Virus-Induced gene silencing of *TaAdnr1* 61
3.3.1 Gene silencing vectors 62
3.3.2 Preparation of RNA transcripts from BSMV vectors. 62
3.3.3 Plant inoculation with prepared viral transcripts. 63
3.3.4 Aphid reproduction measurements 64
3.3.5 Phenotypical observation of gene knockdown 64
3.3.6 Knockdown levels of *TaAdnr1* obtained through VIGS 65
3.3.7 3,3'-diaminobenzidine (DAB) Staining 65
3.3.8 References 67
Chapter 4 69
Results 69
4.1 Full-length sequence analysis of *TaAdnr1* in *Triticum aestivum* L. 70
4.2 RT-qPCR expression analysis of *TaAdnr1* 77
4.3 VIGS Knockdown of *TaAdnr1* 81
Chapter 5 98
Discussion 98
5.1 General discussion 99
5.2 Knockdown induces susceptibility phenotypes on resistant plants 100
5.3 Knockdown alters the oxidative burst 102
5.4 Knockdown increases aphid fecundity and intrinsic rates of reproduction 104
5.5 Knockdown reduces plant biomass 104
5.6 *TaAdnr1* in the aphid resistance response 105
5.7 References 109
Chapter 6 116
Conclusions and future work 116
References 118

Summary **119**

I. Declaration

I, the undersigned, hereby declare that the dissertation submitted herewith for the degree Master of Science at the University of Johannesburg contains my own independent work and has not been submitted for any degree at any other university.

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III. List of Tables

Table 1: The primer sequences used for the full-length amplification and RT-qPCR expression analysis of *TaAdnr1* following generation of the first strand cDNA.....66
Table 2: The mean intrinsic rates (r_m) of the *D. noxia* biotypes on the Gamtoos, Gamtoos-R and the Gamtoos-R BSMV treatments including the standard deviations (*Sd*) 96

IV. List of Figures

Figure 1: Schematic models of NLR activation.....18
Figure 2: Schematic models of BSMV-mediated VIGS.....31
Figure 3: Schematic representation of BSMV-mediated VIGS in barley.....32
Figure 4: The predicted structure of *TaAdnr1* 32
Figure 5: The full-length PCR amplification of *TaAdnr1* from both Gamtoos and Gamtoos-R wheat plants.....70
Figure 6: The colony PCR amplification of insert-containing colonies identified through alpha complementation.....71
Figure 7: The Clustal Omega alignment of the Gamtoos-S and Gamtoos-R full-length nucleic acid sequences of *TaAdnr1* to the Chinese Spring (CS) reference sequence 77
Figure 8: RNA extracted from the Gamtoos wheat cultivars infested with RWASA1 biotype for determining the differential expression of *TaAdnr1* for the different time points.....77
Figure 9: The RT-qPCR standard and melt curve graphs for the differential expression analysis of TaAdnr1 in both Gamtoos wheat cultivars 79
Figure 10: Differential expression of *TaAdnr1* between the susceptible Gamtoos and resistant Gamtoos-R near isogenic lines (NIL) infested with the RWASA1 biotype.....80
Figure 11: The linearised BSMV plasmids resolved on a 1 % agarose gel.....81
Figure 12: The in vitro transcribed RNA products resolved on a 1% native agarose gel.....82
Figure 13: RNA extracted from the Gamtoos-R wheat plants infested with the different RWA (SA1-4, A-D respectively) eleven days after viral inoculation. The RNA was resolved on a 1 % native agarose gel. The lanes 1-3 represents RNA extracted from infested Gamtoos-R plants infested with the different RWA biotypes elen days after viral inoculation 83

Figure 14: The RT-qPCR standard curve graphs for the BSMV-VIGS-mediated knockdown of *TaAdnr1* in Gamtoos-R. 85
Figure 15: The RT-qPCR melt curve graphs for the BSMV-VIGS-mediated knockdown of *TaAdnr1* in Gamtoos-R. 88
Figure 16: Relative levels of knockdown achieved for *TaAdnr1* evaluated using RT-qPCR eleven days post BSMV inoculation (five days post-aphid infestation) 88
Figure 17: The phenotypic effect of *TaAdnr1* knockdown and subsequent *D. noxia* infestation.. 89
Figure 18: Changes to the oxidative burst as measured through H_2O_2 accummulation. 91
Figure 19: Total plant dry weight after sixteen days of aphid feeding.. 93
Figure 20: The mean total aphid production of ten plants per treatment over sixteen days. 95

V. General abbreviations

ABA	Abscisic acid
ARC	Human Apaf-1, plant R proteins and *C. elegans* Ced-4
ATP	Adenosine triphosphate
Avr	Avirulence
BSMV	Barley stripe mosaic virus
CC	Coiled-coil
cDNA	complementary DNA
CNL	Coiled-coil nucleotide-binding leucine-rich repeat
CP	Coat protein
DAB	3,3'-diaminobenzidine
dATP	2-deoxy adenosine triphosphate
DCLs	Dicer-like enzymes
DDT	Dichloro-diphenyl-trichloroethane
Dn	*Diuraphis noxia*
dNTP	Deoxyribonucleotide triphosphate
dsRNA	Double stranded ribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ET	Ethylene
ETI	Effector-triggered immunity
GA	Gibberellic acid
GOI	Gene of interest
GS	Gamtoos
GR	Gamtoos-R
H_2O_2	Hydrogen peroxide
HMA	Heavy-metal-associated domain
Hpi	Hours post infestation
HR	Hypersensitive response
IDs	Integrated domains
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JA	Jasmonic acid
LB	Luria-Bertani broth

LRR	Leucine-rich repeats
MAPKs	Mitogen-activated protein kinases
NB	Nucleotide-binding
NIL(s)	Near isogenic line(s)
NLR	Nucleotide-binding leucine-rich repeat
NLR-ID	Nucleotide-binding leucine-rich repeat containing integrated domain
NOD	Nucleotide-binding and oligomerization domain
O_2^-	Super oxide radical
OH	Hydroxyl radical
ORF	Open reading frame
PAMP(s)	Pathogen-associated molecular pattern(s)
PDS	Phytoene desaturase
PTGS	Post-transcriptional gene silencing
PTI	PAMP-triggered immunity
RISC	RNA-induced silencing complex
r_m	Intrinsic rate of increase
RNBS	Resistance nucleotide binding site
ROS	Reactive oxygen species
RWASA	Russian wheat aphid South African biotype
SA	Salicylic acid
SAR	Systemic acquired resistance
siRNA	Small interfering RNA
STAND	Signal transduction ATPases with numerous domains
STE	Sieve tube element
SNP(s)	Single nucleotide polymorphism(s)
TaAdnr1	*Triticum aestivum Associated with Dn resistance 1*
TAE	Tris-acetate-EDTA
TIR	*Drosophila* Toll and mammalian Interleukin-1 receptor
TNL	Toll/Interleukin-1 nucleotide-binding leucin-rich repeat
TRIS	Tris (Hydroxymethyl) aminomethane
UTR	Untranslated regions
UV	Ultraviolet

VIGS	Virus induced gene silencing
X-gal	5-bromo-4chloro-3-indolyl-β-D-galactopyranoside

Chapter 1

Introduction

1.1 Introduction

Bread wheat (*Triticum aestivum* L.) is a globally important cereal crop that contributes as a staple food to at least 30 % of the world's population (Kochhar, 2016). The wheat's grain serves as a source of nutrition that provides important carbohydrates, fats, minerals, vitamins and proteins. Wheat varieties are used for the commercial production of different food types including bread, cookies, biscuits and noodles (Kochhar, 2016; Nhemachena and Kirsten, 2017) . Countries that contribute to the global production of wheat include India, China, the United States of America, Russia, Australia, Pakistan, Iran and South Africa (Kochhar, 2016). However, its cultivation has long been challenged by both biotic and abiotic factors (Burd and Burton 1992; Jan *et al*. 2017; Jankielsohn 2017). Infestation by insect pests such as *Diuraphis noxia* Kurdjumov in South African wheat-growing regions, has contributed to considerable yield losses and economic strain to the small grain industry (Kriel *et al.*, 1986; Robinson, 1992; Jankielsohn, 2017; Botha *et al.*, 2017; Kaplin and Sharapova, 2017). To curtail these losses, *D. noxia* (*Dn*) genes that afford resistance were bred into wheat cultivars commercially available. There are fourteen known *Dn* resistance genes (*Dn1, Dn2, dn3, Dn4, Dn5, Dn6, Dn7, Dn8, Dn9, Dnx, Dny, Dn2414, Dn626580* and *Dn2401*) identified from wheat and its close relatives. South African resistant wheat lines were developed by incorporating a range of these *Dn*-genes. This has resulted in the continuous development of new virulent aphid biotypes that have overcome several of the deployed resistance genes (Marais *et al*., 1994; Jankielsohn *et al*., 2016).

Diuraphis noxia is an invasive specialist pest of important cereal crops such as wheat and barley in cereal growing areas around the globe (Walters *et al*., 1980; Burd and Burton, 1992; Puterka *et al.*, 2007; Yazdani *et al.*, 2018). It is a small green-coloured aphid that reproduces parthenogenetically with only apterous (wingless) females occurring in Southern Africa (Walters *et al*., 1980). The first report of this pest in South Africa was recorded in 1978 and is currently the primary pest of cultivated wheat in the Western Cape and the Free State regions (Walters *et al*., 1980; Jankielsohn, 2011, 2017). Biotypic variation has been detected since *D. noxia* was introduced into South Africa and there are currently five reported resistance breaking biotypes (Russian Wheat Aphid (RWA) SA1-5) (Jankielsohn, 2019). These biotypes differ only in which resistance genes they have overcome to feed on previously resistant plants. *D.*

noxia feeds on their hosts to obtain nutrients through their stylet mouthparts and this result in plant damage and significant yield losses (Robinson, 1992; Saheed *et al.*, 2007). The common phenotypic symptoms associated with *D. noxia* on infested hosts include necrosis, longitudinal chlorotic streaking, severe chlorosis, inward leaf roll and wilt, stunted growth and eventually death (Walters *et al*., 1980; Hewitt *et al.*, 1984; Goggin, 2007). *D. noxia* management strategies including chemical control proved efficient but expensive and host plant resistance was introduced as a feasible option (Walters *et al*., 1980; Smith *et al.*, 1992; Marasas *et al.*, 2005).

In plants, the innate immunity constitutes complex defence mechanisms and pathways that are mainly dependent on host immune receptors that recognise pest- or pathogen-derived molecules during their invasion (Cook *et al*., 2015). Two categories of innate immunity can be distinguished, namely the pathogen-associated molecular patterns (PAMP)-triggered immunity (PTI) and the effector-triggered immunity (ETI) (Cesari *et al.*, 2014a, 2014b; Tsuda and Katagiri, 2010). ETI activation results in the much stronger and faster plant immune response upon suppression of PTI and is distinguished by localised cell death lesions characterised as the hypersensitive response (HR) (Jones and Dangl, 2006). Invading pathogens secrete and deliver effector molecules that interact by either disabling or activating intracellular disease resistance (R)-proteins. The family of proteins that can perceive foreign pathogen elicitor molecules are known as the nucleotide-binding leucine-rich repeat-containing (NLR) proteins and may do so directly or indirectly for the activation of ETI (Cesari, 2018; Dodds and Rathjen, 2010). The NLR genes consist of similar or variable multidomain architectures proposed to contribute different but important roles for their functioning in resistance responses. The further identification of unusual integrated domains (ID) onto NLR proteins (NLR-ID) provided a comprehensive understanding into the evolutionary arms race between NLRs and their respective pathogen effectors (Cesari, 2018). The *Triticum aestivum Associated with Dn resistance 1* (*TaAdnr1*) NLR-ID gene was identified to participate in *Dn1*-mediated resistance during the wheat-*D. noxia* interaction (Nicolis and Venter, 2018). *TaAdnr1* contains an integrated WRKY domain with a very similar architecture to that of the *Arabidopsis RRS1* gene. Transient silencing of *TaAdnr1* indicated a loss of the *Dn1*-mediated resistance against the Russian Wheat Aphid SA1 biotype in the resistant Tugela DN cultivar. Based on this evidence it was postulated that it plays a role in aphid effector recognition. This study aimed to ascertain the role of *TaAdnr1* in *D. noxia* biotype recognition by investigating its role in *Dn7*-mediated resistance against the first four identified biotypes.

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Chapter 2

Literature review

2.1 Wheat

Wheat is the second most commercially dominant cereal in the production of essential dietary proteins and calories for human nutrition (Awika, 2011). It belongs to the grass family Poaceae that includes economically important cereal species. This family consists of 12 subfamilies, 52 tribes, 90 subtribes, 768 genera and 11 506 species (Soreng *et al.*, 2017; Hodkinson, 2018). The tribe Triticeae Dumort. includes 27 genera with 501 species of which three are of major economic importance, namely rye (*Secale cereale* L.), bread wheat (*T. aestivum*) and barley (*Hordeum vulgare* L.) (Bernhardt, 2015). The genus *Triticum* harbours three ploidy levels, namely the diploid, tetraploid and hexaploid species as a result of interspecies and intraspecies hybridisation.

Wheat production in South Africa precedes maize production, with both crops of commercial importance. South Africa cultivates two main types of commercial wheat, namely bread wheat and durum wheat (*Triticum turgidum* L.). These cereals differ in the complexity of their genome, adaptation to environmental conditions and commercial importance (Summers and Brown, 2013; Li *et al.*, 2015; Nhemachena and Kirsten, 2017). They are used to produce a variety of industrial and commercial food products such as bread, biscuits, cookies, noodles, pasta, couscous, bulgur, grain for alcoholic beverages, straw and starch (McKevith, 2004; Kochhar, 2016; Nhemachena and Kirsten, 2017). Bread wheat production is localised to three provinces (the Free State, the Western and Northern Cape) with 32 production regions. The production of wheat in the country ranges between 1.5 to 3 million tonnes annually and 300 000 tonnes is imported per annum (Nhemachena and Kirsten, 2017).

Bread wheat is an allohexaploid (2n = 6x = 42) that consists of the A, B, and D homologous chromosome sets in its genome (Matsuoka, 2011; Goriewa-Duba *et al.*, 2018). The allopolyploid genome of bread wheat (AABBDD) originated through natural hybridisation between the tetraploid *T. turgidum* (AABB, with *Triticum urartu* donating the AA genome) and the diploid *Aegilops tauschii* Coss. (DD) (Dvořák, 2001; Matsuoka, 2011). The large size (ca.17-gigabases) of the bread wheat genome encodes more than 100 000 protein-coding genes and consists of large tracts of repetitive DNA sequences (International Wheat Genome Sequencing Consortium (IWGSC), 2014; Clavijo *et al.*, 2017). The vast size and polyploid complexity of the genome present drawbacks in genetic studies for the development of new

approaches to crop improvement. This also impacts the effort to meet the increased global food demand (Clavijo *et al.*, 2017; IWGSC, 2018).

2.2 The Russian wheat aphid

The Russian Wheat Aphid (RWA) is a significant specialist pest of small grains including wheat, barley, oats and triticale. It also feeds and overwinters on alternative hosts such as rescue grasses, false barley and wild oats. *D. noxia* contributes to economic losses in major small grain-producing regions (Walters *et al*. 1980; Tolmay *et al*., 2013). This pest originated in Afghanistan, Iran, South of Russia, and the countries that border the Mediterranean Sea and was recorded for the first time in South Africa and the United States of America (USA) in 1978 and 1986, respectively (Walters *et al*. 1980). Australia recorded its first report of the aphid in 2016 and has since been regarded as a serious pest (Yazdani *et al.*, 2018).

Diuraphis noxia is a proportionately small and approximately 2 mm in length. It consists of a long spindle-shaped body that is green-to-yellow in colour. In South Africa, two types of female *D. noxia* occur that are either wingless (apterous) or winged (alate) (Walters *et al*., 1980). The adult apterous females reproduce parthenogenetically by giving birth to apterous female nymphs two or more weeks after their initial birth. During favourable conditions, they produce more than four nymphs per day that occur as dense colonies on young and inward rolled leaves (Walters *et al*., 1980). *D. noxia* poses serious challenges and threatens major small grain production areas and novel management strategies need to be elucidated for the development of cultivars with durable resistance (Botha *et al*., 2005; Tolmay *et al*., 2007).

2.2.1 The *D. noxia* biotypification

Biotypic variation within *D. noxia* populations was first detected from populations collected from South Africa, the USA and Eurasia (Puterka *et al.*, 1992). This was based on the considerable differences in virulence status. There are currently eight *D. noxia* biotypes in the USA designated as RWA1-RWA8 (Puterka *et al.*, 2015). The RWA2 biotype was first detected in Colorado in 2002. In 2006, the RWA3 and RWA4 biotypes were reported from Texas and the RWA5 biotype was reported from Wyoming. The RWA6, 7 and 8 biotypes were reported from Colorado in 2008 (Haley *et al.*, 2004; Weiland *et al.*, 2008). The biotypes RWA3, 4, 5

and 7 were found to show no significant differences in virulence profiles among the other biotypes and the classification was modified to only contain five main biotypes namely RWA1, RWA2, RWA3/7, RWA6 and RWA8 (Puterka *et al.*, 2014). There is currently no record of the emergence of a new biotype in the USA (Puterka *et al*., 2015).

There are five *D. noxia* biotypes reported in South Africa, namely RWASA1-RWASA5, with RWASA5 recently reported in 2019 (Jankielsohn, 2019b). The RWASA2 biotype was recorded in 2005 from the Eastern Free State and displayed virulence against the *Dn1* resistance gene used in commercial wheat cultivars (Tolmay *et al.*, 2007). This was the dominant biotype during the 2010-2011 season in the summer rainfall areas (Jankielsohn, 2017). The RWASA3 biotype, virulent against the *Dn4* wheat resistance gene, was identified in 2009 in the Eastern Free State and dominated the summer rainfall area in 2012-2013 (Jankielsohn, 2011, 2017). The RWASA4 biotype was discovered in Bethlehem in the Eastern Free State in 2011 and was found to be virulent against wheat carrying the *Dn5* resistance gene. The dominance of this biotype was also observed in the summer rainfall area in 2014-2016 following reduction of RWASA3 dominance (Jankielsohn, 2017). The Eastern Free State consists of a diverse biotype complex of RWA with all biotypes prevailing in the region. RWASA1 was found to be more prevalent in the winter rainfall areas during 2010-2016 (Jankielsohn, 2017).

Several factors have been suggested to play a role in the biotypic diversity of *D. noxia*. The occurrence of alternative hosts such as rescue grasses, oats, false barley and wild oats for survival has been suggested to aid in the development of new biotypes (Jankielsohn, 2017). These alternative hosts are suggested to influence *D. noxia* biotypic diversity and fluctuating environmental conditions affecting plants, such as soil type, moisture, temperature and wind, may place additional pressure on the pest (Burd *et al.*, 2006; Jankielsohn, 2017). The deployment of commercial resistant wheat cultivars also places the aphid populations under extreme pressure to adapt and results in the development of more virulent biotypes. The underlying factor or factors contributing to *D. noxia* biotypic diversity have not yet been exclusively identified (Tolmay *et al*., 2007; Jankielsohn, 2011).

2.2.2 Symptoms of *D. noxia* infestation

Upon *D. noxia* infestation, susceptible host plants exhibit phenotypic symptoms such as chlorosis and foliar streaking, necrosis, leaf rolling, trapped heads, stunted growth and eventual death of the susceptible plant (Walters *et al*., 1980; Saheed *et al*., 2007a, 2007b). Saheed *et al*. (2007b), postulated that the symptoms associated with chlorosis and necrosis may result from puncturing of the cells with an induction of the oxidative stress response. Leaf rolling and foliar streaking is associated with the loss of leaf turgor and blockage of the apoplastic and symplastic pathway in the xylem and phloem cells, which prevent nutrient exchange to adjacent parenchyma cells (Burd and Burton, 1992; Saheed *et al.*, 2007a). The damage that results in host cell bleaching may be associated with induced water imbalances (Burd and Burton, 1992).

2.2.3 Feeding by *D. noxia*

Host plant preference by aphids relies on successive events during plant-aphid interaction. The aphid antennae play a major role in the detection of suitable hosts. Upon landing of the aphid on the host leaf surface, their antennae detect plant surface structures and molecules such as waxes, epidermal trichomes, odour and colour (Burd and Burton, 1992; Powell *et al*., 2006; Hewer *et al*., 2011). The aphid stylet mouthpart is responsible for the successful penetration and feeding from the plant tissues (Walling, 2000). Following detection of a suitable host, aphids penetrate through the leaf epidermis or stomata using their stylet mouthparts. The stylet is protected by gelling saliva that is secreted during penetration and hardens by oxidation of sulphydryl-groups to form a canal structure as the stylet grows (Will *et al.*, 2012; van Bel and Will, 2016) . The stylet then travels through the inter- and intracellular routes of the mesophyll cells until it reaches the vascular bundle. Penetration of the vascular bundle becomes mainly intracellular and the stylets penetrate the sieve tube elements (STE) of the phloem tissue whereby feeding is established (Fouché, 1984; Saheed *et al.*, 2007a). Upon penetration of the sieve tube, aphids secrete watery saliva that aids in ingestion of nutrients in the cells (Saheed *et al.*, 2007b; Will *et al*., 2013).

2.2.3.1 The aphid gelling saliva

The gel saliva of aphids is suggested to serve as a lubricant during navigation of the stylet into the plant tissues and also protects the stylet by forming a protective sheath (Will *et al*., 2013).

During gel salivation, a component called the gel flange is initially secreted into the surface of the host leaf to promote stylet penetration and initiation of navigation into the leaf tissues (Tjallingii, 2006). As the stylet travels intercellularly from the mesophyll cells towards the phloem-feeding site, gel saliva is continuously secreted that subsequently forms the solid salivary sheath that consists mainly of sheath protein. Furthermore, cell wall degrading enzymes and detoxifying proteins are suggested to be constituents of the gel saliva (Carolan *et al.*, 2009; Will *et al.*, 2012).

2.2.3.2 The aphid watery saliva

The watery saliva of aphids contains a variety of proteins that aid in the successful modification and suppression of plant defence responses (Nicholson *et al*., 2012; Furch, 2015). The majority of these salivary proteins identified in a variety of aphid species have no known functions due to limited proteomic studies (Elzinga and Jander, 2013). The predicted proteins with known functions in the watery saliva of most aphid species include the calcium ion (Ca^{2+})-binding proteins, detoxifying enzymes, proteases, endosymbiont-derived proteins and effector proteins (van Bel and Will, 2016). The Calcium ion binding proteins are involved in occlusion of the sieve tube with the proteases playing a role in the detoxification of the plant defence compounds (Furch *et al.*, 2007; Nicholson *et al.*, 2012). The proteins in watery saliva of different aphid species contain both species-specific or universally present proteins (Furch *et al*., 2015). Cooper *et al*. (2011), suggested that saliva from different aphid species resulting in similar host damage contains a similar protein repertoire. Furthermore, aphid species resulting in dissimilar host damage and those that feed on different hosts are suggested to have dissimilar salivary proteins. However, variations occur in the watery saliva within biotypes of the same aphid species (Nicholson *et al*., 2012), implying that the salivary proteome is fluctuating. This may even change within a biotype as it feeds on different hosts.

2.2.4 The aphid salivary effectors

Effectors are small elicitor molecules and/or proteins with enzymatic activity that disrupt the plant defence response. In plant-pathogen interactions, they are delivered inside plant cells of their hosts using the Type III secretion system by pathogenic bacteria and haustoria by biotrophic fungi and oomycetes (Hahn and Mendgen, 2001; Panstruga, 2003; Hogenhout *et al.*,

2009) . Aphid species secrete saliva containing effectors from their salivary glands during host penetration (Hogenhout and Bos, 2011). The effectors are responsible for promoting virulence by interacting with the host plant to facilitate feeding and colonisation by invaders (Mutti *et al.*, 2008; Rodriguez and Bos, 2013; Elzinga *et al.*, 2014). Studies conducted on bacterial phytopathogen effectors provided insight into their mode of overwhelming plant defence responses (Cunnac *et al*., 2009; Hogenhout *et al.*, 2009; Dodds and Rathjen, 2010; Rodriguez *et al*., 2017). Aphid salivary effectors should function during virulence as they are functionally similar to effectors found in pathogenic bacteria, oomycetes, fungi and nematodes (Jones and Dangl, 2006; Hogenhout *et al.*, 2009; Bos *et al.*, 2010; Elzinga and Jander, 2013; Wang *et al.*, 2013, 2015).

Aphid effectors suppress plant defences to promote infestation and feeding (Bos *et al.*, 2010). The *Myzus persicae* effectors Mp1, Mp2 and MpC002 improved aphid performance on the *Arabidopsis thaliana* and *Nicotiana benthamiana* hosts (Bos *et al.*, 2010). The *Macrosiphum euphorbiae* effectors Me10 and Me23 were also shown to increase aphid fecundity in *N. benthamiana*. Interestingly, these two effectors were found to have orthologues expressed in *M. persicae* and *Acyrthosiphon pisum* species that have broad and narrow host ranges, respectively. This suggests that effectors may present a general function in a variety of host species (Mondal, 2017). Another possibility exists in which certain aphid effectors play major roles in suppressing plant defence in a species-specific or biotype-specific manner. Nicholson *et al*. (2012), observed high variation in salivary proteomes of *D. noxia* when compared to other aphid species and this suggests a unique host association.

The identified *A. pisum* biotype-specific effectors in the saliva indicated that they may have recently evolved and diversified through duplication events (Boulain *et al.*, 2019). Other effectors with different defence suppression strategies have been identified such as Armet that promotes salicylic acid (SA) accumulation and Mp55 shown to lower the accumulation of both hydrogen peroxide and glucosinolate. Mp55 also inhibits callose deposition in *A. thaliana* (Elzinga *et al.*, 2014). Since some effectors are under negative selective pressure and others are fast evolving, Mp55 and Armet may have evolved to function as new virulence-promoting proteins (Varden *et al.*, 2017; Cui *et al.*, 2019).

The increase in free cellular Ca^{2+} ions in the phloem sieve element is associated with the occlusion mechanism during plant defence (Lecourieux *et al*., 2006; Will *et al.*, 2007). In Fabaceae, the phloem sieve elements contain protein bodies known as the forisomes that disperse in the cell and result in plugging of the sieve plates in response to damage (Furch *et al.*, 2007; Will *et al.*, 2009, 2012). This type of response is activated by an influx of Ca^{2+} into the sieve element that results in the expanded reversible conformational change of forisomes that block sieve plates (Will *et al*., 2013). However, monocotyledonous plants do not have forisomes. The watery saliva of the species *Megoura viciae, A. pisum* and *Sitobion avenae* was shown to include Ca^{2+}-binding proteins that are responsible for suppressing plant defences (Will *et al.*, 2007; Carolan *et al.*, 2009; Zhang *et al.*, 2017). These Ca^{2+}-binding proteins were shown to reverse the occlusion mechanism elicited in the phloem sieve element during plant defence response. This was confirmed by the change in aphid behaviour from phloem sap ingestion to secretion of saliva containing a high number of proteins with Ca^{2+} -binding activity (Furch *et al.*, 2007; Will *et al.*, 2007). Furthermore, Will *et al.* (2007), suggests that the Ca^{2+}-binding proteins may be responsible for preventing clogging of the stylet food canal by sieve element proteins during phloem-feeding. The watery saliva was suggested to be universal across all aphid species for the function of preventing sieve-plate occlusion due to the presence of Ca^{2+}-binding proteins in all species studied (Will *et al.*, 2009). This also indicates that the reverse-occlusion mechanism presented by phloem-feeding insects may be a common strategy against an overwhelming plant defence (Will *et al.*, 2007).

2.3 Plant innate immunity

The plant innate immunity is a complex mechanism of plant defence against pathogen invasion and relies on the recognition of pathogen-derived molecules by plant immune receptors (Cook *et al.*, 2015). Innate immunity induced by the attack can be categorised into two phases PTI and ETI. PTI and ETI differ by the mode of interactions, characteristics and location of the receptors, the interacting molecules, the signalling mechanisms and lastly the strength of the immune response induced (Tsuda and Katagiri, 2010; de Vos and Jander, 2010; Cesari *et al.*, 2014a).

2.3.1 PTI

During the initial PTI phase, the plant cell surface pattern-recognition receptors (PRR) of the targeted cells recognise the conserved pathogen-derived molecules. These PRRs include receptor kinases and receptor-like proteins and the pathogen molecules they perceive include, lipids, peptides, small molecules such as ATP, and cell wall fragments (Dodds and Rathjen, 2010). The PTI exhibits a broader defence response that involves the activation of complex intracellular signalling cascades (; Jones and Dangl, 2006; Mithofer and Boland, 2008; Dodds and Rathjen, 2010). The *Pseudomonas syringae* elicitor flagellin (mainly the N-terminal peptide, flg22) was the first PAMP identified to trigger plant immunity upon its perception by Flagellin Sensing 2 (FLS2) (Felix *et al.*, 1999; Gómez-Gómez and Boller, 2000). The resistance response included accumulation of Pathogenesis-Related protein 1 (PR1) and callose deposition. Further studies are still underway to establish further PAMP-PRR interactions in plant immunity (Čerekovic and Poltronieri, 2017).

