Elucidating functional interactions between the Russian wheat aphid (*D. noxia* Kurjumov) and bread wheat (*Triticum aestivum* L.)

By

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Declaration

By submitting this dissertation electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

This dissertation includes two original papers submitted to peer-reviewed journals and one unpublished publication. The development and writing of the papers (published and unpublished) were the principal responsibility of myself and, for each of the cases where this is not the case, a declaration is included in the dissertation indicating the nature and extent of the contributions of coauthors.

Date: August 2014

English Abstract:

The Russian wheat aphid (*Diuraphis noxia*, Kurdj., Hemipetra, Aphididae, RWA) is an important pest of wheat, causing large-scale damage and yield losses. Various studies have been done at a transcriptomics level, including complementary DNA-amplified fragment length polymorphisms (cDNA-AFLPs), suppressive subtractive hybridization (SSH) and micro-array, which have identified genes putatively involved in RWA resistance. Even though these candidate genes have been identified, their role in host defence still needs to be verified using a functional genetics approach. In this study virus induced gene silencing (VIGS) using a barley stripe mosaic virus (BSMV) vector, has been utilized to knock-down candidate genes of interest in a wheat cultivar with the *Dn1*-resistance gene (TugelaDN). In this study it was hypothesized that genes involved in the hypersensitive response (HR) may contribute towards resistance and were thus targeted for silencing. These include glutathione-*S*-transferase (*GST*), superoxide dismutase Cu/Zn (*SOD*) and thylakoid-associated ascorbate peroxidase (*tAPX*). However, since aphid feeding also results in wounding, the genes were also analyzed under wounding only. Aphid fecundity is considered an indicator of involvement in RWA resistance, as susceptible plants result in higher aphid fertility. Findings in the study suggest that with wounding only, that *Dn1* containing plants produce a greater hypersensitive response than susceptible controls. Ascorbate peroxidase was found to be important for wounding-induced resistance in *Dn1* wheat plants. Under infestation conditions, silencing of superoxide dismutase Cu/Zn (*SOD*) and thylakoid-associated ascorbate peroxidase (*tAPX*) was found not to have an effect on aphid fertility and thus are not directly involved in resistance signaling. Knock-down of a *phi*-class glutathione-*S*-transferase F6 (*TaGSTF6*) transcripts however, had a large effect on aphid nymph numbers and thus may contribute to *Dn1*-resistance. Putative resistance genes silenced under aphid infestation conditions were a nucleotide binding protein (*NBP*) and resistance gene analogue 2 (*RGA2*). Analysis of *NBP* revealed its identity as a part of the iron homeostasis machinery in the cytosol, responsible for Fe-cluster assembly. Silencing of both *NBP* and *RGA2* resulted in the expression of a susceptible phenotype. *T10rga2-1A* is an NBS-LRR protein known to be required for rust resistance in concert with resistance gene *Lr10*. *T10rga2-1D* silenced treatments resulted in susceptibility and plant death after aphid infestation, suggesting that *T10rga2-1D* may be a good up-stream candidate in *Dn1*-resistance.

Afrikaanse Opsomming:

Die Russiese-koringluis (RWA) is ‘n pes wat ‘n belangrike ekonomiese invloed op koring opbrengste het en infestasie kan tot grootskaalse skade en oes verlies lei. Verskeie studies, onder andere komplimentêre DNA amplifiseerde fragment polimorfismes (cDNA-AFLPs), onderdrukkende onderskeidende hibridisaie (SSH) en mikro-reekse wat voorheen op transkriptomiese vlak gedoen is, het moontlike gene wat by RWA weerstand betrokke is, geïdentifiseer. Alhoewel hierdie gene reeds geidentifiseer was, hulle rol is nogtans onbekend. Dié gene moet nog getoets word, duur funksionele genetiese benaderingste maak. In hierdie studie is ‘n gars streep mosaïek virus vektor (BSMV) gebruik om kandidaat-gene van belang in ‘n *Dn1*-weerstandige geen-bevattende kultivar (TugelaDN) te onderdruk. Ondrukking van gene het deur middel van virus geïnduseerde geen onderdrukking (VIGS) plaasgevind. In hierdie studie is die hipotese gestel dat die gene betrokke by die hipersensitiewe reaksie (HR) ‘n invloed op plantweerstand kan hê en is dus geteiken vir geen-onderdrukking-studies. Hierdie gene het die volgende ingesluit: glutatioon-*S*-transferase (GST), superoksied dismutase Cu/Zn (SOD) en askorbien peroksidase (APX). Egter, omdat luisinfestasie ook tot verwonding aanleiding gee, is die onderdrukte gene ook onder alleenlik verwondingstoestande getoets. Luis vrugbaarheid is gebruik as indikator van betrokkenheid omdat meer vatbare plante ‘n hoër luis vrugbaarheid tot gevolg het. In die studie is gevind dat onder alleenlik verwondingkondisies, plante wat *Dn1* bevat, ‘n groter hipersensitiewe respons vertoon, as vatbare kontroles. Daar is verder gevind dat askorbien peroksidase ‘n belangrike rol tydens verwondings-geïnduseerde weerstand in *Dn1*-plante speel. Daar is verder bevind dat die onderdrukking van superoksied dismutase *Cu/Zn (SOD)* en ‘n tilakoïed-geassosïeerde askorbien peroksidase (*tAPX*). Onder luis-infestasie kondisies, geen effek op luisvrugbaarheid gehad het nie en dus nie direk by die weerstandsrespons betrokke is nie. Die onderdrukking van ‘n phi-klas glutatioon-*S*-transferase F6 (*TaGSTF6*) het egter ‘n groot invloed op luis-vrugbaarheid gehad en kan dus ‘n rol in *Dn1*-weerstand speel. Die moontlike weerstands gene, geïdentifiseer as nukleotied bindings proteïen (*NBP*) en weestandsgeen anoloog 2 (*T10rga2-1D*), is getoets onder luis-infestasie kondisies. Die analise van *NBP* het getoon dat dit ‘n integrale deel van die yster homeostase meganisme in die sitosol, wat vir Fe-kluster samestelling verantwoordelik is, vorm. Onderdrukking van beide die *NBP* en *T10rga2-1D* het tot die uitdrukking van ‘n vatbare fenotipe aanleiding gegee. *T10rga2-1A* is ‘n NBS-LRR proteïen wat bekend is om noodsaaklik te wees tydens roes weerstandigheid in teenwoordigheid van die weerstandsgeen *Lr10*. *T10rga2-1D*-onderdrukte behandelings het tot vatbaarheid aangeiding gegee en daartoe gelei dat plante na luis-infestasies doodgaan. Hierdie resultate dui dus ‘n rol vir *T10rga2-1D* in *Dn1*-weerstandigheid aan, en suggereer verder dat hierdie geen ‘n goeie stroom-op kandidaat in *Dn1*-weerstandigheid is.

List of Abbreviations

APX	ascorbate peroxidase
BSMV	barley stripe mosaic virus
CC	coiled coil
DAB	3,3-diaminobenzidine
Dn	*Diuraphis noxia*
ETI	effector triggered immunity
Fe	iron
GPX	glutathione peroxidase
GST	glutathione-*S*-transferase
H_2O_2	hydrogen peroxide
HR	hypersensitive response
JA	jasmonic acid
LRR	leucine rich repeat
MAMP	microbial associated molecular pattern
MeJA	methyl jasmonate
MeSA	methyl salicylate
NBP	nucleotide-binding protein
NBS	nucleotide-binding site
NILs	near isogenic lines
ODA	octadecanoid pathway
OGA	oligogalacturonide acids
PAMP	pathogen associated molecular pattern
PI	proteinase-inhibitor
PR	pathogenesis-related
PTGS	post-transcriptional gene silencing
PTI	PAMP triggered immunity
RGA	resistance gene analogue
RdRp	RNA-dependant RNA poymerase
RT-qPCR	quantitative real-time PCR