2.3.2 ETI

The ETI is activated as the second phase of plant immune response upon suppression of PTI by virulent pathogens. It results in a much stronger and faster plant immune response linked with a localised programmed cell death known as the HR (Jones and Dangl, 2006). This immune response is induced by the recognition of secreted pathogenic effector molecules that interact with intracellular disease resistance (R)-proteins. Plant R-proteins are diverse and vary in both structure and mode of action. The NLR family of R-proteins contributes to the perception of pathogen effector molecules and activation of the ETI. The mechanism by which these NLR proteins convert effector-recognition into immunity remains unclear (Cesari, 2018). Furthermore, the NLR genes consist of mostly conserved or variable multidomain architectures that have constantly evolved through an arms race between their respective pathogen effector genes. Insight into the structure and functions of these domains provides clues to the mechanisms by which NLR proteins contribute to ETI.

2.3.2.1 Plant NLRs and their domains

The NLRs are an important superfamily of R-proteins that recognise pathogen and insect effectors and induce ETI (Cesari, 2018). These receptor proteins share structural homology and

functional similarity to the nucleotide-oligomerisation domain-like (NOD-like) receptors of mammals.

2.3.2.1.1 The TIR and CC domains

The NLRs are separated mainly into three classes on the basis of the differences at their N-terminal domains. The one NLR class contains a Toll/Interleukin-1 (TIR) domain and is predicted to share homology to *Drosophila* and mammalian immune receptors. The other two classes consist of a coiled-coil (CC) or RPW8 domain, respectively, at their N-terminus (Burch-Smith and Dinesh-Kumar, 2007; Shao *et al.*, 2016; Hu *et al.*, 2017; Cesari, 2018). Dicotyledonous plants have NLRs with either the CC or TIR N-terminal domain and monocotyledonous plants contain NLRs with only the CC N-terminal domain (Meyers *et al.*, 2003). Studies conducted on the barley NLR receptor, MLA, showed that the N-terminal CC-domain of this receptor triggers cell death in *N. benthamiana* (Maekawa *et al.*, 2011). Bernoux *et al*. (2011), showed that the TIR domain of the L6 receptor protein is crucial for immune signalling. A recent study on the CNL of *A. thaliana* and *N. benthamiana* ZAR1 suggested that the function of its CC was to stabilise the inactive form of the NLR and also structural remodelling and fold switching during its activation (Wang *et al.*, 2019a; 2019b). Therefore, the variable N-terminal domains of several NLRs play an important role in plant defence signalling, although the subsequent biochemical mechanisms of signal induction have long been unclear (Cesari, 2018). The structural differences at the N-terminal domains of NLRs between plants and animals suggest different NLR signalling mechanisms. The signalling mechanisms of CNL and TNL have long been thought to rely on downstream signalling pathways (Cesari, 2018). However, the recent study on ZAR1 activation showed that the CC domain does not depend on downstream signalling pathways and exerts cell death directly through membrane perturbations (Wang *et al.*, 2019a; 2019b).

The CC domains of most NLRs have been shown to self-associate by forming a homodimer and thereby induce cell death (Meunier and Broz, 2017). The structure of the CC domain of plant NLRs plays an important role in defence signalling since properly folded CC homodimers activate cell death. The crystal structure of the *H. vulgare* CC domain of MLA10 is helical and forms a helix-loop-helix structure. This is formed by two antiparallel alpha-helices that are linked by a short loop that is responsible for the homodimerisation of the CC domain (Maekawa

et al., 2011). The wheat Sr33 and Sr50 CC domains were also shown to be necessary for induction of cell death following their homodimerisation (Cesari *et al.*, 2016). Thus, homodimerisation of the CC domain of plant NLRs plays a crucial role in defence signalling.

2.3.2.1.2 The NB-ARC domain

Following the N-terminal domain of plant NLRs is the highly conserved and large NB-ARC (Nucleotide Binding, Apaf-1 Resistance proteins, and CED-4) domain that belongs to the signal transduction ATPases with numerous domains (STAND) family of NTPases (Takken *et al*., 2006; Lukasik and Takken, 2009). The NB-ARC domain of human apoptotic protease-activating factor 1 (APAF-1) and the *Caenorhabditis elegans* homologue CED- 4 was shown to share homology with the NB-ARC domain of plant NLRs (van der Biezen and Jones, 1998; Takken *et al*., 2006). As such, this domain is predicted to consist of three subdomains, namely the NB, ARC1 and ARC2. Studies conducted on CNLs such as Rx predicted how these subdomains convey initial immune signalling. The NB subdomain conforms a P-loop NTPase fold and consists of the conserved Walker A (P-loop) and the Resistance nucleotide binding site (RNBS)-A motifs predicted to be involved in ATP binding. The Walker B motif is located at the C-termini of the NB subdomain and is also predicted to be involved in ATP hydrolysis (Takken *et al*., 2006). The ARC-1 subdomain conforms to a four-helix bundle and was shown to act as the main scaffolding domain for binding the Leucine Rich Repeat (LRR) domain (Lukasik and Takken, 2009). The ARC-2 subdomain was shown to assume a winged-helix fold and consists of the RNBS-D motif of unknown function and the highly conserved MHD-motif with suggested activity in nucleotide-dependent conformational changes. Further functional ATP binding activity was predicted in the loop connecting the ARC1 and ARC2 subdomain (Takken *et al*., 2006; Lukasik and Takken, 2009).

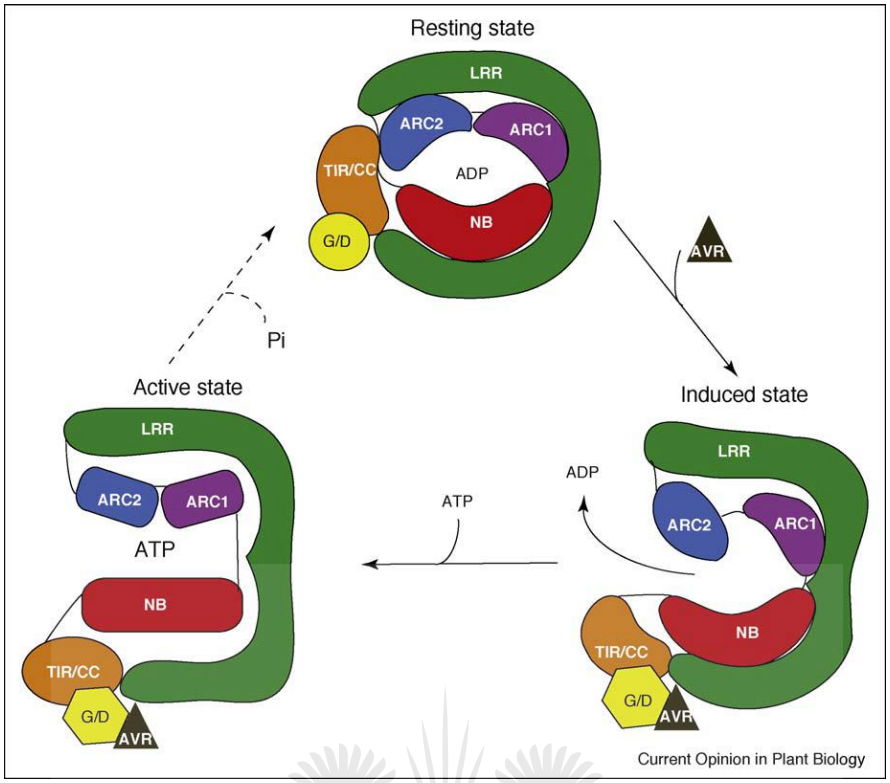

Figure 1: Schematic models of NLR activation (Lukasik and Takken, 2009).

The NB-ARC domain of APAF-1 and CED-4 was predicted to undergo conformational changes that function as a molecular switch to regulate signalling pathways. The biochemical and structural analyses of plant NB-ARC domains proposed a similar switch model to APAF-1 and CED-4 (Takken *et al*., 2006). This model is illustrated in figure 1, in which the resting state represents the stable form of the NRL protein in the absence of an elicitor. The presence of an elicitor results in the conformational change of the NB-ARC domain that triggers the release of the bound ADP nucleotide. Subsequently, nucleotide exchange occurs in which the ATP molecule binds to the NB-ARC domain following the release of the ADP molecule and a second conformational change resulting in the active state is triggered. This active form of the protein potentially relays an immune signal and subsequently returns to its initial resting state (Takken *et al*., 2006; Lukasik and Takken, 2009).

2.3.2.1.3 The LRR domain

The arc-shaped C-terminal Leucine-Rich Repeat (LRR) domain following the NB-ARC domain of plant NLRs was determined to be highly irregular, consisting of variable repeats and

non-canonical motifs (Padmanabhan *et al*., 2009; Takken and Goverse, 2012; Cui *et al*., 2015). Studies conducted on a majority of NLR proteins such as Rx, MLA and L showed that the LRR domain is the most variable although other NLRs such as Lr10 indicated that the LRR is the most conserved domain (Sela *et al.*, 2012; Takken and Goverse, 2012). Structural classes of NLR proteins are proposed to exist due to variations that occur in the number of LRR repeats in the LRR domain (Takken and Goverse, 2012).

The LRRs are divided into two sections in which the N-terminal section consists of a cluster of positively charged residues and a C-terminal section consisting of aromatic amino acids (Takken and Goverse, 2012). The two LRRs consist of a conserved core that forms a β-strand tailed by a more variable region (Padmanabhan *et al*., 2009). They were identified as key role players in specific effector recognition in NLR mutational and domain swap studies (Cui *et al*., 2015). The LRR domain of the *Arabidopsis* resistance protein RPP1-WsB indicated that this domain was important for interaction with the oomycete ATR1 effector (Krasileva *et al*., 2010).

Effector recognition by the LRR domain was first identified in the direct recognition interaction between the LRR domain of the *Oryza sativa* NLR known as Pi-ta and its respective *Magnaporthe grisea* effector AvrPita (Jia *et al.*, 2000). This was also observed for the flax rust resistance protein in which recognition specificity was determined by the direct interaction with the LRR domain (Ellis *et al*., 2007). Takken and Goverse (2012), suggests that the N-terminal section of the LRR is involved in intramolecular signalling and the C-terminal section is a key determinant for recognition specificity. Another key role of the LRR domain is suggested to be involved in keeping an autoinhibited or stable state of the NLR protein in the absence of an effector. Upon effector recognition by the LRR domain, the conformation of the NLR protein is suggested to switch from the stable state into an active state through interactions with the CC/TIR and NB-ARC domains of the NLR (Takken and Goverse, 2012; Slootweg *et al.*, 2013).

Lukasik and Takken (2009), identified that the CC and LRR domains both bind the NB–ARC domain. This interaction provides the recognition of the infectious viral coat protein (CP) and subsequent activation of defence signalling upon its disruption by the CP. Bernoux *et al*.

(2016), demonstrated that the interaction of the TIR and the NB domain of L6 and L7 are important for the activation of these receptors. These findings provide the basis for the structural interactions of these three domains of most NLR proteins and the possible mechanistic activation of defence signalling.

2.3.2.1.4 The WRKY integrated domain

Several NLRs contain additional incorporated C-terminal domains of which the WRKY-domain (WRKYGQK) is one. The WRKY proteins are plant transcription factors that mediate biotic and abiotic stresses and also regulate the expression of defence-related genes (Bekir, 2004; Huang *et al.*, 2016; Singh *et al.*, 2019). Upregulation of WRKY genes are further linked to pathogen infection and wounding in plants (Eulgem *et al.*, 2000). The known WRKY proteins are classified into three groups (I, II and III) based on the number of WRKY domains present and the pattern of zinc-finger motifs. Furthermore, phylogenetic analysis of the WRKY domains of Group II divide these WRKY members into five subgroups (IIa-IIe). They consist of four-stranded beta-sheet and a zinc finger pocket that plays a role in the recognition of the DNA W-box sequence motif and autoregulation of gene expression by self-binding *cis*-elements in their promoters (Eulgem *et al.*, 2000; Huang *et al.*, 2016; Ning *et al.*, 2017; Singh *et al.*, 2019). Studies conducted on the WRKY proteins such as the *A. thaliana* WRKY4, *N. tabacum* WRKY6, 7, 8, 9 and SPF1 have shown that the C-terminal WRKY domain was responsible for binding specific DNA sequences (Maeo *et al.*, 2001; Yamasaki *et al.*, 2005). The function of the N-terminal part of the WRKY domain of many WRKY proteins has long been unknown and a recent study on *A. thaliana* WRKY1 showed that the N-terminal part also contributes to DNA binding (Eulgem *et al.*, 2000; Xu *et al.*, 2020).

Several NLRs containing atypical domains such as WRKY domains, with characteristic and functional features similar to plant transcription factors, and Ankyrin repeat domains, responsible for protein-protein interactions, have been documented (Deslandes *et al.*, 2003). The incorporated C-terminal WRKY domain of *Arabidopsis* RRS1-R together with its cognate NLR partner RPS4 binds DNA and plays a role in the transcriptional activation of defence-related genes (Narusaka *et al.*, 2009; Sarris *et al.*, 2015). This NLR domain together with other host WRKY transcription factors play crucial roles in the plant innate immune system (Le Roux *et al.*, 2015). Therefore, WRKY proteins and NLRs with incorporated WRKY domains are

important role players in the regulation of plant defence responses (Bakshi and Oelmüller, 2014; Ellis, 2016; Sarris *et al.*, 2015).

2.3.2.1.5 The ankyrin repeat domain

The ankyrin repeat domain of many plants, animals and protozoa are predicted to consist of 33 highly conserved amino acid residues that form a protein-binding interface. The repeats of this domain are suggested to form a beta-hairpin or a long loop preceded by two-folded antiparallel alpha-helices (Mosavi *et al.*, 2004) and is known to occur as a single protein. The *Arabidopsis* Ankyrin repeat-containing protein, NPR1, was shown to play a major role in the regulation of the SA signalling pathway and transcriptional regulation of plant defence response (Mou *et al.*, 2003).

The wheat CNL-WRKY, YrU1, consists of an N-terminal ankyrin repeat domain responsible for protein-protein interactions during stripe rust resistance response (Wang *et al*., 2020). It was suggested that the integration of ankyrin domains in plant NLRs may have resulted from fusion with single ankyrin-containing proteins. The ankyrin and CC domains of YrU1 were suggested to individually self-associate and form homodimers that are important for the functioning of this receptor. Nicolis and Venter (2018), proposed that the ankyrin repeats may be involved in binding defence signalling cascade proteins through intermolecular interactions.

2.3.2.2 NLR pairs co-acting in conferring resistance

Various studies have shown that plant NLRs are individually sufficient for sensing of pathogen effectors and initiate a signalling cascade. There is currently growing evidence in cases where a pair of NLRs that are genetically linked are required for pathogen recognition and defence signalling (Le Roux *et al.*, 2015; Baggs *et al*., 2017). The interesting feature shared by most NLR pairs is that they are genetically linked in inverse orientation and they both share a common promoter (Baggs *et al.*, 2017). The paired NLR proteins are suggested to interact together through heterogeneous complexes and function cooperatively in the induction of a downstream defence signal following effector recognition (Cesari *et al.*, 2014b; Baggs *et al.*, 2017). Williams *et al.* (2014), demonstrated that the *Arabidopsis* NLR pair, RPS4 and RRS1, interact through the formation of homo- and heterodimers at their TIR domains. Several studies

have provided evidence that one NLR is commonly responsible for perceiving or binding the pathogen or plant host defence elicitor. The other NLR is responsible for recognising conformational changes in the first NLR that are caused by interacting with the elicitor and subsequent initiation of defence signalling (Baggs *et al.*, 2017). RPS4 induces a resistance signal and RRS1 (containing a C-terminal WRKY domain) perceives and binds pathogen effectors and also represses the TIR signalling activity of RPS4 (Cesari *et al.*, 2014b; Williams *et al.*, 2014). This was also shown to be similar for the NLR pair RGA4 and RGA5 (Cesari *et al.*, 2014c). These NLR pairs functioning cooperatively contribute to providing an understanding of NLR evolution and mechanisms for mediating the resistance response.

2.3.2.3 Proposed mechanisms of pathogen perception by NLRs

The gene-for-gene concept, states that plant disease resistance is governed by two complementary genes, one from the pathogen and the other from the plant. The pathogen contains an avirulence (*Avr*) gene and the host plant contains a matching resistance (*R*) gene that plays a role in plant-pathogen interaction (Flor, 1971). NLR proteins are proposed to recognise pathogen effectors (previously the avirulence proteins) directly by physical association or indirectly through a host protein commonly known as the accessory protein. This protein forms part of the NLR protein complex capable of inducing a defence response (Dodds and Rathjen, 2010).

2.3.2.3.1 The direct model

Direct effector recognition has been reported for various NLR proteins and in some cases, this mechanism is sufficient to trigger plant immunity (Baggs *et al.*, 2017). This model is well demonstrated by Pi-ta in rice that binds to the *M. grisea* effector AvrPita and the tomato Pto protein that interacts with the *Pseudomonas syringae* pv. *tomato* effector avrPto (Salmeron *et al.*, 1996; Jia *et al.*, 2000). In both cases, the direct association between the NLR and the effector resulted in the activation of defence response. However, this model failed to describe a large number of interactions and was adapted into several other models explaining how indirect association plays a role in interactions (Dodds and Rathjen, 2010).

2.3.2.3.2 The indirect model

Plant NLR proteins can recognise pathogen effectors indirectly through a host accessory protein that is a target of the effector and also part of an NLR protein complex (Dodds and Rathjen, 2010). This model is suggested to occur more often in many plant-pathogen interactions and may have evolved to acquire a broad recognition spectrum for various pathogens (Cesari, 2018).

2.3.2.3.2.1The guard hypothesis

The guard hypothesis postulates that a host protein targeted by pathogen effectors is monitored by an NLR protein that "safeguards" it and is activated upon recognition of the modifications caused by the effectors on the host protein (van der Biezen and Jones, 1998; Dangl and Jones, 2001). For example, the *Arabidopsis* RIN4 protein and the NLR proteins RPM1 and RPS2, form exclusive complexes. Upon proteolytic degradation of RIN4 by AvrRpt2, the NLR associated with RIN4 activates RPS2. Furthermore, phosphorylation of RIN4 by the effectors AvrB or AvrRPM1 results in the activation of RPM1. In both cases, activation of the plant NLR results in the induction of a defence signal (Dodds and Rathjen, 2010). This example also explains how a virulence target can be guarded by multiple host factors and how multiple unrelated effectors are recognised for activation of immunity.

2.3.2.3.2.2 The decoy hypothesis

The decoy hypothesis postulates that the NLR protein recognises modifications of the host protein that is a duplicate or a mimic (decoy) of the target protein of effectors (van Der Hoorn and Kamoun, 2008). The sole function of the decoy protein is to drive indirect effector recognition by the NLR for subsequent activation of an immune response. For example, the accessory protein Pto kinase family can diversify and broaden the spectrum for effector recognition. Therefore, the NLR protein Prf that complexes with Pto monitors the decoy and activates defence upon pathogen perception (Gutierrez *et al.*, 2010).

2.3.2.3.2.3 The integrated decoy hypothesis

The integrated decoy model was first proposed by Cesari *et al.* (2014a), following the discovery and identification of unusual domains integrated onto NLR proteins. These integrated domains (IDs) were shown to play key roles in the recognition of pathogen effectors. For example, the rice NLR RGA5 containing integrated heavy metal-associated (HMA) domains physically interacts with pathogen effectors to activate plant immune response (Cesari, 2018). Another example is the *Arabidopsis* RRS1 protein that contains a WRKY integrated domain. Since the effector PopP2 targets WRKY transcription factors, RRS1 has acquired the ability to recognise PopP2 through its WRKY integrated domain (Le Roux *et al*., 2015; Sarris *et al*., 2015). Furthermore, the IDs are suggested to arise from the duplication of genes targeted by the effector gene. This results in the integration of these domains into NLR genes resulting in acquired effector target sites.

2.4 Plant wound responses

The plant PTI and ETI are both associated with various cellular events related to intracellular signalling pathways and downstream responses. These include mechanisms such as the rapid influx of calcium ions, a burst of cellular reactive oxygen species (ROS), activation of mitogen-activated protein kinases (MAPKs), gene expression reprogramming, Sieve tube element (STE) occlusion by callose deposition and/or protein plugging, systemic acquired resistance (SAR), and a localised cell death response also known as the HR (Dodds and Rathjen, 2010).

2.4.1 The reactive oxygen species

In plants, the ROS occur as ubiquitous molecules produced mainly in the chloroplast and other organelles during various cellular metabolic pathways and includes hydrogen peroxide (H_2O_2) and superoxide (O_2^-). These molecules accumulate in a tightly regulated manner as a result of unfavourable environmental conditions and invasion by pathogens or pests (Alvarez *et al.*, 1998; Kotchoni and Gachomo, 2006). During plant-pathogen interactions, these molecules are suggested to play a role in plant defence signalling and cell wall reinforcement (Brisson *et al*. 1994; Kotchoni and Gachomo, 2006).

The oxidative burst is the rapid production of high levels of ROS, mainly H_2O_2 in plants. This burst is regarded as one of the early events that occur during pathogen perception and contributes to the HR (Morel and Dangl, 1997). The HR caused by a pathogen or pest is characterised by tissue damage or cell death at the feeding or infection site (Fath *et al.*, 2001; Kotchoni and Gachomo, 2006). The oxidative burst in plants is important for the cross-linking of cell wall proteins that strengthen the cell wall to prevent penetration by pathogens (Brisson *et al.*, 2010).

The HR activation is linked to receptor-ligand recognition of effectors (Morel and Dangl, 1997). The generation of the ROS in plants is also suggested to be associated with the NADPH oxidase system during interaction with invading pathogens (Bolwell, 1999). Studies have described the influx of Ca^{2+} to be involved in the activation of the HR via the NADPH oxidase system that results in the production of ROS such as O_2^- and H_2O_2 (Doke *et al.*, 1996). The interaction between *Arabidopsis* RPM1 and avrRpm1 and avrB results in the accumulation of H_2O_2 and an increase in the cytosolic Ca^{2+} (Grant *et al.*, 2000). The oxidative burst plays an important role in defence against pest invasion as well. Moloi and van der Westhuizen (2006), showed that the resistance response in wheat during *D. noxia* interaction was associated with the accumulation of H_2O_2 upon activation of the NADPH oxidase system.

2.4.2 Sieve tube occlusion

The STE occlusion is a defence mechanism exhibited against phloem-infecting pathogens and phloem-feeding insect pests. The STE is occluded by intracellular phloem proteins and callose in response to wounding (Will *et al.*, 2009, 2007). This mechanism is important for preventing the loss of sap from distraction by invaders at the phloem-feeding site (Liu *et al.*, 2013; Will *et al.*, 2007, 2013). Occlusion through protein plugging occurs much more rapidly than through callose deposition. STE occlusion through callose deposition is observed as a universal mode across plant species (Furch *et al.*, 2007; Will *et al.*, 2013). Callose is a 1,3-β-glucan consisting of 1,6 branches that are synthesised by a membrane-bound protein 1,3-β-glucan synthase commonly known as callose synthase (Luna *et al.*, 2011). Callose production occurs in the STE cell wall outside the plasma membrane and forms callose plugs around plasmodesmata that are responsible for indirectly blocking sieve pores (Will and Van Bel, 2006; Will *et al.*, 2007). The

formation of callose is activated upon the influx of Ca^{2+} ions into the phloem cell (Will *et al.*, 2009).

2.4.3 Protein plugging

Higher plants are known to consist of a variety of a specific group of phloem proteins known as P-proteins that are involved in the rapid occlusion of sieve tubes (Will *et al*., 2013). Sieve tube element occlusion in the Fabaceae is associated with the dispersal of proteins known as forisomes during wounding (Furch *et al.*, 2009). These proteins are deposited around sieve plates and aggregate to form plugs that block sieve pores (Will *et al.*, 2013). This process is known as protein plugging and is dependent on the influx of Ca^{2+} ions. The sieve tubes of grass family members appear to be empty but may present an occlusion mechanism involving soluble proteins that solidify to form aggregates (Will *et al*., 2009). However, there are no P-proteins present in the grasses. In many plants, the occlusion of the sieve plates involves two events in which protein plugging is followed by callose deposition. The increase in Ca^{2+} influx into sieve tubes is implicated in the induction of STE occlusion mechanisms and reversible through the removal of these ions (Furch *et al.*, 2007).

2.4.4 Systemic acquired resistance

Plants are able to defend themselves through a mechanism known as SAR induced by pathogen infection and pests feeding on their leaves or stems. This mechanism is effective against a wide range of pathogens and pests and occurs at distant locations from the two phases of innate immunity. SAR is associated with the production of hormones such as jasmonic acid (JA), SA, ethylene (ET), abscisic acid (ABA) and gibberellic acid (GA) that play roles in the regulation of disease resistance responses (de Wit, 2007; Goggin, 2007). These hormones are induced based on the type of pathogen or insect pest and may be released separately or in combinations (de Wit, 2007; Botha, 2013). The roles of the different hormones may also be dependent on the aphid species that infest the plant (Giordanengo *et al*., 2010) Induction of SAR in wheat is associated with the induction of the JA and SA pathways during *D. noxia* interaction depending on the presence of the type of resistance gene and the *D. noxia* biotype (Botha, 2013; Botha *et al*., 2014).

2.5 Existing wheat R-genes against *D. noxia*

Diuraphis noxia (*Dn*) resistance genes in wheat and its close relatives have been categorised into three main groups based on the type of resistance response. Antibiosis is the ability of the resistant plant to affect the life cycle or the biology of the insect resulting in unsuccessful colonisation (Starks and Burton, 1977; Painter, 1958). This includes various abnormal behaviours exhibited by the insect such as the significant reduction in the insect's reproduction, altered development resulting in an abnormally short life, adverse effects on insect metabolism and eventual death after the first instar (Painter, 1958; du Toit, 1989; Smith *et al.*, 2004). Antixenosis, or non-preference, is the exhibition of behavioural characteristics that result in rejection of the host plant by the invading insect (Kogan and Ortman, 1978). These include morphological characters such as the thickness of plant tissue and availability of leaf trichomes and surface waxes. The presence of chemical factors may also play a role by acting as insect repellents (Funderburk *et al*., 1993). Tolerance is the potential of the host plant to continue normal growth, development and reproduction despite infestation. The plant does not exhibit adverse effects under conditions in which the levels of insect infestations are similar to those observed on susceptible plants (Kogan and Ortman, 1978; du Toit, 1989; Botha *et al*., 2005).

To date, there are fourteen known wheat *Dn* resistance genes against *D. noxia* with varying levels of specificity to the different aphid biotypes. The *Dn1* and *Dn6* resistance genes exhibit antibiosis against *D. noxia* (du Toit, 1987, 1988, 1989; Liu *et al*., 2001; Saidi and Quick, 1996). The recessive *dn3* gene originated from *T. tauschii* (Nkongolo *et al*., 1991a). The *Dn5* and *Dnx* genes exhibit antixenosis (du Toit, 1987, *et al*., 1995; Harvey and Martin, 1990; Marais and du Toit, 1993; Liu *et al*., 2001) and the *Dn2* and *Dny* genes exhibit tolerance (du Toit, 1987, 1988, 1989; Liu *et al*., 2001; Smith *et al*., 2004). The *Dn4* and the *Dn7* genes originated from rye and exhibit both antibiosis and antixenosis (Nkongolo *et al*., 1991b; Ma *et al*., 1998; Liu *et al*., 2001). Finally, the *Dn8*, *Dn9*, *Dn626580*, *Dn2414* and *Dn2401* genes display various types of resistance (Fazel-Najafabadi *et al*., 2015; Liu *et al*., 2001; Peng *et al*., 2007; Valdez *et al*., 2012).

Dn2401 is the first, and currently the only, cloned *D. noxia* resistance gene from wheat (Tulpová *et al.*, 2019). The winter wheat accession CI 2401 originating from Tajikistan (Porter *et al*., 1993) exhibits both tolerance and an antibiosis effect against *D. noxia* biotype 2 and

imparts resistance against *D. noxia* 1, 2, 6, 7 and 8 (Qureshi *et al*., 2006; Voothuluru *et al.*, 2006; Weiland *et al.*, 2008). It was suggested that the resistance observed in CI 2401 was controlled by two dominant genes of which one was *Dn4* (PI 372129) and the other, an unidentified gene but independent from *Dn5* and *Dn6* (Dong *et al*., 1997). The identified candidate gene of *Dn2401* responsible for imparting resistance was characterised as an epoxide hydrolase enzyme that plays a role in cutin biosynthesis during plant defence against pathogens and pests. Therefore, unravelling the genes and functions of their products playing a role in a host's natural resistance is important in contributing to understanding the host's mechanism of defence and also enables efficient transfer of these resistance genes into susceptible cultivars (Tulpová *et al.*, 2019).

2.6 The wheat-*D. noxia* interaction in South Africa

Diuraphis noxia was first detected in South Africa during 1978. In 1979, it was detected as a pest of wheat in the Eastern and Western Orange Free State, including Lesotho (Walters, 1980). Control measures became necessary and insecticidal control was deployed upon detection of *D. noxia*. However, this control method was detrimental to both the environment and humans and also proved costly. The strategy was considered less efficient as it did not provide a complete solution to the problem. The development of resistant cultivars was initiated upon discovery of existing natural resistance in bread wheat to *D. noxia* and this has been the main focus and the current management practice for wheat (du Toit, 1987; Marasas, 2005; Jankielsohn, 2017; Nhemachena and Kirsten, 2017). Tugela DN was the first resistant wheat cultivar against *D. noxia* in South Africa and also the first in the world (du Toit, 1987, 1988, 1989). The release of several resistant wheat cultivars, controlled *D. noxia* until resistance breaking biotypes developed due to adaptation to the selective pressure applied by the single gene containing wheat populations (Tolmay and Prinsloo, 2000).

Diuraphis noxia is capable of adapting to specific host populations and thereby overcome the resistance presented by these plants by differentiating into multiple biotypes (Jankielsohn, 2017). This biotypification in wheat-growing regions poses a serious challenge to the wheat industry (Jankielsohn, 2011). Factors such as the varying levels of resistance response during antibiosis and antixenosis, plant structural changes that protect aphids such as leaf rolling and

the changes in environmental conditions, all create selective pressure on the aphids that result in the development of more virulent biotypes (Hawley *et al*., 2003; Jankielsohn, 2017, 2019a).

Recently, wheat plants containing the *Dn3* resistance gene exhibited susceptibility to RWASA1 infestations. Wheat containing the *Dn1*, *Dn2*, *Dn3*, *Dn8, Dn9* and *Dny* genes showed susceptibility to RWASA2 infestations. The biotype RWASA3 caused susceptible damage to wheat containing the *Dn1*, *Dn2*, *Dn3*, *Dn4*, *Dn8, Dn9* and *Dny* genes. The RWASA4 biotype caused susceptibility in wheat carrying the *Dn1*, *Dn2*, *Dn3*, *Dn4, Dn5, Dn8, Dn9* and *Dny* genes (Jankielsohn, 2019b). This biotype was also proposed to be the most virulent and possesses the potential to replace other biotypes occurring in the Eastern Free State region (Jankielsohn, 2017). However, the recently identified biotype, RWASA5, was shown to result in similar susceptibility of all eight different *Dn* resistance genes excluding the *Dn7* gene (Jankielsohn, 2019b). The observation that resistance breaking RWA biotypes keep emerging calls for additional resistance sources for managing RWAs in the future (Jankielsohn, 2016, 2019b).

2.7 Virus-induced gene silencing

Reverse genetics is a method of evaluating the specific function of a gene that has previously been identified from sequencing data. This can be through the creation of an endogenous loss-of-function mutation and thereby analysing the phenotypic result (Bouchez and Ho, 1998). The naturally occurring plant post-transcriptional gene silencing (PTGS) mechanism degrades specific messenger RNAs and is an important tool employed in reverse genetic studies. This results in the reduction of the expression of a specific gene and can be manipulated to provide important clues about the function of gene products (Bouchez and Ho, 1998; Stokes, 2000; Unver and Budak, 2009).

One technique utilised in the down-regulation of a specific endogenous gene of interest (GOI) is the use of engineered viruses and is commonly known as virus-induced gene silencing (VIGS) (Unver and Budak, 2009; Broderick and Jones, 2014). VIGS exploits the plant's natural defence systems used to protect against virus invasion (Ramegowda *et al*., 2014). This technique is very efficient in that it is cost-effective and rapid, phenotype developments are transient with no major effects on the host, homologous genes may be silenced with a single

construct and finally, it is suitable to use in transformation of recalcitrant species (Gilchrist and Haughn, 2010; Ben-Amar *et al.*, 2016). Several engineered viruses have been developed for use in VIGS to target host genes (Unver and Budak, 2009). The Barley stripe mosaic virus (BSMV) is a naturally occurring plant virus of the genus Hordeivirus and infects two members of important cereal crops, barley and wheat (Scofield *et al.*, 2005; Hein *et al.*, 2005; Jackson *et al.*, 2009). This virus is a single-stranded positive-sense RNA, each carrying a 5'- terminal cap and a 3'-terminal tRNA-like structure, that contains a tripartite genome designated as *α*, *β* and *γ* (Unver and Budak, 2009).

The mechanism of BSMV-mediated VIGS involves the mechanical entry of the virus through cell wall openings or abrasions, followed by the release of the virus genomic RNAs into the cytoplasm (Jackson *et al.*, 2009). Generally, the complementary DNA (cDNA) of viral genomes are cloned into binary vector systems that are under the control of the CaMV35S promoter along with the multiple cloning sites (Hein *et al.*, 2005; Ramegowda *et al.*, 2014; Scofield *et al.*, 2005). A short section of the plant GOI is cloned in the antisense orientation into the RNAγ downstream of the stop codon of the γb open reading frame (ORF). Following entry of the virus into the host cell, the RNAα encodes a replicase protein while the RNAβ encodes the movement protein from αa and αb, respectively. The RNAγ is responsible for encoding the polymerase component of replicase and viral pathogenicity proteins from γa and γb respectively. The formation of long double-stranded RNA (dsRNA) during viral replication results in their recognition by the host Dicer-like enzymes (DCLs), responsible for cleaving the dsRNA into 21-22 nucleotide siRNAs. The one siRNA strand following cleavage is then incorporated into the host RNA-induced silencing complexes (RISC). This complex is responsible for the endonucleolytic cleavage of single-stranded RNAs with complementary sequences to the siRNA strand in the RISC complex (Figure 2). Therefore, those siRNAs generated from the plant GOI cloned in the antisense orientation into the BSMV genome will also incorporate with RISC and target host mRNAs with complementary sequences for silencing (Lee *et al*., 2012).

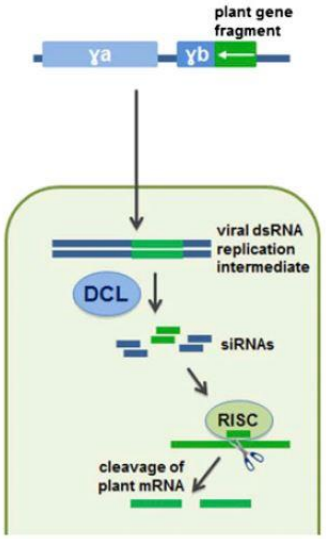

Figure 2: Schematic models of BSMV-mediated VIGS (Lee *et al*., 2012).

The modified BSMV system was developed for gene silencing studies in barley and wheat in which the γ genome was modified for cloning of the GOI and removing genes from the β genome that induce viral infection symptoms. The mechanism involves the development of modified RNA transcripts that are transcribed separately *in vitro* and mixed in a 1: 1: 1 ratio in an appropriate buffer. Leaf tissue at the approximate stage are then infected and phenotypic symptoms analysed following an incubation period (Figure 3). The delivery of the modified virus results in the downregulation of transcript of the GOI through its natural homology dependent mechanism of degradation (Hein *et al.*, 2005; Scofield *et al.*, 2005; Unver and Budak, 2009; Ramegowda *et al*. 2014).

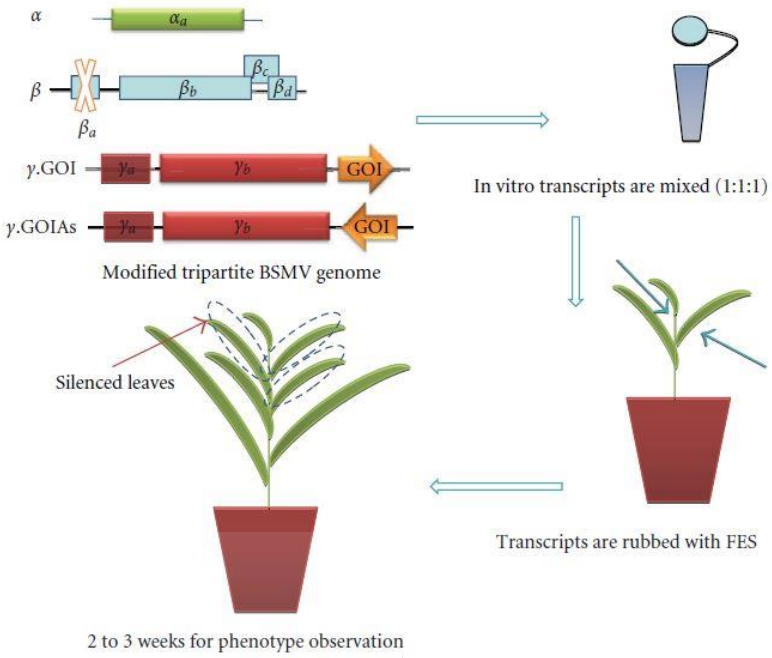

Figure 3: Schematic representation of BSMV-mediated VIGS in barley (Unver and Budak, 2009).

BSMV-VIGS in hexaploid wheat was successfully demonstrated for the first time in the study of plant-pathogen interaction. Scofield *et al*., (2005), showed that the knockdown of the wheat leaf rust resistance gene *Lr21* resulted in host conversion from a resistant to a susceptible interaction. The development of simultaneous gene silencing with a single BSMVVIGS vector provided the possibility to silence genes permitting visual identification of silenced tissue in combination with a second gene selected for experimental analysis of their function (Scofield *et al.*, 2005).

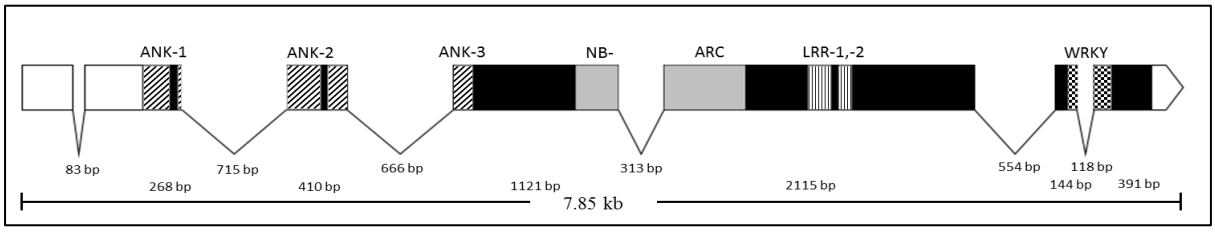

Figure 4: The predicted structure of *TaAdnr1* that encodes seven exons and six introns with the predicted untranslated regions (indicated as empty boxes), ankyrin, NB-ARC, LRR and WRKY domains indicated (Nicolis and Venter, 2018).

Several studies have used a VIGS approach to elucidate gene function in the wheat-*D. noxia* interaction. The first study to report the successful use of BSMV-mediated VIGS to investigate plant resistance genes involved in the wheat-*D. noxia* interaction was performed by investigating two differentially up-regulated candidate genes *WRKY53* and *PAL*. Their roles were investigated in resistant Gamtoos near-isogenic wheat line by knocking down their transcription using VIGS (van Eck *et al.*, 2010). A recent study on BSMV-mediated VIGS knockdown of *TaAGO5* in *D. noxia* susceptible wheat investigated the gene's role in the regulation of stress-related genes (Sibisi and Venter, 2020). The study on VIGS-mediated knockdown of *TaAdnr1*, an integrated domain NLR containing a C-terminal WRKY and additional terminal ankyrin repeats (Figure 4), indicated that it imparts possible effector recognition in the wheat-*D. noxia* interaction (Nicolis and Venter, 2018). Therefore, the use of VIGS has proven to be a useful technique for assessing the function of candidate genes in functional genomics for *D. noxia*-wheat interactions.

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2.9 Conclusion

The mechanism of the antibiotic resistance response in *Dn1* and *Dn7* resistance genes against *D. noxia* in wheat is not fully understood. The unique domain architecture of *TaAdnr1* and the ability to elicit a resistance response upon recognition of *D. noxia* feeding, motivated investigation of its role during the wheat-*D. noxia* interaction based on four of the known South African *D. noxia* biotypes. The fifth biotype was not known when the study was conducted. We tested this theory under the null hypothesis that the multidomain containing NLR-ID *TaAdnr1* does not play a role during the ETI in wheat carrying the *Dn7* resistance gene against *D. noxia.*

Chapter 3

Materials and Methods

3.1 Analysis of the full-length *TaAdnr1* sequence in *Triticum aestivum* L.

3.1.1 Plant Material

All the experiments were conducted on near-isogenic lines (NILs) of hexaploid wheat (*T. aestivum*) Gamtoos (*D. noxia* susceptible, *Dn0* containing the non-translocated *1RS/1BL* chromosome) and Gamtoos-R (*D. noxia* resistant, containing the *Dn7 S. cereale*, gene accession 94M370, that was translocated onto the *1RS/1BL* chromosome of the susceptible Gamtoos cultivar) (Marais *et al*., 1994; Anderson *et al*., 2003). These cultivars were obtained from the Agricultural Research Council-Small Grain (ARC-SG, Bethlehem, South Africa).

For the four VIGS experiments performed for each *D. noxia* biotype (RWASA1-4), Gamtoos (GS) and Gamtoos-R (GR) cultivars were used. In each of the experiments, 20 seeds were sown in four pots and a total of 15 plants were selected and treated as independent biological repeats, (n = 15) and used per treatment at their second-leaf stage (Zadoks stage 12) (Zadoks and Board, 1999).

3.1.2 RWA biotype maintenance and infestation

The four *D. noxia* South African biotypes (RWASA1-4) were obtained from the ARC-SG. Commercially available RWA susceptible PAN3434 wheat (Pannar Seeds) was sown in autoclaved potting soil in plastic pots and placed in separate insect cages (Bugdorm). The aphids used in the experiments were maintained by starting a colony through infesting one adult aphid on young leaves and allowed it to feed and produce more offspring. These were then maintained under controlled conditions in the greenhouse at 20 °C with 12-hour photoperiods and watered three times a week before they were utilised. For determination of the full-length of *TaAdnr1* gene, plants were mass infested with a total of 50 adult RWASA1 biotype at the two-leaf stage. After twenty-four hours, the third leaf was harvested into liquid nitrogen cooled 50 ml Falcon tubes using sterile scissors following removal of aphids on the leaf with a small paintbrush. The harvested plant material was then stored at -80 °C.

3.1.3 Analysis of the full-length *TaAdnr1* sequence

The full length of *TaAdnr1* was PCR amplified from the Gamtoos and Gamtoos-R NILs using the produced complementary DNA and cloned into a pCR2.1-TOPO plasmid vector

(ThermoFisher) for analysis of the sequence. TRIAE_CS42_5AL_TGACv1_374266_AA1195550.1 (November 2014 *T. aestivum* Ensemble Plants release; *TaAdnr1*) (Nicolis and Venter, 2018) was identified as an NLR-ID with conserved multidomain architecture and unique non-conserved additional domains similar to NLR-ID decoys that have effector recognition. The unique domain architecture of *TaAdnr1* and the ability to recognise RWA feeding possibly through effector recognition prompted this study to report its role during the *D. noxia*-wheat interaction.

3.1.4 RNA extraction and cDNA synthesis

The plant RNA extractions were all performed using the TRIzol reagent (ThermoFisher) and followed proper microbiological aseptic techniques. For each extraction, approximately 100 mg plant material was ground with sterile mortars and pestles in liquid nitrogen. Once the tissue sample was finely ground, it was transferred into Eppendorf tubes containing 1 ml of TRIzol reagent and mixed by vortexing. The sample solutions were incubated for 5 minutes at room temperature and 0.2 volume of chloroform was added. The samples were centrifuged for 15 minutes at 12 000 x g at 4 °C following incubation for 3 minutes at room temperature. The aqueous phase containing the RNA was transferred into Eppendorf tubes of 1.5 ml by pipetting it out at an angle. Isopropanol of a 0.5 volume was added to the RNA-containing aqueous solution and the samples were incubated at room temperature for 10 minutes. Following centrifugation at 12 000 x g for 10 minutes at 4 °C, the supernatant was drawn out with a micropipette. The resulting pellet was resuspended in 1 ml of 75 % ethanol (w/v), vortexed briefly and then centrifuged at 7 500 x g for 5 minutes at 4 °C. The RNA pellet was allowed to air dry for approximately 10 minutes following removal of the supernatant. The resulting RNA pellet was resuspended in 30 µl of water (nuclease-free), mixed through pipetting and then incubated at approximately 50-60 °C for 15 minutes in a water bath.

The extracted RNA concentrations were determined by diluting 2.5 µl of the extracted RNA into 47.5 µl water (nuclease-free) for each sample to a total volume of 50 µl. The absorbance was measured at 260 nm and 280 nm with a SmartSpec plus spectrophotometer (BioRad). An optical density (A_{260}) of 1 corresponded to approximately 40 µg/ml of RNA and the sample purity was determined by using the A_{260}/A_{280} ratio. The integrity of the RNA was determined on a 1 % (w/v) agarose gel (Whitesci) prepared using the 1 X TAE (40 mM Tris-acetate pH

8.0, 1 mM ethylenediaminetetraacetic acid (EDTA)) running buffer. A volume of 5 µl RNA was mixed with 2 µl of 5X DNA loading buffer blue (Bioline) and resolved on the agarose gel at 2 V cm^{-1}. The ethidium bromide-stained gel was visualised with a Spectroline ultraviolet transilluminator.

The RNA was reverse transcribed into cDNA using SuperScript IV (SSIV, ThermoFisher) following the instructions by the manufacturer. A high amount (5 µg) of starting RNA was utilised in the cDNA synthesisThe RNA primer annealing reaction was performed in PCR tubes of 0.2 ml using 2 µM of the gene-specific reverse primer from primer set 1 (Table 1), 2 mM final concentration of dNTPs, and water (nuclease-free) to a final volume of 13 µl. The above components of the reaction were gently mixed by flicking the tube and briefly centrifuged. The RNA-primer mix was heated for 5 minutes at 65 °C and incubated on ice for 1 minute. The reverse transcription mix consisting of 10 U of SuperScript IV Reverse Transcriptase, 1 µl of 100 mM DTT, 1 µl RNaseOUT Recombinant RNase Inhibitor (Invitrogen) and 4 µl of the 5X SSIV Buffer was prepared in a separate PCR tube of 0.2 ml volume. The contents were mixed, centrifuged briefly and aliquoted to the prepared RNA template tubes. This was mixed and incubated for 10 minutes at 55 °C. Finally, inactivation of the reaction was performed by incubating for 10 minutes at 80 °C and the produced cDNA was stored at -80 °C until required.

3.1.5 Determination of the full length of *TaAdnr1*

Amplification of *TaAdnr1* using PCR was performed with 1X Phusion High Fidelity Master Mix (0.5 U polymerase, 1.5 mM $MgCl_2$, and 0.2 mM of each dNTP) (NEB), 300 nM of each primer (primer set 1, Table 1), 5 µl of the produced cDNA template (1:19 dilution of the synthesized cDNA reaction) and water (nuclease-free) to a 25 µl final volume. The above reaction was assembled on ice adding 0.5 U of Phusion DNA Polymerase last, mixed gently and centrifuged briefly. Thereafter, it was transferred to a pre-heated thermocycler to the initial denaturation temperature of 98 °C. The PCR amplification reaction was performed according to optimised reaction conditions by Nicolis and Venter (2018), which constituted an initial step of denaturation at 98 °C for 1 minute and then followed by 30 cycles comprising of a step of denaturation at 98 °C for 20 seconds, 63 °C for 15 seconds and 72 °C for 2 minutes and 30 seconds. A final step of elongation consisted of a 72 °C incubation for 5 minutes. The PCR product was resolved on a 1 % (w/v) agarose gel as described.

Following confirmation of amplification, the remaining 20 µl blunt-end reaction was purified using the QIAquick gel extraction kit, (QIAGEN), following instructions by the manufacturer. The DNA fragment from the agarose gel was excised with a sterile scalpel. The excised gel was placed inside a 1.5 ml microcentrifuge tube and weighed. Thereafter, three volumes of QG buffer were added to one volume of the excised gel. The sample was incubated for 10 minutes at 50 °C with brief vortexing every 2 minutes. After the gel slice dissolved completely, one gel volume of isopropanol was added to the sample and mixed. The mixture was applied to a QIAquick column fitted in a collection tube and centrifuged at 17 900 x g for 1 minute. The collected flow-through in the collection tube was thrown away and the QIAquick column was returned in this tube. The column was washed sequentially using 0.5 ml of the QG buffer and 0.75 ml PE buffer with a 1-minute centrifugation step after each buffer addition. The resulting flow-through in the collection tube was discarded and the QIAquick column was dried through a 17 900 x g centrifugation for an additional 1 minute. The QIAquick column-bound DNA was eluted into a clean 1.5 ml microcentrifuge tube by adding 30 µl of EB Buffer to the centre of the column's membrane. The sample was incubated for 1 minute and then centrifuged for 1 minute. The purified DNA product was analysed on a 1% (w/v) agarose gel to ensure that minimal loss occurred.

Phusion is a high-fidelity polymerase and therefore does not add non-templated adenosine nucleotides to the ends of PCR amplicons, thus the amplicons were A-tailed for cloning. Seven microlitres of the cleaned amplified fragment were combined with 1 µl of 10X DreamTaq buffer (20 mM $MgCl_2$), 0.2 mM dATP, 5 U DreamTaq DNA Polymerase (ThermoFisher), and water (nuclease-free) up to a final volume of 10 µl. Following mixing and brief centrifugation, the reaction was incubated at 70 °C for 30 minutes.

The purified PCR fragments from Gamtoos were A-tailed and ligated into the pGEM-T Easy plasmid vector (Promega) following the instructions from the manufacturer. The ligation reaction was performed by ligating 2 µl of the A-tailed PCR product to 50 ng PGEM-T easy. This reaction consisted of 5 µl of the 2X Rapid ligation buffer (60 mM Tris-HCl pH 7.8, 20 mM $MgCl_2$, 20 mM DTT, 2 mM ATP, 10 % polyethylene glycol (MW8000, ACS Grade) and 3 U of T4 DNA ligase in a final volume of 10 µl. The ligation reaction was incubated overnight at 4 °C and subsequently cloned into competent *E. coli* cells. PCR fragments from Gamtoos-R

were difficult to clone using the above-mentioned kit possibly due to the large fragment size of *TaAdnr1* and numerous failed optimisation attempts. Therefore, the TOPO® TA Cloning® Kit (Invitrogen) was selected due to its ability to clone large PCR fragments efficiently. The PCR products from Gamtoos-R were A-tailed and ligated into pCR2.1-TOPO plasmid vector and following the manufacturer's instructions. The ligation reaction was set up by ligating 4 µl of the A-tailed PCR product to 10 ng pC2.1-TOPO vector (50% glycerol v/v, 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM DTT, 0.1% v/v Triton X-100, 100 µg ml^{-1} bovine serum albumin, phenol red), in a reaction containing 1 microlitre of salt solution (1.2 M NaCl and 0.06 M $MgCl_2$) and nuclease-free water to a final volume of 6 µl. The ligation reaction was incubated at room temperature for 2 hours followed by cloning into competent *E. coli* cells.

The ligation reactions were transformed into high-efficiency competent *E. coli* JM109 (Promega) cells by following the manufacturer's instructions. Four Luria-Bertani (LB) plates (1.5 % w/v agar (Merck) containing 1 % w/v tryptone (Oxoid), 0.5 % w/v yeast extract (Merck), 1 % NaCl w/v (Sigma), pH to 7.0), 100 µg ml^{-1} ampicillin (Sigma), 0.5 mM isopropyl β-d-1-thiogalactopyranoside (IPTG, Bioline), and 80 µg ml^{-1} of 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal, Bioline) were prepared and equilibrated to room temperature. Two microlitres of each ligation reaction was added to a sterile microcentrifuge tube that was preplaced on ice. The frozen JM109 cells were thawed in an ice bath, gently mixed and 50 µl of the cells were transferred into each prepared ligation reaction. This was mixed gently by flicking the tubes and incubated on ice for 20 minutes. Following incubation, the cells were heat-shocked in a water bath at 42 °C for 45 seconds and immediately returned to ice for 2 minutes. Nine hundred and fifty microlitres of room-temperature SOC medium (2 % w/v tryptone, 0.5 % w/v yeast extract, 10 mM $NaCl_2$, 2.5 mM KCl, 10 mM $MgCl_2$, 2 M filter-sterilised glucose) (Promega) was added to the cells transformed with the ligation reactions. The cells were incubated on an orbital shaker for 1.5 hours at 37 °C and 150 rpm. Following incubation, the room temperature-equilibrated plates containing LB/ampicillin/IPTG/X-Gal were inoculated by pipetting 100 µl of each transformation reaction onto the plates in a laminar flow cabinet. The plates were then incubated upside-down overnight (approximately 24 hours) at 37 °C. Following overnight incubation, the plates were incubated at 4 °C for 2 hours to facilitate the development of the blue colour.

Insert-containing colonies were identified through alpha complementation and screened using colony PCR. The plates containing the selected white colonies were incubated at 37 °C for 1 hour to facilitate the growth of the cells. Following proper sterile techniques in a laminar flow cabinet, all individual white colonies were carefully picked by gently touching the colony using a sterile toothpick and rub-inoculated on the bottom of each corresponding labelled PCR tube. Colony PCR was performed in a reaction containing 1 µl 10X DreamTaq buffer, 0.3 µM M13 forward primer (5'- GTAAAACGACGGCCAG - 3'), 0.3 µM M13 Reverse primer (5'- CAGGAAACAGCTATGAC - 3'), 0.2 mM of each dNTP and 0.2 U DreamTaq DNA polymerase, to a final volume of 10 µl. The reaction cycles of the colony PCR consisted of an initial denaturation step of 95 °C for 3 minutes, followed by 25 cycles consisting of a denaturation step at 95 °C for 30 seconds, an annealing step at 50 °C for 30 seconds and an elongation step at 72 °C for 4 minutes and 30 seconds. The final elongation step of the colony PCR was 72 °C for 10 minutes. Successful amplicons were evaluated on a 1 % (w/v) agarose gel as described previously.

The original positive colonies were streaked out on LB plates containing 50 µg ml^{-1} ampicillin and incubated overnight. After incubation, a single colony was isolated and inoculated into 3 ml LB liquid culture containing 50 µg ml^{-1} ampicillin. The liquid cultures were incubated for 14 hours at 37 °C with shaking at 150 rpm. Glycerol to a final volume of 15 % (v/v) was added to the liquid cultures containing the positive clones and stored for long term at -80 °C. The rest of the liquid cultures were used to isolate the plasmids from the cells.

The plasmids were isolated using the Zyppy Plasmid Miniprep Kit (Zymo Research) following the manufacturer's instructions. The bacterial culture was pelleted through centrifugation and the supernatant discarded. The resulting bacterial cell pellet was completely resuspended with 600 µl nuclease-free water and the cells were lysed by addition of 100 µl 7X Lysis buffer and mixed by inversion. After complete lysis, 350 µl cold Neutralisation buffer was added and the solution was mixed thoroughly by inversion. Additionally, the solution was further inverted three times for complete neutralisation. Following centrifugation at 16 000 x g for 4 minutes, 900 µl of the supernatant was transferred into a Zymo-Spin IIN column containing a collection tube, centrifuged and the flow-through was discarded. Endo-Wash buffer (200 µl) was added to the column and the sample was centrifuged for 30 seconds, where after Zyppy Wash buffer

(400 µl) was added to the column and the sample was centrifuged for 1 minute. Following centrifugation, the column was transferred into a clean 1.5 ml microcentrifuge tube and 30 µl of Zyppy Elution buffer was added directly to the column matrix and incubated for 1 minute at room temperature. Finally, the sample was centrifuged for 30 seconds to elute the plasmid DNA. The plasmid DNA concentration was determined by diluting 2.5 µl of the isolated plasmid DNA into 47.5 µl of water to a total volume of 50 µl. The absorbance was measured at 260 nm and 280 nm with a SmartSpec plus spectrophotometer. The optical density (A_{260}) of 1 corresponded to approximately 50 µg ml^{-1} and the sample purity was determined by using the A_{260}/A_{280} ratio.

Following purification of the plasmids containing the cloned *TaAdnr1* full-length transcript from Gamtoos, sequencing was performed at the Inqaba Biotec sequencing facility on an ABI 3500XL Genetic Analyser (Applied Biosystems) with the initial use of T7 (5'-TAATACGACTCACTATAGGG - 3') and Sp6 (5'- ATTTAGGTGACACTATAG - 3') primers. The purified plasmid from Gamtoos-R containing the cloned *TaAdnr1* full-length transcript was sequenced using the M13 forward and reverse primers. To produce sequence reads that span the full length of the gene, primer sets 3, 4, 5 and 6 were used for both Gamtoos and Gamtoos-R (Table 1).

The sequence reads obtained for both NILs were analysed with VecScreen (https://www.ncbi.nlm.nih.gov/tools/vecscreen/) to identify and remove nucleic acid segments that may be of vector origin. BioEdit (*version 7.0.5*) was used to analyse and manually edit regions of uncertainty or ambiguity by replacing misread nucleotide bases on sequences and truncating sequence regions that were of low quality. The generated sequences were then aligned using Clustal Omega (http://www.clustal.org/omega/) with TRIAE_CS42_5AL_TGACv1_374266_AA1195550.1 (November 2014 *T. aestivum* Ensemble Plants release) to identify the presence of polymorphisms.

3.2 RT-qPCR expression analysis of *TaAdnr1*

Regulation of *TaAdnr1* was compared with the internal reference genes and was analysed using RT-qPCR at four time points (0, 6, 12 and 24 hours post infestation (hpi)) in both wheat NILs (Gamtoos and Gamtoos-R). These time points were selected based on results from a previous

study and the *GAPDH* and *18S* genes were chosen as reference genes (Table 1) as they are not regulated during the interaction (Nicolis and Venter 2018).

For *TaAdnr1* expression analysis, cDNA was synthesised from 2 µg total RNA as described before using 2 µM oligo $d(T)_{20}$ primer (5' - d PO_4 [(T)20] - 3', Invitrogen). The qPCR reactions were performed in triplicate on a CFX Connect (BioRad) using Brilliant III Ultra-fast SYBR Green qPCR Master Mix (Agilent) according to the manufacturer's protocols. The reaction consisted of 2 µl of template cDNA (1:19 dilution), 0.5 µM forward and reverse primers, primer set 3, 5 µl of 2X SYBR Green qPCR master mix and nuclease-free water to a final volume of 10 µl. The reaction conditions for the qPCR consisted of an initial denaturation step at 95 °C for 2 minutes, subsequently 40 cycles consisting of a denaturation step at 95 °C for 10 seconds, annealing step at 54 °C for 10 seconds and an elongation step at 72 °C for 15 seconds. The melt curve analysis consisted of a denaturation step at 95 °C, followed by a cooling step at 65 °C with an increment of 0.5 °C per 2 seconds. The reaction conditions for the reference genes *GAPDH* and *18S* were similar as outlined above for *TaAdnr1* expression analysis except for the specific annealing temperature of 52 °C for both *GAPDH* and *18S*.