ROS	reactive oxygen species
RWA	Russian wheat aphid
SA	salicylic acid
SAR	systemic acquired resistance
SOD	superoxide dismutase-Cu/Zn
SWRPs	systemic wound response protein
TIR	toll/interleukin-1/resistance
VIGS	virus induced gene silencing

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Table of contents

Chapter 1
Introduction

1.1 General introduction

Wheat (*Triticum aestivum* L.) is cultivated in many countries around the world such as China, India, the USA, France, Russia and Australia. A record 675 mil. metric tonnes of wheat was produced for the 2012-2013 season worldwide, 100 mil. metric tonnes more than reported a decade ago. As wheat is cultivated as a monoculture, it is not only vulnerable to abiotic stressors such as drought or flood, but also to pests and pathogens. Stress brought about by climate change, such as heat and water availability will ultimately result in yield losses (Schlenker and Lobell, 2010) and susceptibility to emerging infectious diseases (Anderson *et al*., 2004).

One of the insect pests that cause economic losses in wheat crops in the USA and South Africa at present is *Diuraphis noxia* (Kurdj., Hemipetra, Aphididae), commonly known as the Russian wheat aphid (RWA). In warmer, drier conditions, *D. noxia* is known to thrive, while under good rainfall conditions, population growth is diminished (Merril and Peairs, 2012). Outbreaks of *D. noxia* populations lead to large scale yield and economic losses. Even though *D. noxia* resistant germplasm is available, new biotypes resistant to existing cultivars have emerged rendering proven resistant wheat cultivars containing single resistance genes susceptible (Haley *et al*., 2004; Jankielsohn 2011; Jyoti and Michaud, 2005; Tolmay *et al*., 2007; Randolph *et al*., 2009). In order to breed sustainable resistant cultivars, causal agents and biological pathways involved in resistance need to be elucidated.

In previous studies the *D. noxia* resistance gene *Dn1* is characterized as conveying an antibiosis-mode of resistance, as well as expressing a hypersensitive response, in response to *D. noxia* biotype SA1 (RWA-SA1) infestation (Du Toit 1989; Van Der Westhuizen *et al*., 1998a,b). Also, using transcriptomic studies, regulated genes have been identified within resistant cultivars during *D. noxia* infestation (Botha *et al*., 2008, 2010; Smith *et al*., 2010). These studies suggested the involvement of glutathione-*S*-transferase F6 (GST), superoxide dismutase Cu/Zn (SOD), ascorbate peroxidase (APX), or potential resistance genes encoding a nucleotide binding protein (NBP) and Fe-S sulphur cluster assembly protein (T10RGA2-1D) in *Dn1* resistance against RWA-SA1. Genes regulated during the *Dn1* resistance response need to be functionally characterized before the level of involvement of each can be established. It is for this reason that the genes previously mentioned were assessed employing virus induced gene silencing (VIGS), within the 'TugelaDN' cultivar, as well as a RWA-SA1 infestation treatment.

1.2 Aim

The aim of our study was thus to silence hypersensitive response related genes glutathione-*S*-transferase F6 (*GSTF6*), superoxide dismutase Cu/Zn (*SOD*), ascorbate peroxidase (*APX*), or potential resistance genes nucleotide binding protein (*NBP*) and Fe-S sulphur cluster assembly protein (*T10RGA2-1D*) in order to guage involvement in *D. noxia* resistance in TugelaDN.