The resulting cycle quantification (Cq) values were imported into the REST 2009 Software (v2.0.13). The reference genes were used to normalise the target gene expression, resulting in calibrated normalised relative quantities (CNRQ) of the expressed target genes for each biological replicate. The CNRQ values were exported into Microsoft® Excel 2010 (Microsoft, Wa) and SPSS software ($p \leq 0.05$) was used for univariate general linear model analysis using the post hoc test for multiple comparisons for the observed means assuming Tukey equal variance. This was performed to identify significant differential regulation of the combined biological replicates for the above-mentioned time points amongst the susceptible and resistant cultivars, as well as between the susceptible and resistant cultivars.

3.3 Virus-Induced gene silencing of *TaAdnr1*

Gene silencing treatments of the target gene were conducted on the RWA resistant Gamtoos-R plants and were infested with *D. noxia*. The control gene silencing treatments that did not consist of the insert target gene were conducted on the Gamtoos-R plants and were infested with *D. noxia*. Control gene silencing treatments for the *phytoene desaturase* (*PDS*) gene was conducted on the Gamtoos plants and were not infested with *D. noxia*. Control Gamtoos and

Gamtoos-R plants in which gene silencing treatments were not conducted were included, in which one group (Gamtoos and Gamtoos-R) was infested with *D. noxia* and the other (Gamtoos and Gamtoos-R) was not infested.

3.3.1 Gene silencing vectors

The region downstream of the WRKY domain was chosen for targeted gene silencing of *TaAdnr1* based on the uniqueness of the sequence (Nicolis and Venter, 2018). Ensembl Plants was used to BLAST™ the sequence of *TaAdnr1* against the *T. aestivum* genome to ensure the uniqueness of the region. Further analysis of this region using wheat-specific and cross-specific BLAST™, showed that non-target transcripts were not available for potential silencing.

The BSMV-mediated VIGS system was employed for this study (Scofield *et al*., 2005). The system comprised of a positive sense single-strand RNA virus with a tripartite genome (α, β, and γ RNAs) that can infect wheat. The three DNA plasmids carrying the full-length cDNA clone of BSMV RNAs (BSMVα, BSMVβ, BSMVγ) were used for the study. The gene silencing fragment of 270 bp was amplified by PCR and inserted into the pSL038-1 BSMV plasmid in the reverse orientation. The fragment was previously cloned by Nicolis and Venter (2018) and used for this study.

3.3.2 Preparation of RNA transcripts from BSMV vectors.

The BSMV-mediated VIGS experiments consisted of the α, β, and γ *in vitro* transcripts of BSMV RNAs. One *in vitro* transcription reaction of each of the α and β RNAs was used for all the VIGS experiments and one transcription reaction of the recombinant γ pSL038-1- *TaAdnr1* ($BSMV_{TaAdnr1}$) was used. The viral vector infection control reaction consisted of γ pSL038-10-empty vector ($BSMV_0$), with no plant gene inserted, to ascertain the phenotypic effects of BSMV infection. The positive control for successful production of transcripts capable of silencing was through the observance of a photobleaching phenotype upon silencing the *phytoene desaturase* (*PDS*) gene using the γ pSL039B-1- *PDS* ($BSMV_{PDS}$). This construct encompassed a 185 bp fragment from barley that targets the *PDS* gene.

The BSMVα, β and γ silencing plasmid vectors were purified from the bacterial cells and linearised using SpeI for plasmid BSMVβ and MluI for all the BSMVγ and α plasmids. To

inoculate 15 plants, 1 µg of plasmid DNA was linearised for each of the three BSMV RNAs. The restriction enzyme digest with SpeI consisted of 1 µg of BSMVβ plasmid DNA, 10 U of SpeI restriction enzyme (NEB), 2 µl of 10X CutSmart buffer and nuclease-free water to a final volume of 20 µl. Digestion with MluI consisted of 1 µg of BSMV plasmid DNA, 10 U of MluI restriction Enzyme (NEB), 2 µl of 10X NE buffer 3.1 and nuclease-free water to a final volume of 20 µl. The restriction enzyme digestion reaction was incubated for 2 hours at 37 °C. Linearisation was evaluated on a 1% (w/v) agarose gel using 1 µl of each of the digests combined with 1 µl of 5X DNA loading buffer and nuclease-free water to a 20 µl final volume. Following confirmation of complete digestion, the reaction was inactivated at 65 °C for 20 minutes.

The *in vitro* synthesis of capped RNA was performed using the T7 mMESSAGE mMACHINE kit (Life Technologies) following the manufacturer's protocol. The transcription reaction was assembled at room temperature. One microgram linearised plasmid template was added to a final volume of 20 µl nuclease free water, 10 µl of 2X NTP/CAP and 2 µl of 10X reaction buffer was added, respectively. Lastly, 2 µl of the enzyme mix was added and the reaction mixed through pipetting up and down gently followed by brief centrifugation. The reaction was incubated for 2 hours at 37 °C. The *in vitro* transcription reaction was verified by running a 1 % (w/v) agarose gel with 1 µl of each transcript diluted with 9 µl Nuclease-free water and 10 µl gel loading buffer provided in the T7 mMESSAGE mMACHINE kit.

3.3.3 Plant inoculation with prepared viral transcripts.

Fifteen wheat plants for each control and gene silencing construct were inoculated by equal mixing of the three *in vitro* transcription reactions (one for each BSMV genomic RNA) and each plant was considered to be a biological repetition. The inoculation buffer consisted of 1 µl of the α, β, γ (with the γ construct varying between the silencing constructs and controls) BSMV *in vitro* transcribed RNA added to 22.5 µl of the FES buffer (0.1 M glycine, 0.06 M K_2HPO_4, 1% w/v $Na_4P_2O_7$, 1 % w/v bentonite as abrasive, 1 % w/v celite, pH 8.5). Care was taken to ensure that the inoculation buffer was thoroughly mixed before each inoculation to keep the bentonite in suspension. This mixture was used to inoculate the wheat plants at the second leaf stage. Inoculation was performed by pipetting 25.5 µl of the inoculation mixture

between the index finger and thumb of a gloved hand. The second leaf was pinched between the two fingers and slid towards the tip of the leaf, whilst the leaf was stabilised at the base with the other gloved hand. This motion was repeated two or more times.

3.3.4 Aphid reproduction measurements

The reproduction of aphids can be used as a measure of their absolute fitness and the antibiosis effect contributed by *Dn7* that negatively impacts the reproductive ability of the aphids. Aphid reproductive measurements were determined as previously published (Thordal-Christensen *et al.* 1997; van Eck *et al.* 2010; Daudi and O'Brien 2012). A total of 15 plants (n=15) were used per treatment (controls or silencing construct) with each plant considered as a biological repeat. Each treatment included infestation by one of the four RWASA biotypes, together with all the necessary controls. Seven days after BSMV inoculation, one adult apterous aphid of each biotype (SA1-4) per experiment was caged inside a clip cage on the emerged third leaf of each of the BSMV-infected and control plants. Subsequently, each plant was mass infested with a total of 50 adult apterous aphids on the emergent third leaf. The following day, each adult aphid from the clip cage was removed using a small paintbrush and one newborn nymph was left in each cage. This nymph was considered as the foundress and this was assessed as the initial date of birth. The birth of new aphid nymphs from the foundress was monitored daily on clip cages by removing the newly born aphids and leaving behind the foundress in the cage. The aphid measurements were taken for 14 days from foundress birth. The mean total number of nymphs born was calculated as a measure of fertility. The intrinsic rate of increase (r_m) of each foundress was calculated using the formula $r_m = (0.738 \times \ln (Md))/d$, where Md is the number of nymphs produced in a period equal to the pre-nymphipositional period (d) (Wyatt and White, 1977; Randolph, Merrill and Peairs, 2008).

3.3.5 Phenotypical observation of gene knockdown

Viral infection symptoms were monitored until the end of the experiments on the third leaf of each plant following the death of the infected second leaf after BSMV inoculation. Classical aphid infestation symptoms and *Dn7*-mediated development of hypersensitive lesions (resistance response) were also monitored at the end of the experiment. Symptoms of

photobleaching resulting from silencing the *PDS* gene and aphid infestation symptoms resulting from susceptibility or resistance were recorded at the end of the experiment.

The plant biomass was assessed for the effects of silencing *TaAdnr1* and aphid feeding on plant growth for both the above-ground and below-ground as previously described (Unver and Budak, 2009). At the end of the VIGS experiment, the shoots and roots of all the remaining plants were harvested. The aphids feeding on the remaining plants were removed by soaking in water containing dishwashing detergent. The shoots and roots of each plant were placed in separate and labelled brown envelopes. The plant samples were then oven-dried at approximately 37-43 °C for 48 hours and weighed to determine dry shoot and root biomass. The mean total weight from analysis of variance for both above and below ground dry plant biomass was analysed. The SPSS software ($p \leq 0.05$) was employed to identify significant differential plant biomass of the combined biological replicates for the VIGS treatments amongst the susceptible controls and resistant control plant cultivars, as well as between the susceptible and resistant control, and also the BSMV-treated plant cultivars.

3.3.6 Knockdown levels of *TaAdnr1* obtained through VIGS

The knockdown of *TaAdnr1* was confirmed using RT-qPCR, 11 days after viral inoculation. RNA extraction and cDNA synthesis reactions were performed as previously described. The RT-qPCR reactions were performed in triplicate on a CFX Connect with an annealing temperature of 52 °C for the *GAPDH* and *18S* reference genes and of 54 °C for primer set 3 (Table 1). The resulting cycle quantification (Cq) values were imported into the REST 2009 Software (v2.0.13) as described above. The SPSS software ($p \leq 0.05$) was employed to identify significant differential regulation of the combined biological replicates for the VIGS treatments amongst the susceptible controls and resistant control plant cultivars, as well as between the susceptible and resistant control, and also the BSMV-treated plant cultivars.

3.3.7 3,3'-diaminobenzidine (DAB) Staining

To test for the accumulation of hydrogen peroxide, 3,3'-diaminobenzidine (DAB) staining was performed at the end of the experiments and three biological replicates of wheat leaves at the fourth leaf stage were chosen at random and sampled from each treatment. Staining was

performed as previously described (Daudi and O'Brien 2012; Thordal-Christensen *et al.* 1997; van Eck *et al*. 2010). The harvested wheat leaves were immersed in 15 ml Falcon tubes containing the DAB staining solution (10 mM DAB pH 3.0, 200 mM Na_2HPO_4, 0.05 % v/v Tween 20), covered with foil and incubated overnight with shaking at 100 rpm on an orbital shaker. The following day, the DAB staining solution was replaced with bleaching solution (3:1:1, ethanol: acetic acid: glycerol) and incubated in boiling water for 20 minutes, where after the bleaching solution was replaced with fresh bleaching solution and incubated at room temperature for 30 min. Finally, the photographs of the samples were taken on a plain white background with uniform lighting under a stereomicroscope (Olympus SZX16, Model SZX2-ILLT).

Table 1: The primer sequences used for the full-length amplification and RT-qPCR expression analysis of *TaAdnr1* following generation of the first-strand cDNA. The primer sets 1,3,4,5,6 and 7 were used to produce sequence reads that span the full length of the gene and also to identify the presence of possible polymorphisms.

Primer	Primer sequence
Primer 1 F	5'- GAGCAAAGACAGCCCCTAGA- 3'
Primer 1 R	5'- AGCCACGATTCGATTTGAGC- 3'
M13 F (Primer 2)	5'- GTAAAACGACGGCCAG - 3'
M13 R	5'- CAGGAAACAGCTATGAC - 3'
Primer 3F	5'- GCCACGTCCACATGCTTCCTAG - 3'
Primer 3R	5'- GAGAGCGGAAGACCGTGCTATG - 3'
Primer 4	5'- AAGACTGGGCAATGTGGAGG - 3'
Primer 5 F	5'- TCCTGCACACTGCATCACATGG - 3'
Primer 5 R	5'- CACTCGCAGACAAGGTTCGTC- 3'
Primer 6	5'- AAGCGGATCACACAGGTGAG- 3'
Primer 7 F	5'- TAATACGACTCACTATAGGG - 3' (T7)
Primer 7 R	5'- ATTTAGGTGACACTATAG - 3' (Sp6)

3.3.8 References

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Chapter 4

Results

4.1 Full-length sequence analysis of *TaAdnr1* in *Triticum aestivum* L.

The full length *TaAdnr1* gene was amplified from both Gamtoos and Gamtoos-R following extraction of pure RNA and reverse transcription into cDNA. Figure 5 indicates the PCR amplicons generated for *TaAdnr1* from both Gamtoos and Gamtoos-R. The amplicons were at the correct size of approximately 4300 bp. These fragments were excised from the gel, purified and cloned into pGEM-T Easy (Gamtoos) and pTOPO (Gamtoos-R) for further analysis and long-term storage.

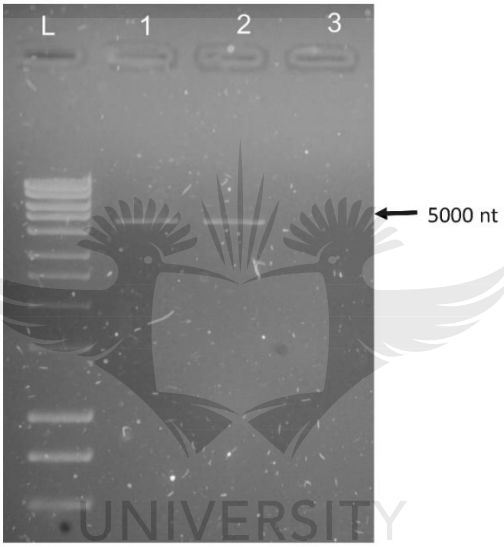

Figure 5: The full-length PCR amplification of *TaAdnr1* from both Gamtoos and Gamtoos-R wheat plants, resolved on a 1 % agarose gel. The lanes 1 and 2 represent the PCR product from Gamtoos and Gamtoos-R that were subsequently selected (< 5 000 bp) for determining the full-length sequence of *TaAdnr1*. Lane L represents HyperLadder 1kb marker and lane 3 contains the no template control (NTC).

The purified *TaAdnr1* PCR fragments from Gamtoos and Gamtoos-R were A-tailed and ligated into the pGEM-T Easy and pCR2.1-TOPO plasmid vectors, respectively. The ligated plasmids were transformed into high-efficiency competent *E. coli* cells and insert-containing colonies were identified through alpha complementation and screening using colony PCR. Figure 6 indicates the colony PCR amplicons with clear and well-defined single bands at the expected size of approximately 4 300-4 400 bp for both Gamtoos (in A) and Gamtoos-R (in B). The

plasmids of the colonies containing positive clones were isolated and sequenced at the Inqaba Biotec sequencing facility for further analysis.

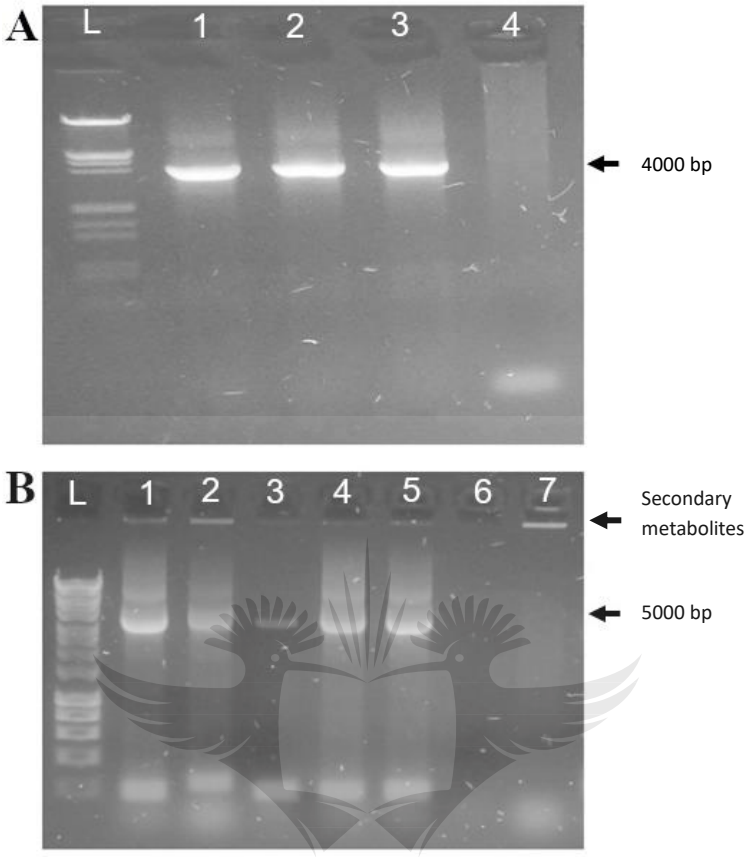

Figure 6: The colony PCR amplification of insert-containing colonies identified through alpha complementation. These were resolved on a 1 % agarose gel. The insert-containing colonies that produced amplicons < 4 361 bp were selected for sequencing for Gamtoos (**A**) and those that produced amplicons < 5 000 bp were selected for Gamtoos-R (**B**). Lane L represents λ EcoRI + HindIII DNA marker (in A) and HyperLadder 1kb marker (in B). Lane 4 (in A) and 7 (in B) represent the NTC. The bands situated above and around the wells of the gel represent possible secondary metabolites.

Following sequencing of the target region on the plasmids, the sequence reads obtained for both NILs were analysed with VecScreen to identify and remove nucleic acid segments that may be of vector origin. Furthermore, the regions of uncertainty or ambiguity were manually edited using BioEdit by replacing misread nucleotide bases on sequences and truncating sequence regions that were of low quality. The size of both the Gamtoos and Gamtoos-R amplified from cDNA for *TaAdnr1* were determined to be 4 449 bp and were aligned to the

Chinese Spring (CS) from the wheat genome (Figure 7). This indicated that no polymorphisms existed between the Gamtoos derived *TaAdnr1* and the one from Chinese Spring.

```
CS TaAdnr1 Exons       ATGGGTGACAGTGCGCTCCACCTTGCAGCAAGGGCCGGAAATGTTGCCCTTGTGCAGAAG   60
Gamtoos_R_TaAdnr1      ATGGGTGACAGTGCGCTCCACCTTGCAGCAAGGGCCGGAAATGTTGCCCTTGTGCAGAAG   60
Gamtoos_S_TaAdnr1      ATGGGTGACAGTGCGCTCCACCTTGCAGCAAGGGCCGGAAATGTTGCCCTTGTGCAGAAG   60
                       ************************************************************

CS TaAdnr1 Exons       ATCTTTGCGGACTGCGATCCGGAGCTGGTCGCGGAATTGACTGACCATCAGAACCAGGAC   120
Gamtoos_R_TaAdnr1      ATCTTTGCGGACTGCGATCCGGAGCTGGTCGCGGAATTGACTGACCATCAGAACCAGGAC   120
Gamtoos_S_TaAdnr1      ATCTTTGCGGACTGCGATCCGGAGCTGGTCGCGGAATTGACTGACCATCAGAACCAGGAC   120
                       ************************************************************

CS_TaAdnr1_Exons       GGTGAGACAGCGCTGTATGTTTCCGCGGAGATGGGGCATGTCGAGGTTGTGTGTGAAATT   180
Gamtoos_R_TaAdnr1      GGTGAGACAGCGCTGTATGTTTCCGCGGAGATGGGGCATGTCGAGGTTGTGTGTGAAATT   180
Gamtoos_S_TaAdnr1      GGTGAGACAGCGCTGTATGTTTCCGCGGAGATGGGGCATGTCGAGGTTGTGTGTGAAATT   180
                       ************************************************************

CS_TaAdnr1_Exons       CTGAAGGTTTGTGATCTGCATTCAGCATTTCTCAAGGCACACAACAGCTTTGATGCATTC   240
Gamtoos_R_TaAdnr1      CTGAAGGTTTGTGATCTGCATTCAGCATTTCTCAAGGCACACAACAGCTTTGATGCATTC   240
Gamtoos_S_TaAdnr1      CTGAAGGTTTGTGATCTGCATTCAGCATTTCTCAAGGCACACAACAGCTTTGATGCATTC   240
                       ************************************************************

CS_TaAdnr1_Exons       CATATTGCAGCAAAGCAGGGCCATCTAGTTGTTTTGCAGGAGCTACTGAAGGCTTTTCCT   300
Gamtoos_R_TaAdnr1      CATATTGCAGCAAAGCAGGGCCATCTAGTTGTTTTGCAGGAGCTACTGAAGGCTTTTCCT   300
Gamtoos_S_TaAdnr1      CATATTGCAGCAAAGCAGGGCCATCTAGTTGTTTTGCAGGAGCTACTGAAGGCTTTTCCT   300
                       ************************************************************

CS_TaAdnr1_Exons       GCATTAGCTATGACAACAAATTCAGTAAATGCCACAGCTTTATACACTGCCGCACTTCAC   360
Gamtoos_R_TaAdnr1      GCATTAGCTATGACAACAAATTCAGTAAATGCCACAGCTTTATACACTGCCGCACTTCAC   360
Gamtoos_S_TaAdnr1      GCATTAGCTATGACAACAAATTCAGTAAATGCCACAGCTTTATACACTGCCGCACTTCAC   360
                       ************************************************************

CS_TaAdnr1_Exons       CGCCACATTGGTATTGTCAATCTTCTACTGGATACAGACCCAAGACTTGCCAGGATTGCA   420
Gamtoos_R_TaAdnr1      CGCCACATTGGTATTGTCAATCTTCTACTGGATACAGACCCAAGACTTGCCAGGATTGCA   420
Gamtoos_S_TaAdnr1      CGCCACATTGGTATTGTCAATCTTCTACTGGATACAGACCCAAGACTTGCCAGGATTGCA   420
                       ************************************************************

CS_TaAdnr1_Exons       AGAAATAATGGGAAGACAGCTCTGCATATAGCAGCAAGACTGGGCAATGTGGAGGTGGTA   480
Gamtoos_R_TaAdnr1      AGAAATAATGGGAAGACAGCTCTGCATATAGCAGCAAGACTGGGCAATGTGGAGGTGGTA   480
Gamtoos_S_TaAdnr1      AGAAATAATGGGAAGACAGCTCTGCATATAGCAGCAAGACTGGGCAATGTGGAGGTGGTA   480
                       ************************************************************

CS_TaAdnr1_Exons       GTGTTGTTGTTGAATAAAGATCCGGCGACTGTTTTCAGAATAGACAGGAAGGGACAAACA   540
Gamtoos_R_TaAdnr1      GTGTTGTTGTTGAATAAAGATCCGGCGACTGTTTTCAGAATAGACAGGAAGGGACAAACA   540
Gamtoos_S_TaAdnr1      GTGTTGTTGTTGAATAAAGATCCGGCGACTGTTTTCAGAATAGACAGGAAGGGACAAACA   540
                       ************************************************************

CS_TaAdnr1_Exons       GCAGTGCACATGGCTTCCAAAGGCCACAATGCTGAAATTCTGCTTGAGCTACTGAAGCCC   600
Gamtoos_R_TaAdnr1      GCAGTGCACATGGCTTCCAAAGGCCACAATGCTGAAATTCTGCTTGAGCTACTGAAGCCC   600
Gamtoos_S_TaAdnr1      GCAGTGCACATGGCTTCCAAAGGCCACAATGCTGAAATTCTGCTTGAGCTACTGAAGCCC   600
                       ************************************************************

CS_TaAdnr1_Exons       GATGTCTCAGTAATCCATTTGGAAGATAACAAGGGGGACAGGCCACTGCATGTTGCAACG   660
Gamtoos_R_TaAdnr1      GATGTCTCAGTAATCCATTTGGAAGATAACAAGGGGGACAGGCCACTGCATGTTGCAACG   660
Gamtoos_S_TaAdnr1      GATGTCTCAGTAATCCATTTGGAAGATAACAAGGGGGACAGGCCACTGCATGTTGCAACG   660
                       ************************************************************

CS TaAdnr1 Exons       CGGAAGGGAAAAACCATTATAGTTCAGACGCTAATATCCATTGAAGAGATTGATATCAAT   720
Gamtoos_R_TaAdnr1      CGGAAGGGAAAAACCATTATAGTTCAGACGCTAATATCCATTGAAGAGATTGATATCAAT   720
Gamtoos_S_TaAdnr1      CGGAAGGGAAAAACCATTATAGTTCAGACGCTAATATCCATTGAAGAGATTGATATCAAT   720
                       ************************************************************

CS_TaAdnr1_Exons       GCAATCAATGGAGCTGGAGAGACCGCTTTTGCCATTGCAGAGAAACTGGGTAATGAAGAG   780
Gamtoos_R_TaAdnr1      GCAATCAATGGAGCTGGAGAGACCGCTTTTGCCATTGCAGAGAAACTGGGTAATGAAGAG   780
Gamtoos_S_TaAdnr1      GCAATCAATGGAGCTGGAGAGACCGCTTTTGCCATTGCAGAGAAACTGGGTAATGAAGAG   780
                       ************************************************************

CS_TaAdnr1_Exons       CTTGTAAACATCCTGAGGGAGGCTGGTGGAGTAACCGCAGAAGAGCAAGTAAATCCTCCG   840
Gamtoos_R_TaAdnr1      CTTGTAAACATCCTGAGGGAGGCTGGTGGAGTAACCGCAGAAGAGCAAGTAAATCCTCCG   840
Gamtoos_S_TaAdnr1      CTTGTAAACATCCTGAGGGAGGCTGGTGGAGTAACCGCAGAAGAGCAAGTAAATCCTCCG   840
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CS_TaAdnr1_Exons       AAATCAATCAAGCGTTTTAAGCAAACACATGATGTCCAATCGCAGATCAAGCAAAAGCGT   900
Gamtoos_R_TaAdnr1      AAATCAATCAAGCGTTTTAAGCAAACACATGATGTCCAATCGCAGATCAAGCAAAAGCGT   900
Gamtoos_S_TaAdnr1      AAATCAATCAAGCGTTTTAAGCAAACACATGATGTCCAATCGCAGATCAAGCAAAAGCGT   900
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CS_TaAdnr1_Exons       CGGACAAATATGCATTTCCACACGATCAGGAAGAGTAGTCAAAAGCTCCACACTGAGGCT   960
Gamtoos_R_TaAdnr1      CGGACAAATATGCATTTCCACACGATCAGGAAGAGTAGTCAAAAGCTCCACACTGAGGCT   960
Gamtoos_S_TaAdnr1      CGGACAAATATGCATTTCCACACGATCAGGAAGAGTAGTCAAAAGCTCCACACTGAGGCT   960
                       ************************************************************

CS_TaAdnr1_Exons       CCAGTCTGCGCTTTGGCAGACGCCATGTTCAGACTTCCTGCAAAGCTTGATGAGCTACTG   1020
Gamtoos_R_TaAdnr1      CCAGTCTGCGCTTTGGCAGACGCCATGTTCAGACTTCCTGCAAAGCTTGATGAGCTACTG   1020
Gamtoos_S_TaAdnr1      CCAGTCTGCGCTTTGGCAGACGCCATGTTCAGACTTCCTGCAAAGCTTGATGAGCTACTG   1020
                       ************************************************************

CS_TaAdnr1_Exons       ATTAGCCACGTCCACATGCTTCCTAGGGGTGCGGAGGATGAGATACCTCTCATCAAGCAA   1080
Gamtoos_R_TaAdnr1      ATTAGCCACGTCCACATGCTTCCTAGGGGTGCGGAGGATGAGATACCTCTCATCAAGCAA   1080
Gamtoos_S_TaAdnr1      ATTAGCCACGTCCACATGCTTCCTAGGGGTGCGGAGGATGAGATACCTCTCATCAAGCAA   1080
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CS TaAdnr1 Exons       GATCTGGAAGAGATAATGGCCATTCTGCAGGAGCACGACCACCCAGGGAGAGCGGAAGAC   1140
Gamtoos_R_TaAdnr1      GATCTGGAAGAGATAATGGCCATTCTGCAGGAGCACGACCACCCAGGGAGAGCGGAAGAC   1140
Gamtoos_S_TaAdnr1      GATCTGGAAGAGATAATGGCCATTCTGCAGGAGCACGACCACCCAGGGAGAGCGGAAGAC   1140
                       ************************************************************

CS TaAdnr1 Exons       CGTGCTATGACGAGCAAGTGCCTGACCAAGGAGGTGCGCGAGCTGTCATACGACATGGAG   1200
Gamtoos_R_TaAdnr1      CGTGCTATGACGAGCAAGTGCCTGACCAAGGAGGTGCGCGAGCTGTCATACGACATGGAG   1200
Gamtoos_S_TaAdnr1      CGTGCTATGACGAGCAAGTGCCTGACCAAGGAGGTGCGCGAGCTGTCATACGACATGGAG   1200
                       ************************************************************

CS_TaAdnr1_Exons       GATAGCGTCGACCAGTACGTGCACGCCGTCGACACCAAGAGAAGGATTGTTCCTCGCCGT   1260
Gamtoos R TaAdnr1      GATAGCGTCGACCAGTACGTGCACGCCGTCGACACCAAGAGAAGGATTGTTCCTCGCCGT   1260
Gamtoos_S_TaAdnr1      GATAGCGTCGACCAGTACGTGCACGCCGTCGACACCAAGAGAAGGATTGTTCCTCGCCGT   1260
                       ************************************************************

CS_TaAdnr1_Exons       AAAAAGTACAAGATCACCTGTCGTAGGGGCAAGACCACTGCGCGGCTCCCGGAGAAGCTT   1320
Gamtoos_R_TaAdnr1      AAAAAGTACAAGATCACCTGTCGTAGGGGCAAGACCACTGCGCGGCTCCCGGAGAAGCTT   1320
Gamtoos_S_TaAdnr1      AAAAAGTACAAGATCACCTGTCGTAGGGGCAAGACCACTGCGCGGCTCCCGGAGAAGCTT   1320
                       ************************************************************

CS_TaAdnr1_Exons       AAGTGGCGTATATGGATGGCCAACAAGATCAGGGAGTTCAGTGTGCGCTCGCAAGAGGCG   1380
Gamtoos_R_TaAdnr1      AAGTGGCGTATATGGATGGCCAACAAGATCAGGGAGTTCAGTGTGCGCTCGCAAGAGGCG   1380
Gamtoos_S_TaAdnr1      AAGTGGCGTATATGGATGGCCAACAAGATCAGGGAGTTCAGTGTGCGCTCGCAAGAGGCG   1380
                       ************************************************************

CS_TaAdnr1_Exons       CTGCAGCGGTACAGCCTATTTAACCACCCTGGTGCTCATGGCATCAGCACGTCTGCTACT   1440
Gamtoos_R_TaAdnr1      CTGCAGCGGTACAGCCTATTTAACCACCCTGGTGCTCATGGCATCAGCACGTCTGCTACT   1440
Gamtoos_S_TaAdnr1      CTGCAGCGGTACAGCCTATTTAACCACCCTGGTGCTCATGGCATCAGCACGTCTGCTACT   1440
                       ************************************************************

CS_TaAdnr1_Exons       TCTACGAGACATGATGTGTGTTTTGGCTCTTGGTATCCCACACCGTGTGGGGAGCTTGTC   1500
Gamtoos_R_TaAdnr1      TCTACGAGACATGATGTGTGTTTTGGCTCTTGGTATCCCACACCGTGTGGGGAGCTTGTC   1500
Gamtoos_S_TaAdnr1      TCTACGAGACATGATGTGTGTTTTGGCTCTTGGTATCCCACACCGTGTGGGGAGCTTGTC   1500
                       ************************************************************

CS_TaAdnr1_Exons       GGTATAGATGGACATTTGAATACTCTTGAAGCGTGGTTGGGTAAGGATGGGGAGCAGCAG   1560
Gamtoos_R_TaAdnr1      GGTATAGATGGACATTTGAATACTCTTGAAGCGTGGTTGGGTAAGGATGGGGAGCAGCAG   1560
Gamtoos_S_TaAdnr1      GGTATAGATGGACATTTGAATACTCTTGAAGCGTGGTTGGGTAAGGATGGGGAGCAGCAG   1560
                       ************************************************************

CS_TaAdnr1_Exons       CTCAAGGTGGTATCTGTTGTTGGATCTGGAGGGGTTGGTAAGACCACACTTTCCAAAGAG   1620
Gamtoos_R_TaAdnr1      CTCAAGGTGGTATCTGTTGTTGGATCTGGAGGGGTTGGTAAGACCACACTTTCCAAAGAG   1620
Gamtoos_S_TaAdnr1      CTCAAGGTGGTATCTGTTGTTGGATCTGGAGGGGTTGGTAAGACCACACTTTCCAAAGAG   1620
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CS_TaAdnr1_Exons       CTGTACCGTAGAATCAGAGGGCAATTCGAGTGCCAGGCATTTGTGAGGACGTCCCGGAAG   1680
Gamtoos_R_TaAdnr1      CTGTACCGTAGAATCAGAGGGCAATTCGAGTGCCAGGCATTTGTGAGGACGTCCCGGAAG   1680
Gamtoos_S_TaAdnr1      CTGTACCGTAGAATCAGAGGGCAATTCGAGTGCCAGGCATTTGTGAGGACGTCCCGGAAG   1680
                       ************************************************************

CS_TaAdnr1_Exons       CCCGACATCAGGAGGCTTCTCATCAGCTTGCTCTCACAAGTCCGGCCACACCAAACCCCT   1740
Gamtoos_R_TaAdnr1      CCCGACATCAGGAGGCTTCTCATCAGCTTGCTCTCACAAGTCCGGCCACACCAAACCCCT   1740
Gamtoos_S_TaAdnr1      CCCGACATCAGGAGGCTTCTCATCAGCTTGCTCTCACAAGTCCGGCCACACCAAACCCCT   1740
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CS TaAdnr1 Exons       CACACTTGGAAATTGCATAGTCTAATTGCCGATATCAGGACACATCTCCACGATAAGAGG   1800
Gamtoos_R_TaAdnr1      CACACTTGGAAATTGCATAGTCTAATTGCCGATATCAGGACACATCTCCACGATAAGAGG   1800
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Gamtoos_S_TaAdnr1       CACACTTGGAAATTGCATAGTCTAATTGCCGATATCAGGACACATCTCCACGATAAGAGG   1800
                        ************************************************************

CS_TaAdnr1_Exons        TACTTGATCGTCATTGATGATGTATGGGCTACACAAACATGGGATATCATTAATCGTGCT   1860
Gamtoos_R_TaAdnr1       TACTTGATCGTCATTGATGATGTATGGGCTACACAAACATGGGATATCATTAATCGTGCT   1860
Gamtoos_S_TaAdnr1       TACTTGATCGTCATTGATGATGTATGGGCTACACAAACATGGGATATCATTAATCGTGCT   1860
                        ************************************************************

CS_TaAdnr1_Exons        TTGCCGGCTGGTAATCTTTGCAGTAGAATTCTAATAACGACAGAAGTCGAAGATGTAGCT   1920
Gamtoos_R_TaAdnr1       TTGCCGGCTGGTAATCTTTGCAGTAGAATTCTAATAACGACAGAAGTCGAAGATGTAGCT   1920
Gamtoos_S_TaAdnr1       TTGCCGGCTGGTAATCTTTGCAGTAGAATTCTAATAACGACAGAAGTCGAAGATGTAGCT   1920
                        ************************************************************

CS_TaAdnr1_Exons        CTGAAATGTTGTGGTTATGACTCTAGGCATGTTCTTATGGTGAAACCACTTGGTTACGAT   1980
Gamtoos_R_TaAdnr1       CTGAAATGTTGTGGTTATGACTCTAGGCATGTTCTTATGGTGAAACCACTTGGTTACGAT   1980
Gamtoos_S_TaAdnr1       CTGAAATGTTGTGGTTATGACTCTAGGCATGTTCTTATGGTGAAACCACTTGGTTACGAT   1980
                        ************************************************************

CS_TaAdnr1_Exons        GATTCAAGCAAATTATTTTTCAGCACAGCTTTTGGACTACAATATGAATGTCCTCCAGAA   2040
Gamtoos_R_TaAdnr1       GATTCAAGCAAATTATTTTTCAGCACAGCTTTTGGACTACAATATGAATGTCCTCCAGAA   2040
Gamtoos_S_TaAdnr1       GATTCAAGCAAATTATTTTTCAGCACAGCTTTTGGACTACAATATGAATGTCCTCCAGAA   2040
                        ************************************************************

CS_TaAdnr1_Exons        CTCTGTGACGCTGCACACAACATTGTGAGGAAATGTGCTGGTTCACCACTAGCAATGGTT   2100
Gamtoos_R_TaAdnr1       CTCTGTGACGCTGCACACAACATTGTGAGGAAATGTGCTGGTTCACCACTAGCAATGGTT   2100
Gamtoos_S_TaAdnr1       CTCTGTGACGCTGCACACAACATTGTGAGGAAATGTGCTGGTTCACCACTAGCAATGGTT   2100
                        ************************************************************

CS_TaAdnr1_Exons        ACTGTTGCTAGTCTTTTAGTAAGCCAGATTGGCAAACCAGAGAAATGGGATTATGTAAAT   2160
Gamtoos_R_TaAdnr1       ACTGTTGCTAGTCTTTTAGTAAGCCAGATTGGCAAACCAGAGAAATGGGATTATGTAAAT   2160
Gamtoos_S_TaAdnr1       ACTGTTGCTAGTCTTTTAGTAAGCCAGATTGGCAAACCAGAGAAATGGGATTATGTAAAT   2160
                        ************************************************************

CS_TaAdnr1_Exons        GAAATCTTTGGTCACGGTTTGAGCACATATCCTAGCTCGGAAGGAATGAAACAAGTACTA   2220
Gamtoos_R_TaAdnr1       GAAATCTTTGGTCACGGTTTGAGCACATATCCTAGCTCGGAAGGAATGAAACAAGTACTA   2220
Gamtoos_S_TaAdnr1       GAAATCTTTGGTCACGGTTTGAGCACATATCCTAGCTCGGAAGGAATGAAACAAGTACTA   2220
                        ************************************************************

CS_TaAdnr1_Exons        AACCTTAGTTACAACAATCTTCCTCATTATTTGAAGGCATGTGTGATGTATCTCAGTATA   2280
Gamtoos_R_TaAdnr1       AACCTTAGTTACAACAATCTTCCTCATTATTTGAAGGCATGTGTGATGTATCTCAGTATA   2280
Gamtoos_S_TaAdnr1       AACCTTAGTTACAACAATCTTCCTCATTATTTGAAGGCATGTGTGATGTATCTCAGTATA   2280
                        ************************************************************

CS_TaAdnr1_Exons        TATGAAGAGGACTACATAATTCAGAAAGATGATTTGGTAAAGCAATGGATAGCTGAAGGT   2340
Gamtoos_R_TaAdnr1       TATGAAGAGGACTACATAATTCAGAAAGATGATTTGGTAAAGCAATGGATAGCTGAAGGT   2340
Gamtoos_S_TaAdnr1       TATGAAGAGGACTACATAATTCAGAAAGATGATTTGGTAAAGCAATGGATAGCTGAAGGT   2340
                        ************************************************************

CS_TaAdnr1_Exons        CTTATCCTAGCAACAGAAGAGAAAGACAAAGAGGAAATATCAAGGAGATATTTTGATGAG   2400
Gamtoos_R_TaAdnr1       CTTATCCTAGCAACAGAAGAGAAAGACAAAGAGGAAATATCAAGGAGATATTTTGATGAG   2400
Gamtoos_S_TaAdnr1       CTTATCCTAGCAACAGAAGAGAAAGACAAAGAGGAAATATCAAGGAGATATTTTGATGAG   2400
                        ************************************************************

CS_TaAdnr1_Exons        CTTATCAGTAGCAGAATGATCCTACCTGTGTATACAAATGACAACGATGATGTTTTGTCC   2460
Gamtoos_R_TaAdnr1       CTTATCAGTAGCAGAATGATCCTACCTGTGTATACAAATGACAACGATGATGTTTTGTCC   2460
Gamtoos_S_TaAdnr1       CTTATCAGTAGCAGAATGATCCTACCTGTGTATACAAATGACAACGATGATGTTTTGTCC   2460
                        ************************************************************

CS_TaAdnr1_Exons        TGCACACTGCATCACATGGTACTTGATTTTATCAAACACAAGTCCTTAGAAGAGAATTTT   2520
Gamtoos_R_TaAdnr1       TGCACACTGCATCACATGGTACTTGATTTTATCAAACACAAGTCCTTAGAAGAGAATTTT   2520
Gamtoos_S_TaAdnr1       TGCACACTGCATCACATGGTACTTGATTTTATCAAACACAAGTCCTTAGAAGAGAATTTT   2520
                        ************************************************************

CS_TaAdnr1_Exons        GTCATCGCAATAGATCATAGTCAGACAACTGCACCACTCGCAGACAAGGTTCGTCGACTG   2580
Gamtoos_R_TaAdnr1       GTCATCGCAATAGATCATAGTCAGACAACTGCACCACTCGCAGACAAGGTTCGTCGACTG   2580
Gamtoos_S_TaAdnr1       GTCATCGCAATAGATCATAGTCAGACAACTGCACCACTCGCAGACAAGGTTCGTCGACTG   2580
                        ************************************************************

CS_TaAdnr1_Exons        TCTCTCCACTTTGGTAATGCAGAAGCAACGCCACCAACAAATATGAGACTATCACAAGTT   2640
Gamtoos_R_TaAdnr1       TCTCTCCACTTTGGTAATGCAGAAGCAACGCCACCAACAAATATGAGACTATCACAAGTT   2640
Gamtoos_S_TaAdnr1       TCTCTCCACTTTGGTAATGCAGAAGCAACGCCACCAACAAATATGAGACTATCACAAGTT   2640
                        ************************************************************

CS_TaAdnr1_Exons        CGGACTCTCGCATTTTTCGGGGTCATTGAGTGTTTGCCTTCCGTTATAGAGTTTCGGCTT   2700
Gamtoos_R_TaAdnr1       CGGACTCTCGCATTTTTCGGGGTCATTGAGTGTTTGCCTTCCGTTATAGAGTTTCGGCTT   2700
Gamtoos_S_TaAdnr1       CGGACTCTCGCATTTTTCGGGGTCATTGAGTGTTTGCCTTCCGTTATAGAGTTTCGGCTT   2700
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CS_TaAdnr1_Exons       CTTCAAGTCCTAATCCTACATCTTTTTGGCGATGATGAAAGTGTCAGTTTTGATCTCACT   2760
Gamtoos_R_TaAdnr1      CTTCAAGTCCTAATCCTACATCTTTTTGGCGATGATGAAAGTGTCAGTTTTGATCTCACT   2760
Gamtoos_S_TaAdnr1      CTTCAAGTCCTAATCCTACATCTTTTTGGCGATGATGAAAGTGTCAGTTTTGATCTCACT   2760
                       ************************************************************

CS_TaAdnr1_Exons       GGAATATCTGAGCTTTTTCGGTTGAGATATTTGCATGTCACATGTAATGCCACCTTAGAA   2820
Gamtoos_R_TaAdnr1      GGAATATCTGAGCTTTTTCGGTTGAGATATTTGCATGTCACATGTAATGCCACCTTAGAA   2820
Gamtoos_S_TaAdnr1      GGAATATCTGAGCTTTTTCGGTTGAGATATTTGCATGTCACATGTAATGCCACCTTAGAA   2820
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CS_TaAdnr1_Exons       GTACCACAAACTCAGATGCGAGGTTTACAATATTTGGAGACACTGAAAATAGATGCAAGA   2880
Gamtoos_R_TaAdnr1      GTACCACAAACTCAGATGCGAGGTTTACAATATTTGGAGACACTGAAAATAGATGCAAGA   2880
Gamtoos_S_TaAdnr1      GTACCACAAACTCAGATGCGAGGTTTACAATATTTGGAGACACTGAAAATAGATGCAAGA   2880
                       ************************************************************

CS_TaAdnr1_Exons       GTAAGTGCAGTTCCGTCGGACATTGTTCATTTGCCGAGCTTGTTGCACCTCAGTCTTCCT   2940
Gamtoos_R_TaAdnr1      GTAAGTGCAGTTCCGTCGGACATTGTTCATTTGCCGAGCTTGTTGCACCTCAGTCTTCCT   2940
Gamtoos_S_TaAdnr1      GTAAGTGCAGTTCCGTCGGACATTGTTCATTTGCCGAGCTTGTTGCACCTCAGTCTTCCT   2940
                       ************************************************************

CS_TaAdnr1_Exons       GTTGGGACAAATCTACCAAATGGTATTGACCATATGACATCGCTTTGCACACTTGAATAT   3000
Gamtoos_R_TaAdnr1      GTTGGGACAAATCTACCAAATGGTATTGACCATATGACATCGCTTTGCACACTTGAATAT   3000
Gamtoos_S_TaAdnr1      GTTGGGACAAATCTACCAAATGGTATTGACCATATGACATCGCTTTGCACACTTGAATAT   3000
                       ************************************************************

CS_TaAdnr1_Exons       TTTGATATAAATGTTAACTCAATGGAGAATGTGCACAGCCTTGGTGAGCTGACCAATCTT   3060
Gamtoos_R_TaAdnr1      TTTGATATAAATGTTAACTCAATGGAGAATGTGCACAGCCTTGGTGAGCTGACCAATCTT   3060
Gamtoos_S_TaAdnr1      TTTGATATAAATGTTAACTCAATGGAGAATGTGCACAGCCTTGGTGAGCTGACCAATCTT   3060
                       ************************************************************

CS_TaAdnr1_Exons       CAGGATCTTCGGCTCACATGTTCTACAGTTCCTTCTTCTTACTTAAAGAGTAAAATCGAT   3120
Gamtoos_R_TaAdnr1      CAGGATCTTCGGCTCACATGTTCTACAGTTCCTTCTTCTTACTTAAAGAGTAAAATCGAT   3120
Gamtoos_S_TaAdnr1      CAGGATCTTCGGCTCACATGTTCTACAGTTCCTTCTTCTTACTTAAAGAGTAAAATCGAT   3120
                       ************************************************************

CS_TaAdnr1_Exons       AGTATGGGCTCTATTCTTGCGAACCTCAGCAACCTCAGGTCTGTAACTCTGAAGTCTTCA   3180
Gamtoos_R_TaAdnr1      AGTATGGGCTCTATTCTTGCGAACCTCAGCAACCTCAGGTCTGTAACTCTGAAGTCTTCA   3180
Gamtoos_S_TaAdnr1      AGTATGGGCTCTATTCTTGCGAACCTCAGCAACCTCAGGTCTGTAACTCTGAAGTCTTCA   3180
                       ************************************************************

CS_TaAdnr1_Exons       GGTATTCTGGAGAGTGAACCTTACAGCATGATCATTTCCTGTGATGGCTTGAGCAGCGTT   3240
Gamtoos_R_TaAdnr1      GGTATTCTGGAGAGTGAACCTTACAGCATGATCATTTCCTGTGATGGCTTGAGCAGCGTT   3240
Gamtoos_S_TaAdnr1      GGTATTCTGGAGAGTGAACCTTACAGCATGATCATTTCCTGTGATGGCTTGAGCAGCGTT   3240
                       ************************************************************

CS_TaAdnr1_Exons       TCCTCTCCTCCAGCTCTTCTTCAAAGATTTGAGTGGTTGCCACGCATTTGTACCTTCTCC   3300
Gamtoos_R_TaAdnr1      TCCTCTCCTCCAGCTCTTCTTCAAAGATTTGAGTGGTTGCCACGCATTTGTACCTTCTCC   3300
Gamtoos_S_TaAdnr1      TCCTCTCCTCCAGCTCTTCTTCAAAGATTTGAGTGGTTGCCACGCATTTGTACCTTCTCC   3300
                       ************************************************************

CS_TaAdnr1_Exons       AGCATCCCTAAGTGGATTAGCCATCTCAACAAGCTCTGCATTTTAAAGATTGGGCTTAGG   3360
Gamtoos_R_TaAdnr1      AGCATCCCTAAGTGGATTAGCCATCTCAACAAGCTCTGCATTTTAAAGATTGGGCTTAGG   3360