1.3 Technical objectives

To address the aims of this functional genetics study, the following technical objectives were set:

- Optimize the VIGS technique in TugelaDN
- Clone VIGS fragments from the genes of interest into the barley stripe mosaic virus (BSMV) vectors
- Create reciprocal RT-qPCR primers for each of the genes of interest
- Knock down transcript levels of the genes of interest in TugelaDN
- Confirm silencing with RT-qPCR
- Measure aphid fertility on silenced and control plants
- Measure peroxidase activity or GST activity when relevent
- Stain leaves to phenotype H_2O_2 presence or absence
- Contribute to knowledge of genes involved in *D. noxia* resistance

1.4 Hypothesis statement

Silencing of putative *D. noxia* resistance genes and hypersensitive response related genes will render TugelaDN susceptible, increase the fertility of the aphid and affect H_2O_2 production. Evidence that the genes are required for *D. noxia* resistance in TugelaDN will be garnered from aphid fertility counts, protein activity assays and phenotyping.

1.5 Layout of thesis

This study utilizes a functional genetics technique that knocks-down available transcripts of target genes in order to investigate the relationship between *D. noxia* and the resistant TugelaDN cultivar. Each chapter has an abstract, introduction, materials and methods, results and discussion. This thesis is divided into the following chapters:

Chapter 2 is a survey of literature focussing on wheat as an economically important crop and the Russian wheat aphid or *Diuraphis noxia*. The plant pest/pathogen resistance systems are discussed in order to place studies on *D. noxia* as an insect pest of wheat into perspective. Studies on the interaction of *D. noxia* and wheat at the genetic and protein levels are discussed.

Chapter 3 focused on the antioxidant enzymes GSTF6, SOD and tAPX that are involved in detoxification during the hypersensitive response. Their requirement during the *D. noxia* resistance response is not known and was evaluated by utilizing the VIGS technique to knock-down transcript levels of each. The treated plants were evaluated utilizing aphid fertility measurements, H_2O_2 staining as well as protein activity assays.

Chapter 4 describes the use of VIGS to investigate two putative resistance genes, *NBP* and *T10RGA2-1D* that are silenced in TugelaDN under infestation conditions. The slienced plants were appraised utilizing aphid fertility measurements, H_2O_2 staining, as well as protein activity assays.

Chapter 5 utilized TugelaDN plants in which the *SOD*, *tAPX* and *TaGSTF6* were silenced. These plants were assessed under abiotic stress conditions by utilizing a wounding treatment. H_2O_2 staining as well as protein activity assays were used to evaluate the differences between the Tugela and TugelaDN NILs, as well as the effects of silencing.

Chapter 6 is a general conclusion to the thesis.

The findings presented in this thesis represent the results of a study undertaken between July 2010 and July 2014 in the Department of Genetics, Stellenbosch University, under the supervision of Prof. AM Botha-Oberholster. Chapter 3 has been accepted for publication in *Physiologia Plantarum* (ISI Web of Knowledge IF = 3.656), while Chapter 4 has been submitted for review to *Functional Plant Biology* (previously known as Autralian Journal of Plant Physiology; ISI Web of Knowledge IF = 2.471). Chapter 5 has been prepared as a manuscript, to be submitted at a later date.

1.7. Outputs related to the study:

Schultz T, Botha A-M. (2010) Characterization of Resistance Pathways activated by *Dn1* and *Dn2* resistance genes in Betta wheat infested by *Diuraphis noxia*. International Plant Resistance against Insects Workshop, SC, 2010. (April 2010, Hampton Inn West Ashley, Charleston, USA) (PAPER)

Schultz T, Botha A-M. (2011) Russian wheat aphid feeding results in cross-talk between SAR and wounding signaling pathways. 1st Cereal Biotechnology and Breeding Congress. (May 2011, IH Events center, Sveged, Hungary) (PAPER)

Schultz T, Botha A-M. (2012) Silencing of Russian wheat aphid resistance response related genes using viral induced gene silencing. International Pant Resistance against Insects Workshop, MN, 2012. (April 2012, University Hotel Minneapolis, Minneapolis, USA) (PAPER)

Schultz T, Botha A-M. (2012) Silencing of hypersensitive response related genes involved in Russian wheat aphid resistance. South African Genetics & Bioinformatics and Computational Biology Society Conference 2012. Pg53 (September 2012, Stellenbosch University, Stellenbosch, South Africa) (PAPER)

1.8. Other outputs

Peer reviewed papers published:

Van Eck L, **Schultz T**, Leach JE, Scofield SR, Peairs FB, Botha A-M, Lapitan NLV. (2010) Virus-induced gene silencing of WRKY53 and an inducible phenylananine ammonia-lyase in wheat reduces aphid resistance. *Plant Biotechnology Journal*. pp 1-10

Book Chapters:

Botha A-M, van Eck L, Jackson CS, Burger NFV, **Schultz T**. (2012) Phloem feeding insect stress and photosynthetic gene expression. *In* Applied Photosynthesis, Edited by Dr. Mohammad Najafpour. ISBN 979-953-307-664-4

Awards

Finalist in the THRIP best black or female student category for the DTI technology awards 2010

Best PhD talk: South African Genetics Society (2012): Silencing of hypersensitive response related genes involved in Russian wheat aphid resistance

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Chapter 2

Literature review

2.1. Wheat

Wheat is a member of the grass family (*Poaceae*), along with amongst others, rice, maize, barley, sorghum and rye. This family therefore contains most of the economically important crop species used as staples around the world. Rice is one of those with high economic importance and has a relatively small diploid genome of 430 Mbp with (2n=24); it was thus chosen as the model species for the *Poaceae*. *Triticum aetsivum* L., or bread wheat, has a hexaploid genome and is an allopolyploid (2n = 6x = 42). The wheat genome is large in comparison to other plant genomes with an estimated 17 000 Mbp (Brenchley *et al*., 2012) and consists of three genomes A, B and D (Arumuganathan and Earle, 1991). The donors of the three genomes are thought to be *Triticum urartu* (AA), *Aegilops speltoides* (BB), and *Aegilops tauschii* (DD) (Feldman and Levy, 2005).

In 2003, Sorrells *et al*., created a comparative map between the rice and wheat genomes using bioinformatics, deletion mapping BAC/ PAC clones and sequence and genome comparisons. Their data however, revealed numerous discontinuities in gene order between the two genomes and came to the conclusion that for genes that may be species specific, direct information from the wheat genome will be necessary.

In 2009 the barley genome sequence provided another base for comparison (Wicker *et al*., 2009). The transposable elements (TE) from the barley genome were compared to diploid wheat (*Triticum monococum*), and results showed that there were TE families that they had in common. There were also families highly represented in wheat such as *Claudia*, *Daniela*, *Fatima* etc. which were almost absent from the barley sequence. Although there was this difference, the overall number of TE remained constant.

Brenchly *et al*. (2012) published the first analysis of the bread wheat genome. The cultivar Chinese spring (CS42) sequence information was compared to *T. monoccocum*, *Ae. speltoides* and *Ae.*

tauschii which revealed reductions in the numbers of genes associated with some of the larger gene families, although transcription factors were found to be conserved. Families of genes involved in growth, defence and domestication were found to be increased. The A, B and D genome analysis concluded that there are approximately 94 000-96 000 genes and 132 000 SNPs were identified. A draft sequence of the D genome progenitor *Ae. tauchii* has also been released (Jia *et al*., 2013). These sequencing projects constitute a valuable resource which has identified a large amount of variation between cultivars which can be used in future studies involving gene discovery and crop improvement (Smith and Clement, 2012).

2.2. Plant defence

Plants are sessile organisms, and with their inability to move they have developed many different defences against attack by pathogens and insects alike. There are two general pathways that a defensive reaction can follow. Some defences are non-specific and are triggered by wounding for example, others are specific and require a recognition event within the plant where an elicitor from the pathogen is recognized and a response is initiated, known as an 'incompatible' reaction. In the event that there is no recognition or resistance response, the plant is deemed susceptible to the attacker and the reaction is known as a 'compatible' reaction (Buchanan *et al*., 2000).