Gamtoos_S_TaAdnr1      AGCATCCCTAAGTGGATTAGCCATCTCAACAAGCTCTGCATTTTAAAGATTGGGCTTAGG   3360
                       ************************************************************

CS_TaAdnr1_Exons       GAATTAGTGAGCAATGATGTCGCTGCTCTGAGAGGATTGCCTGCACTAACTGTTTTGTCG   3420
Gamtoos_R_TaAdnr1      GAATTAGTGAGCAATGATGTCGCTGCTCTGAGAGGATTGCCTGCACTAACTGTTTTGTCG   3420
Gamtoos_S_TaAdnr1      GAATTAGTGAGCAATGATGTCGCTGCTCTGAGAGGATTGCCTGCACTAACTGTTTTGTCG   3420
                       ************************************************************

CS_TaAdnr1_Exons       TTATATGTCCGAGCAAAGCCCGCAGAAAAAATTGTCTTTACTAGGGCAGGATTCTTGGTT   3480
Gamtoos_R_TaAdnr1      TTATATGTCCGAGCAAAGCCCGCAGAAAAAATTGTCTTTACTAGGGCAGGATTCTTGGTT   3480
Gamtoos_S_TaAdnr1      TTATATGTCCGAGCAAAGCCCGCAGAAAAAATTGTCTTTACTAGGGCAGGATTCTTGGTT   3480
                       ************************************************************

CS_TaAdnr1_Exons       CTCAAGTGCTTCAAGTTCAGGTGCAGTGTACCTTGGCTGGAATTTGAGGTGGATGCAATG   3540
Gamtoos_R_TaAdnr1      CTCAAGTGCTTCAAGTTCAGGTGCAGTGTACCTTGGCTGGAATTTGAGGTGGATGCAATG   3540
Gamtoos_S_TaAdnr1      CTCAAGTGCTTCAAGTTCAGGTGCAGTGTACCTTGGCTGGAATTTGAGGTGGATGCAATG   3540
                       ************************************************************

CS_TaAdnr1_Exons       CCTAATCTCTTGAAACTCAAGCTAAGTTTTGATGCCCATGGAGTAGATCAACATCGTACT   3600
Gamtoos_R_TaAdnr1      CCTAATCTCTTGAAACTCAAGCTAAGTTTTGATGCCCATGGAGTAGATCAACATCGTACT   3600
Gamtoos_S_TaAdnr1      CCTAATCTCTTGAAACTCAAGCTAAGTTTTGATGCCCATGGAGTAGATCAACATCGTACT   3600
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CS_TaAdnr1_Exons       ATACCTGTCGGCATGGTGCACTTAACAGGCCTTAAGGAAATCTCTGCAAAAATTTGGGGT   3660
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Gamtoos_R_TaAdnr1      ATACCTGTCGGCATGGTGCACTTAACAGGCCTTAAGGAAATCTCTGCAAAAATTTGGGGT   3660
Gamtoos_S_TaAdnr1      ATACCTGTCGGCATGGTGCACTTAACAGGCCTTAAGGAAATCTCTGCAAAAATTTGGGGT   3660
                       ************************************************************

CS_TaAdnr1_Exons       GCTGGTGCCAATGAAAGAAGGGCTGCAAAATCAGCGCTGATTGATGCTATAAAAATGCAT   3720
Gamtoos_R_TaAdnr1      GCTGGTGCCAATGAAAGAAGGGCTGCAAAATCAGCGCTGATTGATGCTATAAAAATGCAT   3720
Gamtoos_S_TaAdnr1      GCTGGTGCCAATGAAAGAAGGGCTGCAAAATCAGCGCTGATTGATGCTATAAAAATGCAT   3720
                       ************************************************************

CS_TaAdnr1_Exons       TCGGGATGTCCCACCTCCAGCATACAGTGTTTAGATGGGATGTTCAGTGGTAAGGATGAT   3780
Gamtoos_R_TaAdnr1      TCGGGATGTCCCACCTCCAGCATACAGTGTTTAGATGGGATGTTCAGTGGTAAGGATGAT   3780
Gamtoos_S_TaAdnr1      TCGGGATGTCCCACCTCCAGCATACAGTGTTTAGATGGGATGTTCAGTGGTAAGGATGAT   3780
                       ************************************************************

CS_TaAdnr1_Exons       AATAATAGCGGGATACAAGAGGAAGAACACTTGACTCTGCAAAAGCAATACAATATCAAG   3840
Gamtoos_R_TaAdnr1      AATAATAGCGGGATACAAGAGGAAGAACACTTGACTCTGCAAAAGCAATACAATATCAAG   3840
Gamtoos_S_TaAdnr1      AATAATAGCGGGATACAAGAGGAAGAACACTTGACTCTGCAAAAGCAATACAATATCAAG   3840
                       ************************************************************

CS_TaAdnr1_Exons       GAGGAAGACTCCAAGAAACAGCATGATCTTCCAAAGGACTACATGGATGTTGCATACAAA   3900
Gamtoos R TaAdnr1      GAGGAAGACTCCAAGAAACAGCATGATCTTCCAAAGGACTACATGGATGTTGCATACAAA   3900
Gamtoos_S_TaAdnr1      GAGGAAGACTCCAAGAAACAGCATGATCTTCCAAAGGACTACATGGATGTTGCATACAAA   3900
                       ************************************************************

CS_TaAdnr1_Exons       CAAACTTCCAGCAGCAACAATCATAGGAAGTCCAAGCGGATCACACAGGTGAGGATGCAG   3960
Gamtoos R TaAdnr1      CAAACTTCCAGCAGCAACAATCATAGGAAGTCCAAGCGGATCACACAGGTGAGGATGCAG   3960
Gamtoos_S_TaAdnr1      CAAACTTCCAGCAGCAACAATCATAGGAAGTCCAAGCGGATCACACAGGTGAGGATGCAG   3960
                       ************************************************************

CS_TaAdnr1_Exons       GTGAGGGTGGGATCGGTGCAGGACAACAGCGCCCTCGAGGATGGCTTTAGCTGGAGGAAG   4020
Gamtoos_R_TaAdnr1      GTGAGGGTGGGATCGGTGCAGGACAACAGCGCCCTCGAGGATGGCTTTAGCTGGAGGAAG   4020
Gamtoos S TaAdnr1      GTGAGGGTGGGATCGGTGCAGGACAACAGCGCCCTCGAGGATGGCTTTAGCTGGAGGAAG   4020
                       ************************************************************

CS_TaAdnr1_Exons       TACGGCCAGAAGGATATCATCGGCTCCATGCACCCAAGAGCTTATTTCCGGTGCACGCAC   4080
Gamtoos_R_TaAdnr1      TACGGCCAGAAGGATATCATCGGCTCCATGCACCCAAGAGCTTATTTCCGGTGCACGCAC   4080
Gamtoos_S_TaAdnr1      TACGGCCAGAAGGATATCATCGGCTCCATGCACCCAAGAGCTTATTTCCGGTGCACGCAC   4080
                       ************************************************************

CS_TaAdnr1_Exons       AGGCACGTTAAGGGCTGCCCAGTGACCAAGCAGGTGCAGCGCACGTCTACTGACCCGCTG   4140
Gamtoos_R_TaAdnr1      AGGCACGTTAAGGGCTGCCCAGTGACCAAGCAGGTGCAGCGCACGTCTACTGACCCGCTG   4140
Gamtoos_S_TaAdnr1      AGGCACGTTAAGGGCTGCCCAGTGACCAAGCAGGTGCAGCGCACGTCTACTGACCCGCTG   4140
                       ************************************************************

CS_TaAdnr1_Exons       CTCTTTGACGTCGTGTATCACGGGGAGCACACGTGCTTGGACTCTGTCGGATCCCCTGCG   4200
Gamtoos_R_TaAdnr1      CTCTTTGACGTCGTGTATCACGGGGAGCACACGTGCTTGGACTCTGTCGGATCCCCTGCG   4200
Gamtoos_S_TaAdnr1      CTCTTTGACGTCGTGTATCACGGGGAGCACACGTGCTTGGACTCTGTCGGATCCCCTGCG   4200
                       ************************************************************

CS_TaAdnr1_Exons       ACGTCTTGTGGCCATGTCGCCGGCGTGGAGGTGATGAGCAGAAGTAGGCCCGGAGTTGGA   4260
Gamtoos_R_TaAdnr1      ACGTCTTGTGGCCATGTCGCCGGCGTGGAGGTGATGAGCAGAAGTAGGCCCGGAGTTGGA   4260
Gamtoos_S_TaAdnr1      ACGTCTTGTGGCCATGTCGCCGGCGTGGAGGTGATGAGCAGAAGTAGGCCCGGAGTTGGA   4260
                       ************************************************************

CS_TaAdnr1_Exons       TTCGTGTCCCAGTCCCAGGCGGCGTGCAGTAGCCAGGTTATGTCCTCCGAGGTGGTAAGC   4320
Gamtoos_R_TaAdnr1      TTCGTGTCCCAGTCCCAGGCGGCGTGCAGTAGCCAGGTTATGTCCTCCGAGGTGGTAAGC   4320
Gamtoos_S_TaAdnr1      TTCGTGTCCCAGTCCCAGGCGGCGTGCAGTAGCCAGGTTATGTCCTCCGAGGTGGTAAGC   4320
                       ************************************************************

CS_TaAdnr1_Exons       GGAAGCGGGAGTACGGCGGGACTTTGGGGTGACGAGATTGACATGCCGGACCCGGACCGC   4380
Gamtoos_R_TaAdnr1      GGAAGCGGGAGTACGGCGGGACTTTGGGGTGACGAGATTGACATGCCGGACCCGGACCGC   4380
Gamtoos_S_TaAdnr1      GGAAGCGGGAGTACGGCGGGACTTTGGGGTGACGAGATTGACATGCCGGACCCGGACCGC   4380
                       ************************************************************

CS_TaAdnr1_Exons       GATGACACCGGCATCAGTGCTGACTACCTCGGTGGCTACGAATTTGATGTCAGCGCGTTT   4440
Gamtoos_R_TaAdnr1      GATGACACCGGCATCAGTGCTGACTACCTCGGTGGCTACGAATTTGATGTCAGCGCGTTT   4440
Gamtoos_S_TaAdnr1      GATGACACCGGCATCAGTGCTGACTACCTCGGTGGCTACGAATTTGATGTCAGCGCGTTT   4440
                       ************************************************************

CS_TaAdnr1_Exons       TTTGCCTAG      4449
Gamtoos_R_TaAdnr1      TTTGCCTAG      4449
Gamtoos_S_TaAdnr1      TTTGCCTAG      4449
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Figure 7: The Clustal Omega alignment of the Gamtoos-S and Gamtoos-R full-length nucleic acid sequences of *TaAdnr1* to the Chinese Spring (CS) reference sequence. There were no single-nucleotide polymorphisms (SNPs) identified on either sequence from the wheat plants.

4.2 RT-qPCR expression analysis of *TaAdnr1*

The Gamtoos and Gamtoos-R NILs were infested with the *D. noxia* RWASA1 biotype for analysis of the regulation of *TaAdnr1* at four time points (0, 6, 12 and 24 hpi) using RT-qPCR. These time points were selected based on the previous study by Nicolis and Venter (2018). Figure 8 indicates the RNA extractions performed for Gamtoos (in A) and Gamtoos-R (in B). The A_{260}/A_{280} ratio of the samples in both NILs was between 1.8-2. Two distinct bands representing the 28S and 18S rRNA subunits were observed in all the samples of both cultivars.

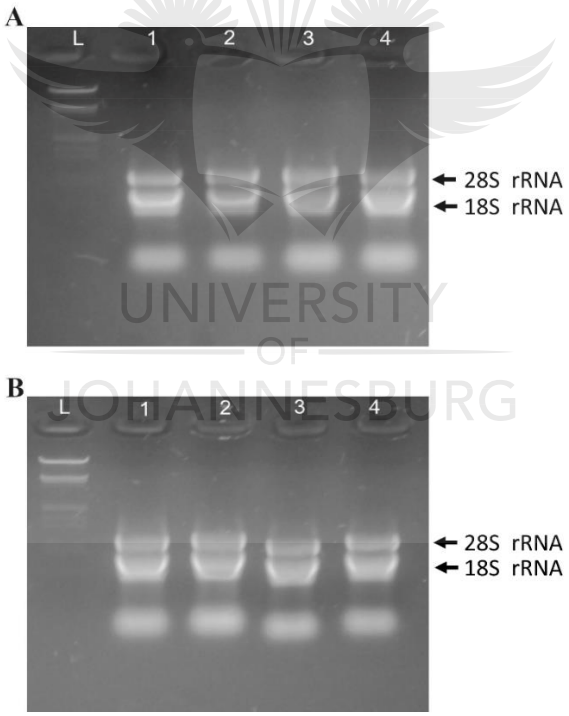

Figure 8: RNA extracted from the Gamtoos wheat cultivars infested with RWASA1 biotype for determining the differential expression of *TaAdnr1* for the different time points. The RNA was resolved on a 1 % native agarose gel. The lanes 1-4 represents RNA extracted from Gamtoos (**A**) and Gamtoos-R (**B**) for 0, 6, 12 and 24 hpi, respectively. Lane L represents λ EcoRI + Hind III DNA markers.

The synthesised cDNA samples were used for RT-qPCR reactions performed in three technical replicates for each sample on a CFX Connect and normalised using the *GAPDH* and *18S* reference genes. The standard curves were generated for *TaAdnr1* and the reference genes and all the primers were optimised to amplify within the 90-105 % efficiency range and high R^2 values of approximately 0.99. The standard curves produced for Gamtoos *GAPDH*, *18S* and *TaAdnr1* indicated efficiency of 104.9%, 101.3% and 98.2% and R^2 value of 0.998, 0.979 and 0.982 respectively (Figure 9. A i), B i) and C i)). Gamtoos-R indicated an efficiency of 91.7%, 101.5% and 93.3% and R^2 value of 0.997, 0.982 and 0.962, respectively (Figure 9. A ii), B ii) and C ii)). The melt curves for both Gamtoos and Gamtoos-R indicated the amplification of a single gene product for *TaAdnr1* (Figure 9. C). The melt curves for *GAPDH* and *18S* indicated the presence of primer-dimer or secondary structures that did not, however, affect the quantification (Figure 9. A and B). Sequencing of the *TaAdnr1* RT-qPCR product was performed and indicated a single fragment of the correct size of 118 bp. The melting temperature for the *GAPDH* and *18S* reference genes was 85 °C and 89 °C, respectively for both Gamtoos and Gamtoos-R cultivars (Figure 9. A and B). The melting temperature for the *TaAdnr1* gene fragment was 80 °C in both cultivars (Figure 9. C).

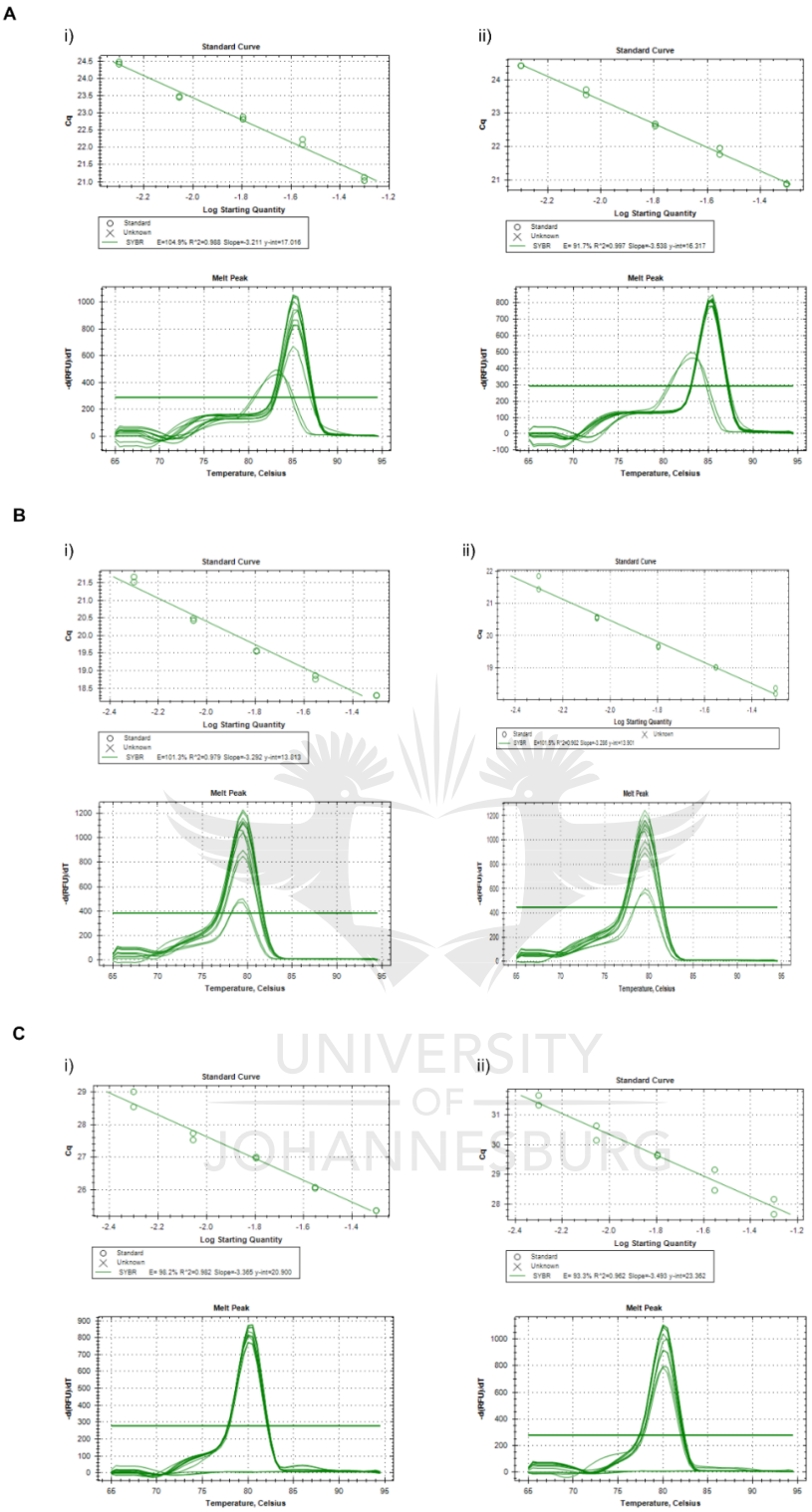

Figure 9: The RT-qPCR standard and melt curve graphs for the differential expression analysis of *TaAdnr1* in both Gamtoos wheat cultivars. The standard and melt curves are shown for *GAPDH* (A), *18S* (B**)** and *TaAdnr1* (C) in Gamtoos (i) and Gamtoos-R (ii) respectively. The PCR efficiency, R^2 values and the slope are indicated for the different samples.

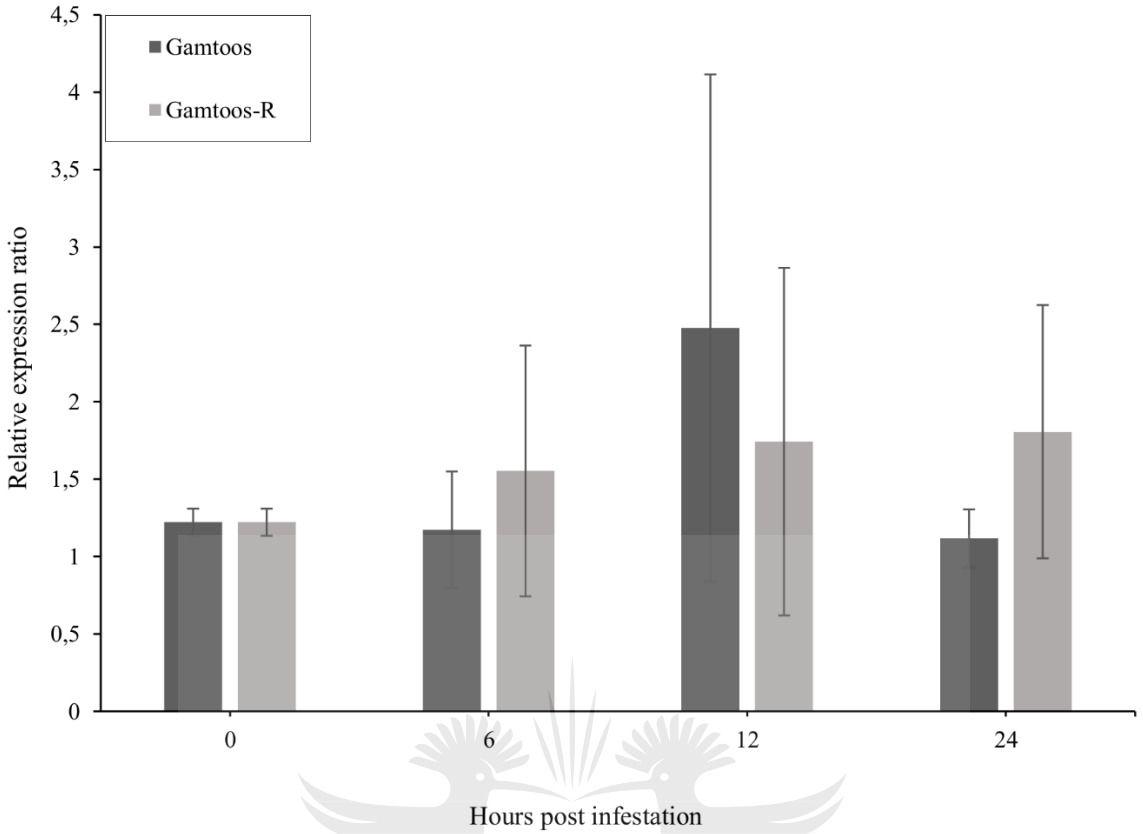

Figure 10: Differential expression of *TaAdnr1* between the susceptible Gamtoos and resistant Gamtoos-R near-isogenic lines (NIL) infested with the RWASA1 biotype. The fold change *TaAdnr1* normalised with the internal reference genes (*GAPDH* and *18S*) was analysed using RT-qPCR at four time points (0, 6, 12 and 24 hpi) in both wheat NILs. The means of the biological replicates (n = 3) per treatment were used to determine the fold change. There was no statistical significance observed between the genotypes ($p \leq 0.05$, ANOVA and Tukey's HSD post hoc test).

Differential expression of *TaAdnr1* was observed between the susceptible and resistant NILs (Figure 10). There were no significant differences between the regulation of transcript levels in the susceptible Gamtoos cultivar in all the time points investigated (0-24 hpi). Furthermore, there was no significant upregulation of *TaAdnr1* in both the susceptible Gamtoos and resistant Gamtoos-R NIL at all the tested time points ($p \leq 0.05$).

4.3 VIGS Knockdown of *TaAdnr1*

The purified α, β and γ BSMV knockdown plasmid vectors were linearised with restriction enzyme digestion using SpeI for plasmid BSMVβ and MluI for all the BSMVγ and α plasmids. Figure 11 indicates the linearised BSMV plasmids that were used for RNA transcription. The DNA concentration on the agarose gel was estimated based on known amounts of marker DNA and the A_{260}/A_{280} value was not evaluated due to the presence of a significant amount of RNA in the plasmid preparations that would have interfered with accurate spectrophotometric quantification of the DNA (Scofield and Brandt, 2005).

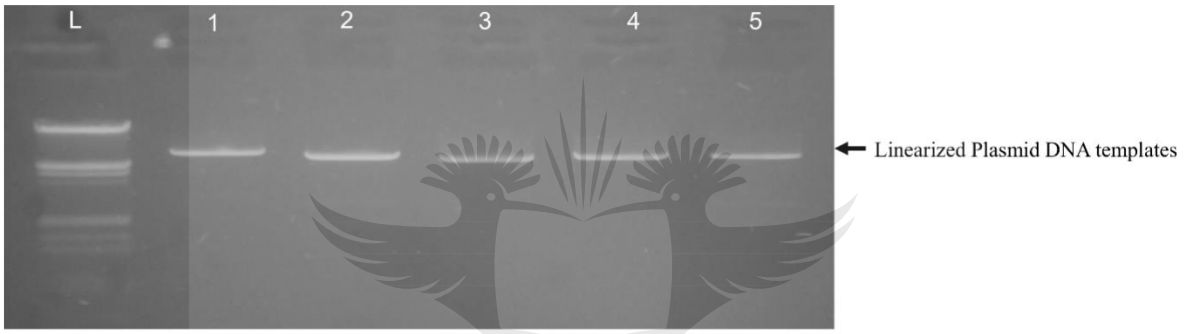

Figure 11: The linearised BSMV plasmids resolved on a 1 % agarose gel. The lanes 1-2 represent the BSMV α and β linear plasmids, respectively. Lanes 3-5 represent the BSMV γ plasmid with no fragment cloned at the cloning sites (empty vector, $BSMV_0$), the BSMV γ plasmid containing the cloned *PDS* gene ($BSMV_{PDS}$) and the BSMV γ plasmid containing the 270 bp cloned *TaAdnr1* silencing fragment ($BSMV_{TaAdnr1}$), respectively. Lane L represents λ EcoRI + Hind III DNA markers.

The *in vitro* transcripts were produced from the linearised BSMV α, β and γ plasmids and evaluated on a 1 % (w/v) agarose gel to confirm successful transcription. Faint bands representing the linearised plasmid DNA at approximately 10 000 bp and distinct bands of a high degree of integrity representing the *in vitro* transcribed RNA at approximately 3 000 bp were observed for each of the *in vitro* transcription reactions (Figure 12). A total of 15 Gamtoos-R wheat NILs were inoculated by equal mixing of 1 µl of each of the three *in vitro* transcription reactions.

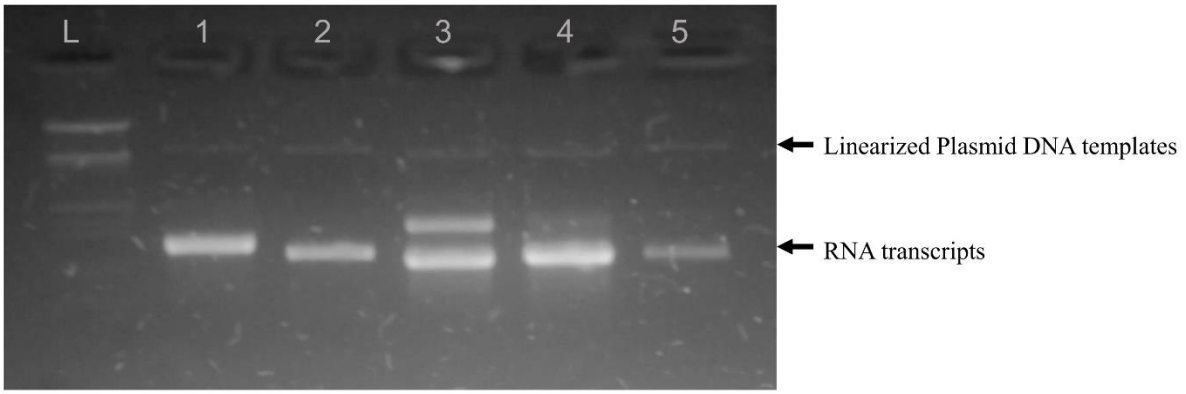

Figure 12: The *in vitro* transcribed RNA products resolved on a 1% native agarose gel. Lanes 1 and 2 represent the BSMV α and β *in vitro* transcription reaction, respectively. Lanes 3 represents the BSMV γ plasmid with no fragment cloned at the cloning sites (empty vector, $BSMV_0$), lane 4 represents the BSMV γ plasmid containing the 270 bp cloned *TaAdnr1* silencing fragment ($BSMV_{TaAdnr1}$) and lane 5 represents the BSMV γ plasmid containing the cloned *PDS* gene ($BSMV_{PDS}$), respectively. Lane L represents λ EcoRI + Hind III DNA markers. Faint bands were observed at the 10 000 bp size range that represent the linearised plasmid DNA and distinct bright bands of high integrity representing the *in vitro* transcribed RNA were observed at the 3 000 bp size range for each of the *in vitro* transcription reactions (Lane 1-5).

RNA extractions were performed from three biological replicates on each of the Gamtoos-R NIL infested with the *D. noxia* RWASA1-4 biotypes **(**Figure 13. A-D) eleven days after viral inoculation (5 days post-aphid infestation) for analysis of the VIGS knockdown levels of *TaAdnr1*. The A_{260}/A_{280} ratio of the samples in both NIL was between 1.8-2. Two distinct bands of a high degree of integrity representing the 28S and 18S rRNA subunits were observed in all the samples. The RNA of all the samples was then reverse transcribed for equimolar concentrations into cDNA and then used for RT-qPCR to determine the knockdown levels of *TaAdnr1*.

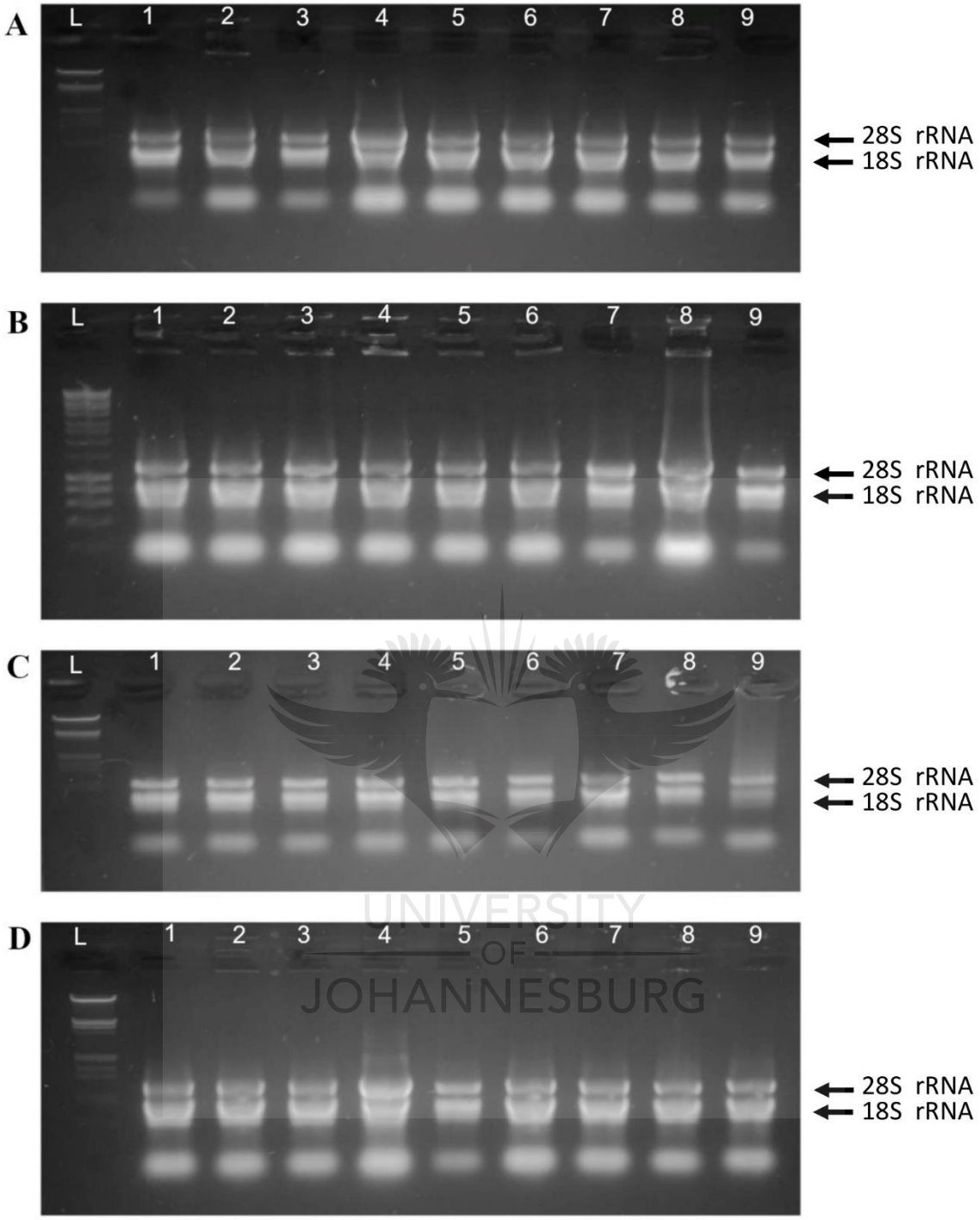

Figure 13: RNA extracted from the Gamtoos-R wheat plants infested with the different RWA (SA1-4, A-D respectively) 11 days after viral inoculation. The RNA was resolved on a 1 % native agarose gel. Lane 1-3 represents RNA extracted from infested Gamtoos-R plants, lanes 4-6 represent RNA extracted from $BSMV_{TaAdnr1}$-treated Gamtoos-R plants and finally lanes 7-9 represents RNA extracted from $BSMV_0$-treated control Gamtoos-R plants. Lane L represents λ EcoRI + HindIII DNA markers.

The synthesised cDNA samples were used for RT-qPCR analysis of *TaAdnr1* knockdown and the reactions were performed in three technical replicates for each sample normalised using the *GAPDH* and *18S* reference genes on a CFX Connect. The standard curves produced for *GAPDH* in the RWASA1-4 experiments indicated an efficiency of 86.9 %, 94.2 %, 87.5 % and 106.5 % and R^2 values of 0.998, 0.990, 0.997 and 0.995 respectively (Figure 14. A, i-iv). The standard curves produced for *18S* in the same experiments indicated efficiency of 91.8 %, 95.6 %, 85.3 and 93.4 % and R^2 values of 0.996, 0.992, 0.999 and 0.99 respectively (Figure 14. B, i-iv). The standard curves produced for *TaAdnr1* in the same experiments indicated an efficiency of 103.5 %, 95.6 %, 109.8 % and 95.1% and R^2 values of 0.860, 0.992, 0.936 and 0.995, respectively (Figure 14. C, i-iv). The melt curves for all the experiments indicated the amplification of a single gene product for *TaAdnr1* (Figure 15. A-C). The melt curves for *GAPDH* and *18S* did not indicate the presence of primer-dimer or secondary structures (Figure 15. A-C). The melting temperature for the *GAPDH* and *18S* reference genes was 82 °C and 85 °C respectively for the RWASA1, RWASA3 and RWASA4 experiments (Figure 15. A-B). A melting temperature of 81 °C and 84 °C for *GAPDH* and *18S* respectively was obtained for the RWASA2 experiment (Figure 15. A-C, ii). The melting temperature for the *TaAdnr1* gene fragment was 77 °C, 84 °C, 77 °C and 80 °C for the four experiments, respectively (Figure 15. C).

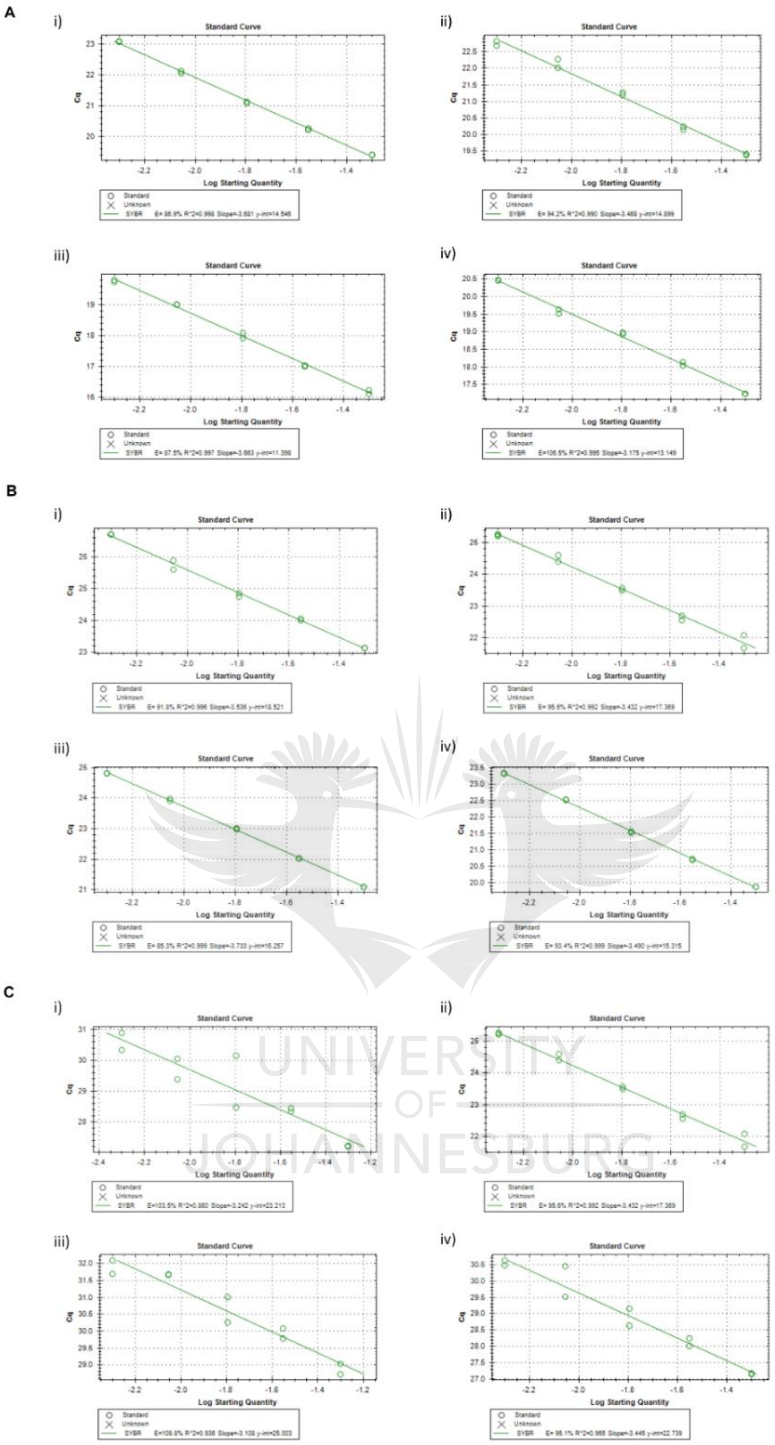

Figure 14: The RT-qPCR standard curve graphs for the BSMV-VIGS-mediated knockdown of *TaAdnr1* in Gamtoos-R. The standard curves are shown for *GAPDH* (A), *18S* (B**)** and *TaAdnr1* (C) for the different BSMV-VIGS experiments against the respective RWA biotypes (SA1-4, i-iv respectively) in Gamtoos-R. The PCR efficiency, R^2 values and the slope are indicated for the different samples.

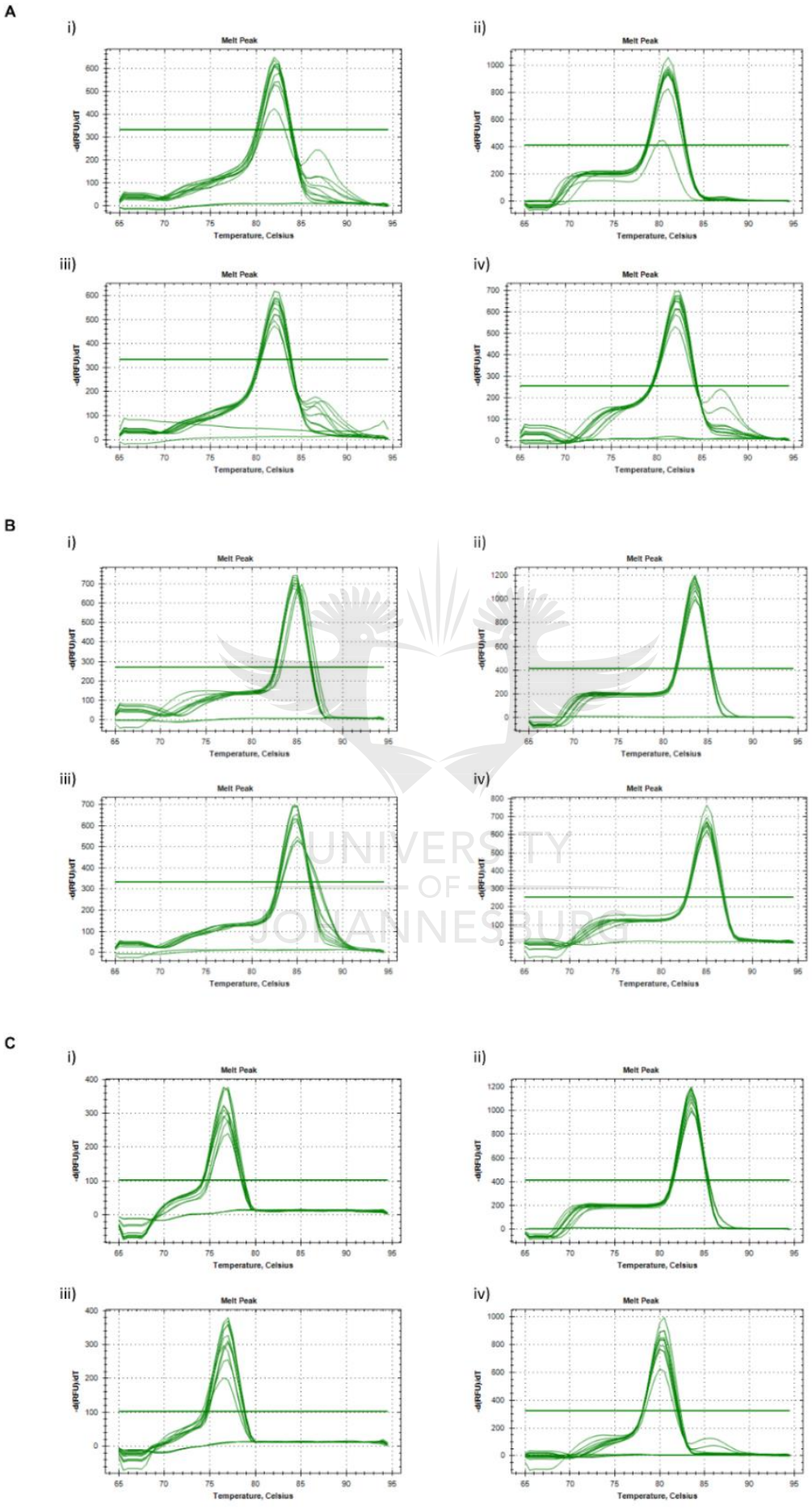

Figure 15: The RT-qPCR melt curve graphs for the BSMV-VIGS-mediated knockdown of *TaAdnr1* in Gamtoos-R. The melt curves are shown for *GAPDH* (A), *18s* (B) and *TaAdnr1* (C) for the different BSMV-VIGS experiments against the respective RWA biotypes (SA1-4, i-iv respectively) in Gamtoos-R.

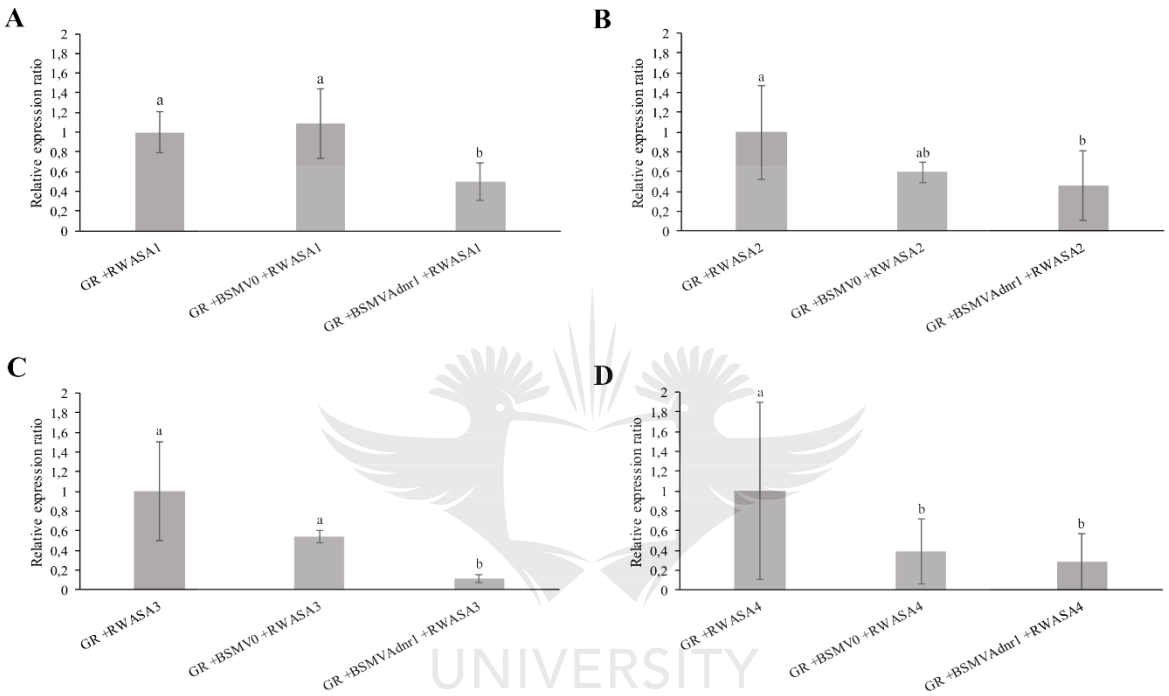

Figure 16: Relative levels of knockdown achieved for *TaAdnr1* evaluated using RT-qPCR 11 days post BSMV inoculation (5 days post-aphid infestation) in the resistant Gamtoos-R NIL. A) Gamtoos-R (GR) and BSMV-treated GR plants infested with the RWASA1, B) with RWASA2, C) with RWASA3 and D) with the RWASA4 biotype. The means of the biological replicates (n = 3) per treatment were used to determine the fold change in the relative expression. The *TaAdnr1* fold change was normalised with the internal reference genes *GAPDH* and *18S*. The different letters indicate the statistical difference between the samples ($p \leq 0.05$, ANOVA and Tukey's HSD post hoc test).

In the RWASA3 and RWASA4 experiments, the GR+BSMV$_{TaAdnr1}$ plants showed the highest knockdown levels with a mean decrease of 89 % and 72 %, respectively ($p \leq 0.05$) (Figure 16. C and D). The GR+BSMV$_{TaAdnr1}$ plants in the RWASA1 and RWASA2 experiments showed a

decrease of 50 % and 54 %, respectively ($p \leq 0.05$), in the knockdown levels (Figure 16. A and B). The GR+BSMV$_0$ plants in the RWASA2, RWASA3 experiment indicated a 40 % and 46 % ($p \leq 0.05$) decrease in expression, while a 9 % upregulation was observed in the RWASA1 experiment compared to the GR+RWA control plants (Figure 16. B and C). Furthermore, a 61 % downregulation in the GR+BSMV$_0$ plants was observed in the RWASA4 experiment compared with the GR+RWA control and the GR+BSMV$_{TaAdnr1}$ (72 % knockdown) plants (Figure 16. D).

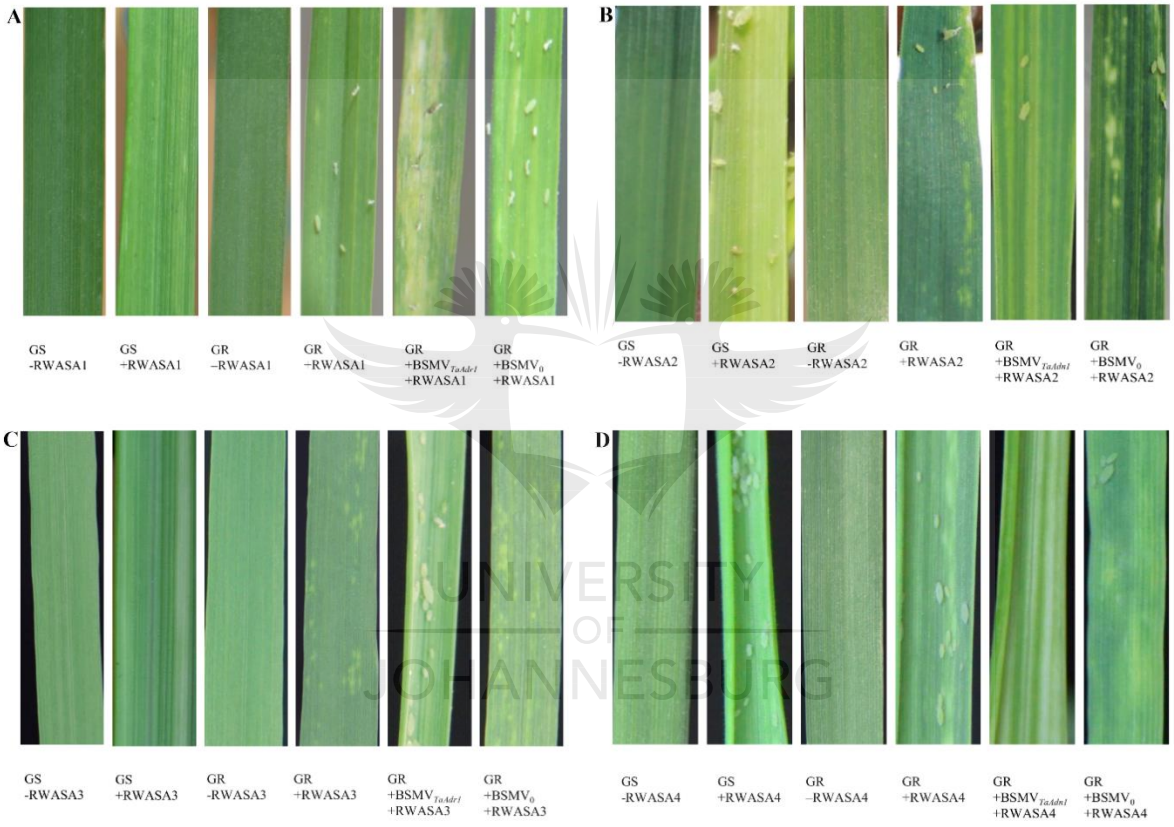

Figure 17: The phenotypic effect of *TaAdnr1* knockdown and subsequent *D. noxia* infestation. A) Gamtoos (GS), Gamtoos-R (GR) and BSMV-treated plants infested with the RWASA1, B) RWASA2, C) RWASA3, D) RWASA4 biotypes. The phenotypes of representative plants per treatment were observed 19 days after viral infection. The GS and GR (-RWA) controls represent uninfested plants and GS and GR (+RWA) infested plants. The resistant controls (GR+RWA and GR+BSMV$_0$+RWA) infested with the different RWA biotypes (SA1-4) exhibited localised cell-death lesions (A-D). The susceptible control (GS+RWA) and the

TaAdnr1 knockdown (GR+BSMV$_{TaAdnr1}$+RWA) treated plants resulted in the development of leaf yellowing, chlorotic streaking and leaf rolling (A-D).