2.2.1 Recognition of pests

There are several genetic reasons why a pathogen may succeed in colonizing a host: The plant supplies its nutritional needs, there are no barriers or toxins that restrict infection or colonization; there is no recognition event by the plant and thus no defences are initiated. The lack of these leads to what is known as a compatible interaction, in which a pest or pathogen will successfully infest/infect a host. The opposite is true for the non-compatible interactions. In that case the pest/ pathogen growth is restricted and is not successful in using the host (Buchanan *et al*., 2000).

In a non-compatible interaction, the plant must discern that there is a pathogen/ pest that is attacking or is harmful, and then launch a defence response. Plants recognize either microbial-, pathogen- or herbivore associated molecular patterns (MAMPS; PAMPs; HAMPs) that come into contact with the cell in the form of conserved molecules or "effectors" (Gomez-Gomez and Boller, 2002; Hogenhout *et al*., 2009; Hogenhout and Bos, 2011; Jones and Dangle, 2006).

These defence responses are highly coordinated both spatially and temporally and require a level of signal transduction. Resistance is the result of a plant R-protein recognizing an effector from the pathogen that results in effector triggered immunity (ETI; Jones and Dangle, 2006). The hypersensitive response (HR) response and other amplified resistance responses are thought to be activated by ETI in what is known as the gene-for-gene resistance response model (Flor, 1971). The gene-for-gene hypothesis predicts that a resistance response will only occur if a plant has the genetic material to encode for a dominant/ semi dominant resistance gene (*R*-gene) that recognizes an avirulence factor encoded by an *Avr* gene, within the insect/ pathogen. Avr-proteins are thought to have important roles within the pathogen/ pest in terms of either its pathogenicity, or other important functional roles such as its reproduction or spread. R-proteins take the role of both receptor and signal transmitter, provide race-specific resistance, and contain similar structural domains. One of these is the leucine rich repeat (LRR) motif, which most R-proteins possess, and that is involved in signal transduction. This repeated motif contains leucine and other hydrophobic amino acids, and has been shown to mediate protein-protein interactions (Jia *et al*., 2000), and may function in recognition specificity (Dodds *et al*., 2001; Ellis *et al*., 1999; Wang *et al*., 1998). The consensus sequence xxLxLxx forms a beta strand/ beta turn motif of which the x residues have been found to be hyper variable with many substitutions, allowing the motif plasticity (Wang *et al*., 1998). Other R-protein motifs are the nucleotide binding site (NBS), leucine zipper (LZ) and coiled coil (CC) domain as well as the

Toll/interleukin-1/resistance (TIR) domain. There is also a serine/ threonine kinase domain found in a few of the R-proteins such as *Pto* and *Xa21* (Pan *et al*., 2000; Belkhadir *et al*., 2004).

After a specific recognition event, one of the first lines of defence is the creation of reactive oxygen species (ROS) which can cause localized tissue death in the form of necrotic lesions as part of the HR. These necrotic lesions form at the site of attack and obstruct the pathogen's ability to cause infection (Tenhaken *et al*., 1995). In order for ROS to form, the attack needs to be recognized as such, and antibiotic molecules such as hydrogen peroxide (H_2O_2) and superoxide anions (O_2^-) are released in a burst called the HR. Many enzymes are involved in this process as well as in the detoxification of ROS molecules in surrounding tissues in order to limit the spread of the HR and resulting tissue death (Apel and Hirt, 2004). One of these enzymes is NADPH oxidase, which is involved in the production of O_2^- (Babior *et al*., 1976) which in turn is used by superoxide dismutase (SOD) in order to form H_2O_2 (Klug-Roth *et al*., 1972). H_2O_2 is able to cross plant plasma membranes, thus although it can be toxic to pathogens and plants at high concentrations, it can also act as a signaling molecule (Orozco-Cárdenas *et al*., 2001).

Initial responses are localized around the site of infection/ attack where after the signal may be spread systemically in the form of systemic acquired resistance (SAR). SAR is characterized by the production of both salicylic acid (SA) and the production of the pathogenesis related (PR) proteins. A recognition event has to have occurred in order for SAR to be activated. The type of resistance conveyed by the SAR response is broad-spectrum and once initiated can protect the plant from subsequent attacks by the same pest/ pathogen, or others (Buchanan *et al*., 2000). Ethylene also has a role to play here as well, as co-expression of Ethylene and SA acts to augment PR protein expression (Buchanan *et al*., 2000).

2.2.2 Wounding

Herbivory can initiate a systemic defence response in plants called the wounding response. This response involves an increase in jasmonic acid (JA) that induces the elicitation of various proteins such as protease inhibitors (PI) and other molecules such as polyphenol oxidase (PPO). The PIs are induced by damage caused by herbivory and wounding (Green and Ryan, 1972; Moore *et al*., 2003) and these small proteins interfere with digestion within the gut of the insect. Proteolysis of food gained by the insect from the plant is thus inhibited, and the nutrient amino acids are not released for absorption. This is to the detriment of the insect and can even cause insect death (Buchanan *et al*., 2000).

PIs have been found to accumulate in distal tissue of plants that have been wounded within hours of the wounding event (Green and Ryan, 1972). The initiators of the wounding response can originate from damaged plant cell walls and are called HAMPS that are made up of a large variety of proteins, polymers, polysaccharides and polypeptides (Cosgrove 2005; Varner and Lin, 1989). One such HAMP-producing molecule is pectin, a complex polysaccharide that makes up the cell wall and is one of the first cell wall components attacked or digested by invading pathogens, resulting in HAMPs (Pagel and Heitifuss, 1990). Pectic oligosaccharides are called oligogalacturonides (OGAs) (Ridley *et al*., 2001) and upon cell wall damage, OGAs are released which initiate a local response, which in turn activates a signaling cascade (Ferrari *et al*., 2013).

H_2O_2 is the result of the production of reactive oxygen species (ROS) by the plant in response to wounding or attack. H_2O_2 acts both at the site of wounding via enzymes such as peroxidases and NADPH oxidase (Liu *et al*., 2008), as well as acting as a secondary messenger which affects transcription of defence related genes (Avsian-Kretchmer *et al*., 2004; Bienert *et al*., 2006; Orozco-Cárdenas *et al*., 2001). It is produced locally and systemically after wounding and is activated by

methyl-jasmonate, systemin, OGA, chitosan and all of the inducers of polygalacturonases (PGs) (Orozco-Cárdenas and Ryan, 1999).

Another role for H_2O_2 includes initiating cross-linking of glycoproteins and increasing lignin polymer formation, which reinforces plant cell walls (Bradley *et al*., 1992; Iiyama *et al*., 1994), a reaction that is activated in specific defence responses as well as the wounding response (Apel and Hirt, 2004). Lignin also has the ability to cross-link with other cell wall constituents such as polysaccharides, carbohydrates and proteins (Iiyama *et al*., 1994). This characteristic is important in defence of fungal attack and the wounding response. The attack or damage, once recognized, is stopped by the formation of a cross-linked lignin plug and the gathering of hydroxyproline rich glycoproteins and other anti-microbial proteins, the combination of which stops infection (Bradley *et al*., 1992; Corbin *et al*., 1987; Hammerschmidt *et al*., 1984). Detoxifying enzymes such as ascorbate peroxidase and glutathione peroxidase remove H_2O_2 by converting it back into water (Buchanan *et al*., 2000).