The susceptible Gamtoos and resistant Gamtoos-R cultivars with no aphid infestations (-RWA) did not show any observable phenotypic symptoms of disease or stress and the plants were all green and healthy (Figure 17). Feeding by the different *D. noxia* biotypes resulted in different phenotypic responses for the resistant cultivar, Gamtoos-R (GR+RWA) and the susceptible cultivar Gamtoos (GS+RWA) (Figure 17, A-D). RWASA1 infestation on the GS+RWA plants resulted in less pronounced symptoms and was observed as minimal yellowing of the leaf with the development of chlorotic lesions. RWASA2 infestation on Gamtoos plants resulted in severe leaf yellowing, RWASA3 infestation caused chlorotic streaking and RWASA4 infestation resulted in both leaf rolling and yellowing, respectively (Figure 17. B-D). The infestation of the Gamtoos-R plants resulted in the development of hypersensitive lesions and for RWASA3 and 4 leaf rolling was also observed. The development of distinct hypersensitive lesions were more pronounced when Gamtoos-R was infested with RWASA2, RWASA3 and RWASA4 (Figure 17. A-D). The resistant control with empty vector (GR+BSMV$_0$+RWA) plants infested with RWASA1 resulted in leaf yellowing and exhibited minimal localised cell-death lesions similar to the GR+RWA (Figure 17. A). The infestation of the GR+BSMV$_0$ plants with RWASA2 and RWASA3 showed distinct and more pronounced hypersensitive cell death lesion compared with the GR+RWA plants (Figure 17. B and C). Infestation with RWASA4 on the GR+BSMV$_0$ plants resulted in an observable hypersensitive response almost similar to the resistant GR+RWA plants (Figure 17. D). This phenotypic disease resistance response is characteristic of *Dn7*-mediated antibiotic effect against *D. noxia*. The knockdown of *TaAdnr1* in the resistant plants (GR+BSMV$_{TaAdnr1}$+RWA) and subsequent infestation with RWASA1 resulted in the development of severe chlorosis on the leaf (Figure 17. A). Infestation with RWASA2 on the GR+BSMV$_{TaAdnr1}$ plants resulted in the development of chlorotic streaking and leaf yellowing, while RWASA3 and RWASA4 both resulted in leaf yellowing, chlorotic streaking and leaf rolling (Figure 17. C-D).

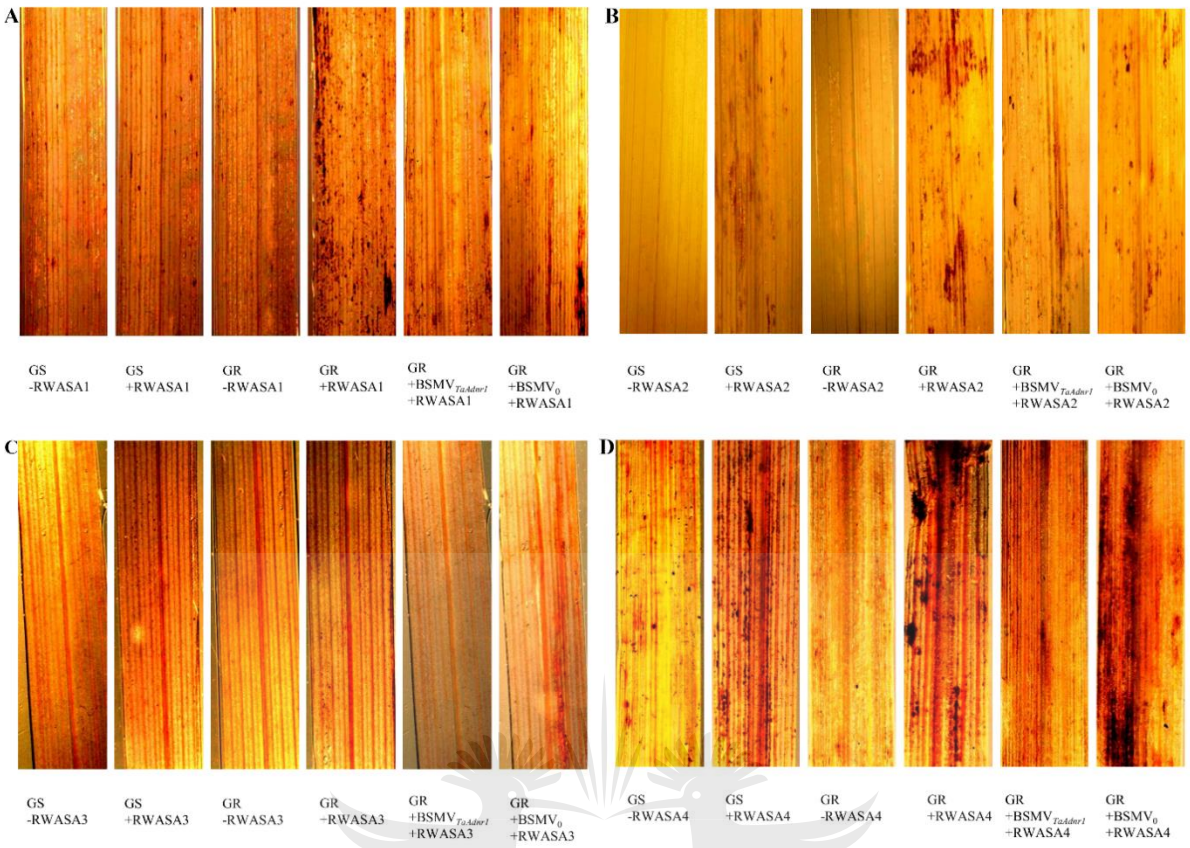

Figure 18: Changes to the oxidative burst as measured through H_2O_2 accumulation. DAB staining of plants after BSMV-mediated knockdown of *TaAdnr1* in resistant plants resulted in different observable patterns after infestation with the different *D. noxia* biotypes. A) Gamtoos (GS), Gamtoos-R (GR) and BSMV-treated plants infested with the RWASA1, B) RWASA2, C) RWASA3 and D) RWASA4 biotype. The resistant Gamtoos-R and Gamtoos-R +BSMV$_0$ DAB stained leaves exhibited mild to intense H_2O_2 accumulation following infestation with the different *D. noxia* biotypes. (A-D). A loss in the level of staining was observed in Gamtoos-R +BSMV$_{TaAdnr1}$ compared with the resistant controls Gamtoos-R and Gamtoos-R +BSMV$_0$ DAB stained leaves. This was comparable to that observed for the susceptible control Gamtoos (A-D). The DAB staining phenotypes of representative plants per treatment were observed at the end of the experiments.

The change in the oxidative burst was observed by staining with DAB for the presence of H_2O_2 in the RWA infested and non-infested susceptible Gamtoos (GS), resistant Gamtoos-R (GR) cultivars including the Gamtoos-R +BSMV$_0$ and Gamtoos-R +BSMV$_{TaAdnr1}$ treatment for *TaAdnr1* knockdown. (Figure 18. A-D). These changes were investigated by DAB staining the leaves where aphid feeding occurred at the end of the experiments. The Gamtoos and Gamtoos-

R control plants that were not infested with the RWASA2 and RWASA3 biotypes did not show any presence of H_2O_2 accumulation (Figure 18. B and C). However, minimal staining was observed in these plants when RWASA1 and RWASA4 biotypes were not infested (Figure 18. A and D). The Gamtoos plants exhibited mild and distinct H_2O_2 accumulation following infestation with RWASA1 and RWASA3, while infestation with RWASA2 and RWASA4 resulted in high levels of H_2O_2 accumulation in these plants. Infestation with RWASA1, RWASA2 and RWASA4 biotypes (Figure 18. A, B and D) following *TaAdnr1*-knockdown in the resistant Gamtoos-R plants resulted in leaves presenting mild or low levels of H_2O_2 accumulation when compared with the high levels of H_2O_2 accumulation in the GR and GR+BSMV$_0$ controls. Complete loss of detection of H_2O_2 accumulation was observed following infestation with RWASA3 on the GR+BSMV$_{TaAdnr1}$ compared to the minimal accumulated levels observed in the control GR and GR+BSMV$_0$ plants (Figure 18. C). The levels of detected H_2O_2 differed when the different biotypes fed on the resistant control plants. The observed levels were the lowest for RWASA3 and the most severe for RWASA4.

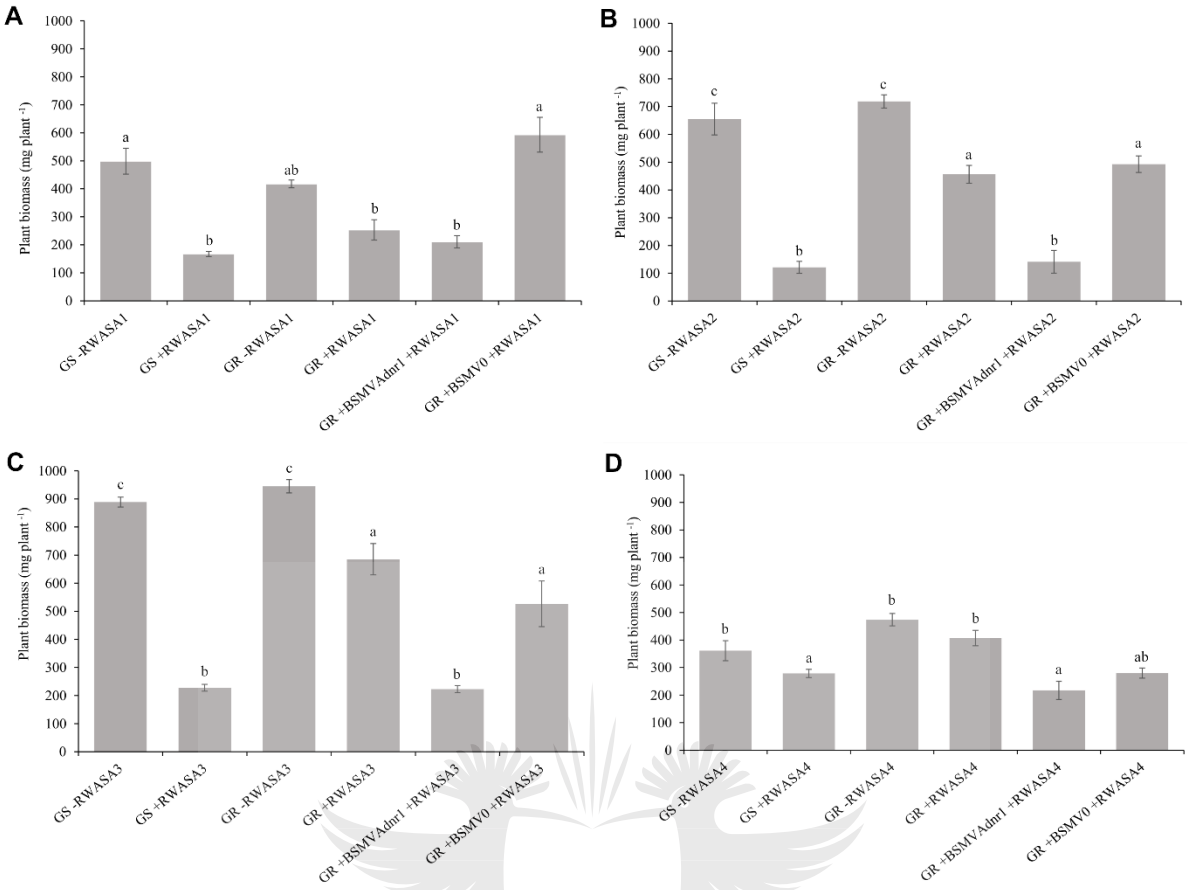

Figure 19: Total plant dry weight after 16 days of aphid feeding. The susceptible Gamtoos (-RWA) and resistant Gamtoos-R (-RWA) controls with no RWA infestation had higher plant biomass compared to the Gamtoos (+RWA) and Gamtoos-R (+RWA) infested with the different *D. noxia* biotypes (A-D). BSMV-mediated VIGS knockdown of *TaAdnr1* in the resistant plants (GR+BSMV$_{TaAdnr1}$+RWA) resulted in reduced total plant biomass compared to the Gamtoos-R (+RWA) plants following infestation with the RWASA2, RWASA3 and RWASA4 biotypes (B-D). However, feeding by RWASA1 on the GR+BSMV$_{TaAdnr1}$+RWA plants resulted in total plant biomass similar ($p \leq 0.05$) to the GR+RWA plants. The levels of plant biomass differed after feeding by the different biotypes. The different letters (a, b and c) indicate significant differences compared with the resistant control GR+BSMV$_0$+RWA with no target gene inserted, with $p \leq 0.05$ as determined by ANOVA and Tukey's HSD post hoc test.

The change in total plant biomass was measured for the infested and non-infested susceptible Gamtoos (GS) and resistant Gamtoos-R (GR) cultivars including the Gamtoos-R+BSMV$_0$ and Gamtoos-R+BSMV$_{TaAdnr1}$ treatment for *TaAdnr1* knockdown (Figure 19. A-D). Feeding by all the *D. noxia* biotypes on the control Gamtoos plants resulted in significantly lower total plant

biomass compared to the non-infested Gamtoos plants (Figure 19. A-D). RWASA1 and RWASA4 (Figure 19. A and D) feeding on the Gamtoos-R plants resulted in a similar ($p \leq 0.05$) total plant biomass compared with the noninfested Gamtoos-R (-RWA), while RWASA2 and RWASA3 (Figure 19. B and C) feeding on the Gamtoos-R plants resulted in significantly different total plant biomass compared with the non-infested Gamtoos-R (-RWA) plants. In the RWASA1 experiment, the total plant biomass of the resistant control GR+BSMV$_0$+RWA was significantly higher compared with the treated plants and similar to the non-treatred control Gamtoos (-RWA) and Gamtoos-R (-RWA) plants ($p \leq 0.05$) (Figure 19. A). In the RWASA4 experiment, the GR+BSMV$_0$+RWA biomass was similar to the GR+BSMV$_{TaAdnr1}$+RWA and the infested Gamtoos-R (+RWA) plants (Figure 19. D). The RWASA2 and RWASA3 experiments resulted in similar ($p \leq 0.05$) total plant biomass between the GR+BSMV$_0$+RWA and the infested Gamtoos-R (+RWA) plants that were both significantly different to the noninfested controls Gamtoos and Gamtoos-R (-RWA). The total plant biomass of the GR+BSMV$_{TaAdnr1}$+RWA plants was severely reduced after feeding by RWASA2 and RWASA3 and was significantly less ($p \leq 0.05$) than that of the infested Gamtoos-R (+RWA) and GR+BSMV$_0$ controls (Figure 19. B and C). Feeding by RWASA1 and 4 also resulted in significantly reduced total dry biomass in the GR+BSMV$_{TaAdnr1}$+RWA plants, however, the biomass was higher and similar compared with the GR+BSMV$_0$ plants following feeding by these biotypes respectively (Figure 19. A and D). Furthermore, infestation with RWASA1 resulted in similar ($p \leq 0.05$) total dry biomass in GR +BSMV$_{TaAdnr1}$+RWA and the infested Gamtoos-R (+RWA) plants (Figure 19. A).

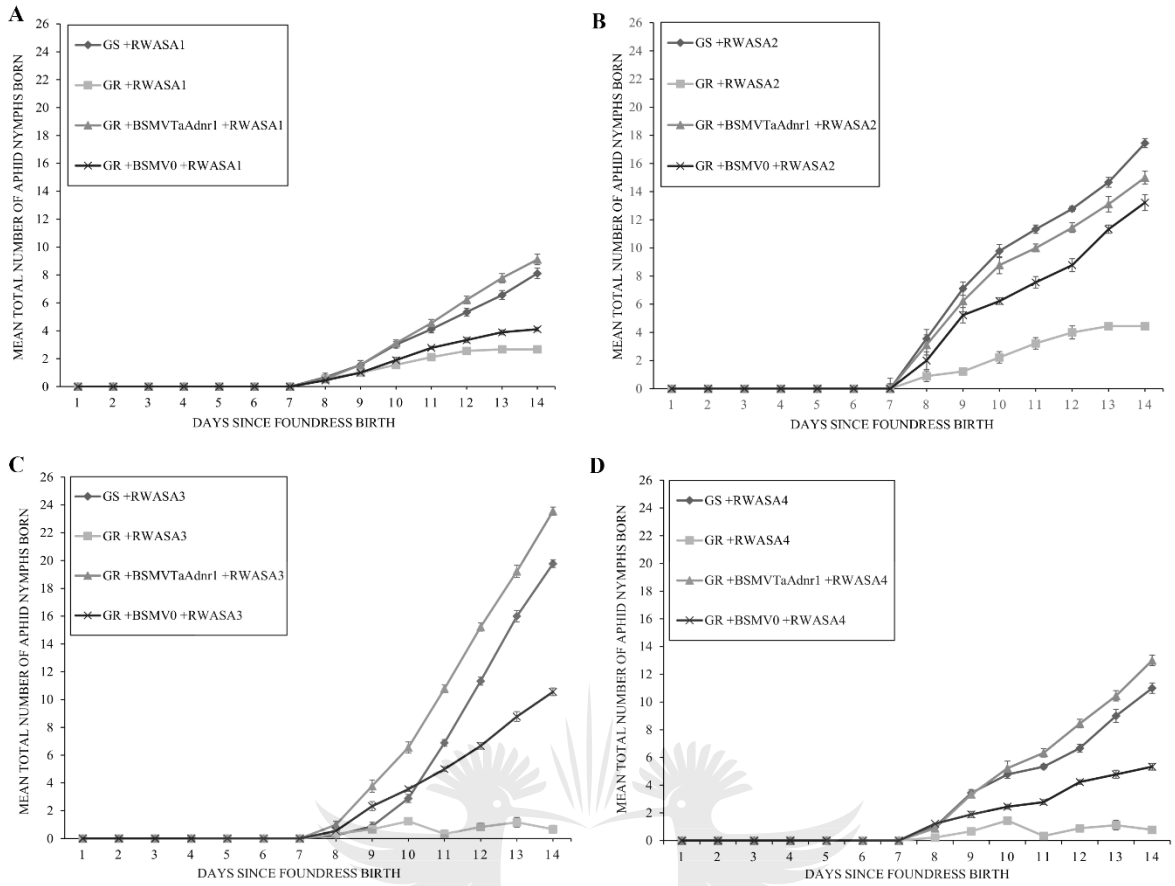

Figure 20: The mean total production of aphids on 10 plants per treatment over 16 days. For all the *D. noxia* biotypes, feeding on the susceptible controls Gamtoos (GS) cultivar and the resistant Gamtoos-R (GR+BSMV$_{TaAdnr1}$) cultivar for which *TaAdnr1* was knocked-down the highest number of mean nymph production was observed compared with the resistant Gamtoos R (GR) and Gamtoos R empty vector (GR+BSMV$_0$) controls plants. A) Represents plants infested with RWASA1, B) RWASA2, C) RWASA3 and D) RWASA4.

The different *D. noxia* biotypes responded differently to feeding on the resistant cultivar, Gamtoos-R (GR+RWASA) (Figure 20. A-D). RWASA1 and 2 recorded a higher mean total number of offspring produced compared with RWASA3 and 4. After 14 days of feeding (twenty-one days after viral infection) the first two biotypes accrued 2.67 and 4.44 offspring. The *Dn7* resistance gene in Gamtoos-R kept the levels of RWASA3 and 4 much lower at 0.67 and 0.78 total offspring (Figure 20. C and D). The biotypes also performed differently on the susceptible control plant with RWASA1 and 4 accrued much lower levels, 6.70 and 10.25, compared with RWASA2 and 3 at 19.00 and 19.77.

High mean totals of 9.11, 17, 23.6 and 13 offspring were observed for the four biotypes on the knockdown GR+BSMV$_{TaAdnr1}$ plants, respectively. These were relatively similar to the levels of offspring observed on the susceptible Gamtoos (GS+RWA) control plants (8.11, 17.4, 19.8 and 11 respectively) for the four biotypes. Furthermore, the *D. noxia* biotypes RWASA2 and RWASA3 responded differently to feeding on the susceptible Gamtoos cultivar maintaining higher levels 17.4, 19.8 of offspring than the RWASA1 and RWASA4 biotypes with 8.11 and 11 offspring. In contrast, the infested resistant Gamtoos-R (+RWA) and empty vector control, GR+BSMV$_0$, produced lower mean total numbers of offspring compared to the infested susceptible control Gamtoos (+RWA) and the knockdown line GR+BSMV$_{TaAdnr1}$. High mean total numbers of 13.2 and 10.6 offspring produced were observed for RWASA2 and RWASA3 on the GR+BSMV$_0$ plants.

Table 2: The mean intrinsic rates (r_m) of the *D. noxia* biotypes on the Gamtoos, Gamtoos-R and the Gamtoos-R BSMV-treatments including the standard deviations (*Sd*). The letters (a and b) indicate treatments with a statistically significant difference from the infested Gamtoos-R (+RWA) control (P<0.05).

	Biotype							
	RWASA1		RWASA2		RWASA3		RWASA4	
Treatment	Mean r_m	*Sd*	Mean r_m	*Sd*	Mean r_m	*Sd*	Mean r_m	*Sd*
GS	0.167b	0.042	0.309b	0.022	0.234b	0.030	0.260b	0.023
GR	0.076a	0.053	0.133a	0.097	0.131a	0.040	0.161a	0.032
GR +BSMV$_{TaAdnr1}$	0.178b	0.064	0.276b	0.097	0.281ab	0.033	0.265b	0.022
GR +BSMV$_0$	0.120a	0.048	0.284b	0.060	0.204c	0.031	0.148a	0.028

Sd represents the standard deviation; GR and GS represent Gamtoos-R and Gamtoos wheat cultivars respectively, BSMV$_0$ represents the empty vector viral inoculation control and BSMV$_{TaAdnr1}$ represents the *TaAdnr1* silencing treatment.

The different *D. noxia* biotypes feeding on the GR+BSMV$_{TaAdnr1}$ plants had a significantly higher mean intrinsic rate (r_m = 0.178, 0.276, 0.281 and 0.265 respectively) compared with the aphids feeding on the infested Gamtoos-R (+RWA) plants (r_m = 0.076, 0.133, 0.131 and 0.161) (Table 2). These high intrinsic rates were not significantly different from aphids feeding on the

infested susceptible control Gamtoos (+RWA) plants (r_m = 0.167, 0.309, 0.234 and 0.260). The RWASA1 and RWASA4 biotypes feeding on the resistant empty vector control, GR+$BSMV_0$, plants also had a low mean intrinsic rate (r_m = 0.120 and 184 respectively) and were not significantly different from aphids feeding on the Gamtoos-R plants for the same biotypes. However, RWASA2 and RWASA3 feeding on the GR+$BSMV_0$ plants indicated an increase in the mean intrinsic rate (r_m = 0.284 and 0.204, respectively) that was significantly different from the aphids feeding on the respective Gamtoos-R plants. The different letters indicate (a, b and c) the statistical difference between the samples ($p \leq 0.05$, ANOVA and Tukey's HSD post hoc test).

Chapter 5

Discussion

5.1 General discussion

In this study, we showed that the knockdown of *TaAdnr1* in wheat harbouring *Dn7*-mediated resistance perturbs incompatible associations during the wheat-*D. noxia* interaction resulting in a classical susceptible phenotype. This is supported by the loss of the *Dn7* antibiotic resistance mechanism including the loss of both the oxidative burst and the hypersensitive response. Furthermore, the increase in aphid fecundity, production of susceptible phenotypic symptoms and loss of plant biomass provided evidence for the loss of the *Dn7* resistance response.

The rye cultivar Turkey 77 harbours a resistance gene, designated as *Dn7*, against *D. noxia*. This gene was transferred to a South African wheat cultivar Gamtoos that consists of a lBL-lRS Veery translocation (Marais *et al.*, 1994, 1998). The resistance response exhibited by the *Dn7* gene is characterised as antibiosis and is linked with the oxidative burst in the HR that is mediated by a rapid accumulation of H_2O_2 (Nkongolo *et al.*, 1991; Ma *et al.*, 1998; Liu *et al.*, 2001; van Eck *et al.*, 2010).

Sequencing of *TaAdnr1* from the susceptible Gamtoos and the resistant Gamtoos-R NIL indicated there were no SNPs compared to the Chinese Spring reference. This was consistent with the data compiled by Nicolis and Venter (2018) where the *TaAdnr1* homologue in a *Dn1* containing background (Tugela DN) was also not different. The *Dn1* and *Dn7* resistance genes both impart resistance against the RWASA1 biotype, with *Dn7* also providing resistance against the subsequent three new aphid biotypes (RWASA 2, 3 and 4) (Jankielsohn *et al.*, 2016). There was no significant upregulation of *TaAdnr1* observed in the Gamtoos-R compared to Gamtoos upon *D. noxia* infestation.

The role of *TaAdnr1* in *Dn7*-mediated *D. noxia* resistance was further investigated using the BSMV-VIGS transcript level knockdown. In the RWASA1 and RWASA2 experiments, 50 % and 54% knockdown levels were achieved respectively compared to the controls. These levels are consistent to those achieved in other VIGS based studies with knockdown levels between

50 % and 85 % (Scofield *et al.*, 2005; van Eck *et al.*, 2010; Schultz *et al.*, 2015). Higher levels of knockdown were achieved in the experiments with RWASA3 and RWASA4 compared to the controls with 89 % and 78 %, respectively. Interestingly downregulation of *TaAdnr1* in the empty vector ($BSMV_0$) treated plants with 46 % and 61 % was observed. However, the phenotypic responses characteristic of the *Dn7* antibiotic resistance was present in all these plants. The mean total dry plant biomass for the Gamtoos-R $BSMV_0$ treatments was not similar to the controls ($p \leq 0.05$) for the RWASA3 and RWASA4 experiments. Therefore, this downregulation did not affect the biomass of the plants. Also, the aphid fecundity and intrinsic rates of reproduction were also not increased. Furthermore, H_2O_2 also accumulated in specific regions of aphid feeding. Taken together, these findings indicate that the Gamtoos-R $BSMV_0$-treated plants may have been affected by the viral inoculation, however, the plants maintained their resistance to *D. noxia* similar to the Gamtoos-R infested control plants.

5.2 Knockdown induces susceptibility phenotypes on resistant plants

The GR+$BSMV_{TaAdnr1}$ +RWA plants displayed leaf rolling phenotypes similar to the infested susceptible Gamtoos +RWA plants. Feeding by *D. noxia* induces two types of leaf rolling phenotypes on susceptible wheat plants. The first is observed in mature and expanded leaves and the second occurs in emerging young leaves in which unfolding is prevented as the leaf develops. These leaf rolling phenotypes are suggested to be caused by *D. noxia* salivary fractions that activate Ca^{2+} and/or K^+ ion fluxes across the plasma membranes resulting in increased turgor and rapid expansion of the cells in the adaxial or abaxial surfaces of leaves (Botha *et al.*, 2005). Therefore, the presence of *TaAdnr1* in the susceptible plants may be responsible for mediating some level of aphid recognition and this can also be linked with the minimal oxidative burst observed in the susceptible plants.

Saheed *et al.* (2007), suggested that *D. noxia* feeding causes damage to the wheat cellular transport system especially in the conducting elements and results in the chlorosis and necrosis phenotype. This was observed in both the infested susceptible Gamtoos plants and also the resistant Gamtoos-R *TaAdnr1*-knockdown plants. Susceptible *D. noxia*-infested wheat suffer a reduced photosynthetic capacity that is caused by damage to the vascular parenchyma cell membranes and chloroplasts resulting in plasmolysed cells (Botha *et al.*, 2006; Saheed *et al.*, 2007). Chlorosis develops following these alterations and is mainly due to the reduction in the

plant photosynthetic pigments and chlorophyll. However, this effect has not been observed in resistant plants exhibiting a *Dn1* and *Dn7* resistance response against *D. noxia* (Heng-Moss *et al.*, 2003; Botha *et al.*, 2006; van Eck *et al.*, 2010).

The infested resistant Gamtoos-R and Gamtoos-R+BSMV$_0$ +RWA plants displayed an HR-like phenotype characteristic of the *Dn7* resistance response (Figure 8. A-D). The HR involves the rapid death of the cells around the area of *D. noxia* feeding. The cytoplasm of the cells of the mesophyll and bundle sheath around this region of feeding collapse as a result of cellular events such as changes in cytoplasmic streaming, shrinking of the protoplast and accumulation of granules (Belefant-Miller *et al.*, 1994; Botha *et al.*, 2005; Morel and Dangl, 1997). This is accompanied by the rapid production of high levels of ROS and polymerisation of phenolics. Taken together, these processes result in the development of necrosis around the aphid feeding site. Furthermore, surrounding cells undergo lignification and cross-linking of cell wall proteins that strengthen the cell wall to prevent further penetration (Brisson *et al.*, 1994; Morel and Dangl, 1997; Fath *et al.*, 2000; Botha *et al.*, 2005). Due to the varying levels of virulence potential of the different *D. noxia* biotypes, the extent of chlorotic and necrotic damage varies (Jankielsohn, 2017).

The plant HR is suggested to be triggered upon the perception of a specific pathogen effector by a corresponding R-protein in the host plant and this is proposed by the original *avr-R* gene product interaction model (Flor, 1971; Scofield *et al.*, 1996; Tang *et al.*, 1996). This was evident in the resistance gene product of tomato, *Pto,* interacting with the *P. syringae* pv. tomato *avrPto* gene product (Scofield *et al*, 1997; Tang *et al*, 1997). The stimulated oxidative burst by the rapid influx of Ca^{2+} is also associated with HR during plant-pathogen interactions (Levine *et al.*, 1996). In combination with this, the NLR protein TIR and CC domains have been determined to activate a hypersensitive cell death response (Cui *et al.*, 2015). A recent study on the CC-domain containing NLR ZAR1 resistosome showed that ZAR1 induced a cell death response and resulted in the subsequent rapid activation of stress-induced defence genes (Wang *et al.*, 2019a, 2019b). Therefore, these results suggest that a link exists between *TaAdnr1* recognition of *D. noxia* and the oxidative burst as well as the HR-linked cell death. This was evident by the loss of the characteristic disease resistance response mediated by the

Dn7 gene that resulted in susceptible phenotype following knockdown of *TaAdnr1* in all instances.

5.3 Knockdown alters the oxidative burst

The ROS, namely H_2O_2, the O_2^- and the hydroxyl radical (OH) are constitutively produced at relatively low levels in plants cells. Following pathogen infection, two phases of ROS accumulation are observed during PTI and ETI respectively. The second phase that occurs during ETI, consists of a much stronger and prolonged production of intracellular ROS (Wojtaszek, 1997; Kadota *et al.*, 2015). Production occurs mainly in chloroplasts and mitochondria and is also mediated by cellular enzymes that participate in redox reactions. The enzymes involved include nicotinamide adenine dinucleotide hydrogen (NADH) oxidases and NADH-dependent cytochrome c reductases (Wojtaszek, 1997). Intracellular ROS play a role in signalling by affecting the expression of genes involved in defence (Apel and Hirt, 2004; Straus *et al.*, 2010). The event involving the rapid accumulation of the ROS known as the oxidative burst results from elicitation by external stimuli from pathogens such as viruses, bacteria and fungi and also herbivore insects (Mehdy, 1994; Apel and Hirt, 2004). It is one of the early events that occur during defence responses against pathogen or insect invaders and is driven by the production of high levels of ROS, mainly H_2O_2 (Wojtaszek, 1997).

DAB staining for detection of H_2O_2 in plant tissue results in the development of dark brown deposits in the presence of redox enzymes such as peroxidases (van Eck *et al.*, 2010; Daudi, 2016). In this study, the controls Gamtoos-R and GR+$BSMV_0$ leaves exhibited elevated production of H_2O_2 following infestation with the different *D. noxia* biotypes (RWASA1-4) in *Dn7*-mediated resistance compared with the infested susceptible control Gamtoos. Moreover, the H_2O_2 accumulation patterns were either located in specific regions of aphid feeding or spread across the veins of the leaf indicating that the stress response was triggered at these sites or could be transported through the leaf vein. This may suggest that either H_2O_2 is produced in these regions and/or may move from different parts of the leaf, into the veins and eventually reach the *D. noxia* feeding site (Schultz *et al*., 2015). This is consistent with the occurrence of higher H_2O_2 accumulation levels observed in the infested control Gamtoos-R and the GR +$BSMV_0$ leaves in *Dn7*-mediated resistance against *D. noxia* (van Eck *et al.*, 2010). However, the RWASA2 and 3 biotypes have since maintained virulence against the *Dn1* gene and there

is currently no report on the level of oxidative burst elicited by these biotypes in wheat plants exhibiting a hypersensitive defence response. Here we observed mild to relatively high levels of H_2O_2 accumulation following infestation with RWASA2 and 3 biotypes respectively. This suggests that both these biotypes elicit the HR observed in *Dn7*-mediated increase linked with the production of H_2O_2. Although the observed H_2O_2 accumulation varied, it is important to note that the *D. noxia* biotypes vary in their virulence potential to different wheat resistant genes (Jankielsohn, 2017).

VIGS knockdown of the wheat defence-related genes *WRKY53* and *PAL* resulted in the disruption of the rapid *Dn7*-mediated production of H_2O_2 following *D. noxia* infestation (van Eck *et al.*, 2010). Knockdown of HR-associated genes using VIGS, resulted in decreased H_2O_2 production in the defence response mediated by the *Dn1* resistance gene (Schultz *et al.*, 2015). Consistent with these studies, we observed reduced levels of H_2O_2 in the infested GR +BSMV$_{TaAdnr1}$ plants compared with the infested Gamtoos-R and GR +BSMV$_0$ controls. This indicated that the rapid *Dn7*-mediated production of H_2O_2 was disrupted in all aphid infestation instances. *D. noxia* infestation of susceptible wheat lines resulted in lower levels of H_2O_2 accumulation compared to the resistant lines (Moloi and van der Westhuizen, 2006). This may be attributed to the first phase of ROS accumulation during PTI that is weaker and occurs for a short period in the apoplast (Kadota *et al.*, 2015). The recognition of pathogen effectors by NLR proteins are associated with the induction of a strong ROS burst and the HR response (Jwa and Hwang, 2017) and increased H_2O_2 levels are linked to the lignification of cells in the xylem, phloem and also in epidermal cells (Jones and Dangl, 1996). It is also directly toxic to invading pathogens, drives oxidative cross-linking of the cell wall for protection, induces the HR and mediates the subsequent SAR. Salivary fractions from aphids induce host reactive oxygen species during plant immunity (Wojtaszek, 1997; Tjallingii, 2006). The results in this study suggest that recognition of aphid infestation was hampered upon knockdown of *TaAdnr1* and the general oxidative stress response at or near the site of feeding was lost. Therefore, *TaAdnr1* may function in cooperation with *Dn7* in mediating the oxidative burst and subsequent signalling of defence-related genes.

5.4 Knockdown increases aphid fecundity and intrinsic rates of reproduction

Resistant plant varieties can deter aphid infestations by affecting their fecundity, natural longevity of life of both adults and offspring, and also population maintenance (Painter, 1985). The *D. noxia* reproductive ability or fecundity was measured by determining the mean total number of nymphs produced over some time during their life and also estimating the mean intrinsic rate of increase (r_m) (Wyatt and White, 1977; Van Eck *et al.*, 2010).

van Eck *et al.* (2010), found that the BSMV-associated physiological changes resulting from viral infection affected aphid behaviour by reducing the reproduction rate but to a lesser extent. Furthermore, Scofield *et al.* (2005), showed that infection with the BSMV control virus that did not contain a silencing fragment exhibited less severe viral symptoms in wheat. Therefore, the increased mean intrinsic rate (Table 1) and the high mean total number of offspring observed for RWASA2 and RWASA3 may be attributed to the effects of viral infection in the control GR +$BSMV_0$ plants. However, other VIGS studies indicated that the BSMV viral infection did not affect gene knockdown (Cloutier *et al.* 2007; van Eck *et al.* 2010). Knockdown of *TaAdnr1* in the resistant plants permitted an increase in both the mean total production of offspring and the mean intrinsic rate that resulted due to increased susceptibility against *D. noxia*. Therefore, the *Dn7*-mediated antibiotic and antixenotic responses against *D. noxia* were negatively affected and the aphid fecundity was enhanced in all instances. This is consistent with the loss of the oxidative burst and the susceptibility symptoms exhibited on all *TaAdnr1* knockdown plants following *D. noxia* infestation. This supports the results of Nicolis and Venter (2018), that showed knockdown of *TaAdnr1* in the *Dn1*-mediated antibiotic resistance response resulted in an increased reproductive ability of *D. noxia*.

5.5 Knockdown reduces plant biomass

D. noxia feeding causes a reduction in total plant dry weight in susceptible genotypes (Burd and Burton, 1992; Smith *et al.*, 1992; Mirik *et al.*, 2009). However, wheat plants harbouring the *Dn1* and *Dn7* resistance genes that exhibit antibiosis and permits accumulation of plant biomass following *D. noxia* infestation (Smith *et al.*, 1992). The total dry biomass of GR +$BSMV_{TaAdnr1}$ +RWA was severely reduced after feeding by RWASA2 and 3 and was significantly less ($P < 0.05$) than that of the infested resistant Gamtoos-R and GR +$BSMV_0$ controls. Feeding by RWASA1 and 4 also resulted in significantly reduced total dry biomass in

the GR +BSMV$_{TaAdnr1}$ +RWA plants, however, the biomass was higher and similar compared with the GR +BSMV$_0$ plants following feeding by these biotypes respectively. Furthermore, infestation with RWASA1 resulted in significantly similar total dry biomass in GR +BSMV$_{TaAdnr1}$ +RWA and infested Gamtoos-R. The VIGS experiments for the different RWA biotypes were conducted in the greenhouse with uneven distribution of light and also at different seasons of the year and these may have contributed to the variations observed in the GR +BSMV$_0$ +RWA and GR +RWA when assessing against the RWASA1 and RWASA4 biotypes. Scofield *et al.* (2005), observed that viral symptoms in BSMV-mediated VIGS increased during the summer season. This caused a reduction of biomass in the virus-infected leaf regions and led to the use of growth chambers to eliminate these effects. This provides an equal distribution of light and masks the viral effects on all the plants. Knockdown of *TaAdnr1* resulted in the reduction of the total dry plant biomass in *Dn1*-mediated resistance against *D. noxia* (Nicolis and Venter, 2018) and this is consistent with the results obtained in this experiment. Therefore, the loss of plant biomass accumulation and the increased *D. noxia* fecundity are associated with loss of the antibiotic and antixenotic defence response exhibited in *Dn7*-mediated resistance.

5.6 *TaAdnr1* in the aphid resistance response

Plant NLR proteins directly perceive pathogen effectors through physical interactions or indirectly through a host target that forms part of the NLR protein complex that is a target of the effector (Dodds and Rathjen, 2010a; Baggs *et al.*, 2017). The indirect model is observed in many plant-pathogen interactions (Cesari, 2018). Following the observation of unusual integrated domains onto NLR proteins that play roles in the recognition of pathogen effectors and induction of an immune response a new model was proposed referred to as the integrated decoy (ID) model (Cesari *et al.*, 2014). However, the mechanism of host plant recognition of *D. noxia* effectors and the activation of downstream signalling response by NLRs remains insufficiently understood.

In some plant CNLs, oligomerisation and/or homodimerisation activity of the N-terminal domains are required to trigger an immune signalling response (Maekawa *et al.*, 2011; Casey *et al.*, 2016; Baudin *et al.*, 2017; Wang *et al.*, 2019a). The inactive form of most CNLs is kept stable by the CC domain and NB-ARC-LRR domain molecular interactions (Rairdan *et al.*,

2008; Wang *et al.*, 2019a, 2019b). Cesari *et al.* (2014), suggest that in the activated state the molecular interactions in the N-terminal domains undergo conformational changes and become exposed to associate with other signalling components for the subsequent induction of immune responses. A recent study indicated that structural remodelling occurs in *Arabidopsis* ZAR1 upon pathogen effector recognition and its CC-domains undergo fold switching to become exposed (Wang *et al.*, 2019a). This may be true with TaADNR1 as it also consists of the integrated N-terminal ANK-domains that may possibly be exposed, together with the CC-domain, to participate in protein-protein interactions with possible cognate signalling components to activate an immune response. This is consistent with the study conducted on the wheat ANK-CNL-WRKY, YrU1, further suggesting that the presence of the ANK-domain in the N-termini indicates possible participation in protein-protein interactions and that homodimerisation in the ANK and CC domains is critical for its function (Wang *et al*., 2020).

NLRs have diverse localisations inside plant cells and upon effector-induced activation, they can accumulate in different parts of the cell such as the cytoplasm, nucleus, plasma membrane and endo-membranes such as the Golgi apparatus and the tonoplast (Qi and Innes, 2013). The *Arabidopsis* CNLs such as RPS2, RPS5, RPM1 and ZAR1 have been shown to localise at the cell membrane upon effector recognition and this requirement is important for the functional activation of the HR. (Elmore *et al*., 2012; Qi, Deyoung and Innes, 2012; el Kasmi *et al*., 2017; Wang *et al*., 2019a). Some NLRs are reported to accumulate in two different cellular parts such as the cytoplasm and nucleus or endo-membranes (Qi and Innes, 2013). The *Arabidopsis* TNL RRS1 that includes an integrated WRKY domain, was shown to interact with the bacterial effectors PopP2 and AvrRps4 in the nucleus to trigger an HR and immunity (Deslandes *et al.*, 2003; Sarris *et al.*, 2015). The localisation of plant NLRs in different parts of the cell may suggest that they activate different signalling pathways and recognise different pathogen effectors in those different locations. Similar to the WRKY containing RRS1 TNL, TaADNR1 which contains a weak nuclear localization signal may have similar nuclear and cytoplasmic localisation mediating different functions in these compartments (Qi and Innes, 2013; Sarris *et al.*, 2015).

WRKY transcription factors in plant ETI regulate the transcriptional reprogramming of genes associated with defence and may act as repressors and also activators of immunity (Eulgem *et*

al., 2000). The barley CNL resistance protein MLA was shown to recognise the fungal AVR10 effector in the cytoplasm and associates with the transcription factors HvWRKY1 and HvWRKY2 in the nucleus (Shen *et al.*, 2007). The rice CNL Pb1 was shown to associate with WRKY45 in the nucleus for resistance activation against the rice blast fungus *M. oryzae* (Inoue *et al.*, 2013). Several NLR proteins with additional C-terminal fused WRKY domains that function in pathogen disease resistance have also been reported. The *Arabidopsis* TNL-WRKY, RRS1-R, was shown to act as a decoy trap for the PopP2 and AvrRps4 effectors. These effectors disable activation of host immunity by disrupting WRKY transcription factor activity. Therefore, the additional WRKY domain acts as a mimic of the targeted WRKY transcription factors of the host and allows the NLR pair to activate immunity (Rushton *et al.*, 2010; le Roux *et al.*, 2015; Sarris *et al.*, 2016). Furthermore, *TaAdnr1* and *YrU1* were proposed to function similar to RRS1-R in recognising and binding of the *D. noxia* and stripe rust effectors on its WRKY domain for subsequent activation of immune responses (Nicolis and Venter, 2018: Wang *et al*., 2020).

Studies have reported that an NLR can fuse with another and function together in the indirect pathogen effector recognition and immune response activation. In monocots, CNLs functioning as pairs such as rice Pik-1 and Pik-2 and also RGA4 and RGA5 has been reported to control resistance to *M. oryzae* (Cesari *et al.*, 2013; Williams *et al.*, 2014). The paired *Arabidopsis* TNL protein, RRS1 and RPS4, was shown to be essential in recognising the bacterial AvrRps4 and PopP2 effectors. These NLRs together form a heterodimer through associations with their TIR domains to form a recognition and immune signalling NLR complex. Furthermore, RRS1 and RPS4 have different functions upon pathogen invasion and one might be involved in pathogen effector perception while the other in immune response signalling (Williams *et al.*, 2014). The NLRs that carries an additional C-terminal ID such as WRKY or heavy metal-associated (HMA) domains are suggested to function in pathogen effector recognition by acting as decoy binding targets (Ellis, 2016; Sarris *et al.*, 2016). We predict that TaADNR1may function to trap aphid effectors and may function in conjunction with a cognate NLR partner in which the pair forms a dimer for the perception of these effectors through the TaADNR1 WRKY domain and defence signalling. Thus, as previously postulated in *Dn1*-mediated resistance, TaADNR1 may dimerise with *Dn7* or another NLR in the resistance pathway to facilitate resistance. These results support rejection of the null hypothesis that *TaAdnr1* does not play a role in the resistance response and we accept the alternative hypothesis that it does

recognise the different biotypes and play a role in the resistance response. Furthermore, we postulate that TaADNR1 recognises the same effector from the different South African RWA biotypes for activation of resistance.

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Chapter 6

Conclusions and future work

The *TaAdnr1* NLR-ID gene plays a role in the *D. noxia*-wheat interaction (Nicolis and Venter, 2018). It contains the atypical integrated WRKY on its C-terminal region and this is interesting since transcription factors such as WRKY10, -12, and -51 and also ankyrin repeat proteins have been linked with resistance against *D. noxia* (Smith *et al*. 2010; van Eck *et al*. 2010). Knockdown of *TaAdnr1* in the wheat cultivar Gamtoos-R carrying *Dn7*-mediated resistance resulted in a highly susceptible phenotype and increased *D. noxia* fecundity from the four South African *D. noxia* biotypes (RWASA1-4). The fact that a similar susceptible phenotype was observed against all the South African RWA biotypes suggests that *TaAdnr1* recognises the same aphid effector in all of the biotypes. This would imply that this effector has not changed in the process of biotypification and that additional effectors are being employed by RWASA2-4 to perturb the resistance response further downstream of the initial recognition. The NLR-ID decoy proteins that heterodimerise to perceive effector proteins and activate defence signalling for immunity is contributing in unravelling complex processes in innate immunity against pathogens and pests. This led to further investigation of the wheat NLR-ID protein, previously reported to contribute a crucial role in the defence response in *Dn1*-mediated resistance during *D. noxia*-wheat interaction.

Future work should include characterising the NLR-ID genes in wheat cultivars carrying the *Dn1* and *Dn7* resistance genes. This will include VIGS to establish their role in various *Dn* gene-carrying cultivars. Identification of possible interaction partners in both wheat and from *D. noxia* is imperative. The additional screening of different wheat cultivars to identify allelic variation would also need to be performed. Identification of the wheat and *D. noxia* interaction partners of *TaAdnr1* will grow our understanding of the wheat defence response against pests and also how *D. noxia* as a specialist pest of wheat adapts to these responses.

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Summary

Wheat commercial production in several major producing regions is challenged by a specialist pest known as the Russian wheat aphid (*Diuraphis noxia,* RWA). Crop losses incurred as a result of aphid infestation have raised concerns in the agriculture industry around the world and also calls for control strategies. Wheat varieties providing resistance against *D. noxia* due to several inbred resistance genes were overcome by the aphid's ability to develop into differentially virulent biotypes. In South Africa, five *D. noxia* biotypes have been reported (RWASA1-5) since its discovery. The nucleotide-binding leucine-rich repeat gene (NLR), *TaAdnr1*, containing the atypical integrated WRKY and Ankyrin repeat domains, was identified to contribute a critical role in the *Dn1*-mediated gene-based resistance response in wheat against *D. noxia*. The study aimed to knock down the transcript level of the NLR receptor (*TaAdnr1*) in a resistant wheat cultivar carrying the *Dn7* resistance gene to determine the level of resistance against the South African *D. noxia* biotypes. VIGS-mediated knockdown of *TaAdnr1* resulted in the typical *Dn7*-mediated resistance response exhibiting a susceptible phenotype. This was determined by the development of the susceptible phenotype, increase in the aphid fecundity and intrinsic rates of reproduction, decrease in dry weights and the loss of the oxidative response in the resistant plants. These findings suggest that knockdown of *TaAdnr1* underlies resistance to the South African *D. noxia* biotypes in *Dn7*-mediated resistance in wheat. Furthermore, this raises the possibility that *TaAdnr1* may recognise the same elicitor/effector from the different *D. noxia* biotypes and that biotypification may rely on additional effectors that target the defence pathway by bypassing initial aphid recognition.