Another molecule that is closely associated with wounding is Jasmonic acid (JA). Systemin initiates the synthesis of JA within distal tissues and is derived from fatty acids, namely α-linolenic acid (LA) as part of the octradecanoid pathway (ODA). Systemin is a short 18 amino acid long polypeptide first found in tomato. It was found that this molecule was able to move across the plant and initiate the synthesis of PI I and PI IIs systemically (Pearce *et al*., 1991). Lipases are activated after wounding that break down membranes to release LA. The LA is utilized in the ODA pathway which starts in the chloroplast. There, lipoxygenase (LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC) use LA to form the JA precursor 12-oxophytodienoic acid (OPDA; Creelman and Mullet, 1997). OPDA is then utilized in the peroxisome by 12-oxo-phytodienoate reductase (OPR3) to form mature JA (Strassner *et al*., 2003). The ODA pathway is also responsible for the release of volatiles for the attraction of predatory insects (Thaler 1999).

The activity of JA is altered by conjugation to a variety of other molecules (Glauser *et al*., 2008; Kramell *et al*., 1995). The methyl-jasomonate (MeJA) conjugate is a highly reactive volatile, capable of both inter-plant signalling (Farmer and Ryan, 1990; Seo *et al*., 2001) and intra-plant signaling, with the ability to move long distances within the plant, possibly playing an important role in the amplification of a systemic wounding signal (Thorpe *et al*., 2007).

JA has also been found to be conjugugated in an ATP-dependant reaction via the JAR1 enzyme to amino acids (Staswick and Tiryaki, 2004). Of the isoleucine (Ile) amino acid conjugates, the (–)-JA-Ile conjugate has been found to be the most active in plants and preferentially formed *in vivo* (Guranowski *et al*., 2007). JA-Ile in turn activates early response genes, and is a key signal for systemic induced wound signaling and defence (Koo *et al*., 2009).

2.3. Russian wheat aphid

The production of wheat is impacted by many endemic pathogens and pests, one of which is the Russian wheat aphid or *Diuraphis noxia* (Kurdjumov). *D. noxia* is thought to have originated in the Carpathean basin, and has spread globally to most wheat producing countries, except Australia (Basky 2003). Due to its ability to reproduce via parthenogenesis, controlling infestation of this insect is difficult, and it has been labelled as one of the highest impacting pests in wheat producing areas (U.S. congress OTA, 1993).

D. noxia is a small, spindle shaped insect that varies from yellow-green to grey-green in colour. This insect was first reported in the Eastern Free State in South Africa in 1978. By the end of 1978 the insect had spread through to the Western Free State and Lesotho (Walters *et al*., 1980). *D. noxia* reproduces via parthenogenesis, a form of asexual reproduction and gives birth to live young (Capinera, 2008). Under favourable conditions the aphid can produce 4-7 nymphs per day (Walters *et al*., 1980). A

nymph can reach maturity within a week, thus a field can go from being 20% infested to 80% infested within two weeks (Walters *et al*., 1980).

D. noxia infestation causes several symptoms in susceptible wheat. Longitudinal streaking or chlorotic streaks as well as leaf rolling are the most common symptoms, subsequently; head trapping, stunted growth and death (Walters *et al*., 1980).

2.3.1 *D. noxia* biotypes

Infestations of *D. noxia* in South Africa were at first confined to the Eastern and South Western Free State, however new infestations have been seen in the Western Cape wheat growing areas, possibly due to dry climatic conditions (Botha, 2013). The first *D. noxia* resistant wheat lines to be cultivated in South Africa contained *Dn1* and later, *Dn2* (Du Toit, 1989). *Dn1* cultivars have been planted in the Free State and used in combination with other insect deterrents such as spraying with insecticides (Walters *et al*., 1980). In reaction to exposure to these resistant cultivars, new *D. noxia* biotypes have developed in South Africa (Jankielsohn, 2011).

The first reports of *D. noxia* in the USA were from the Texan Panhandle in 1986 (Webster *et al*., 1987). In Colorado in 2003, *D. noxia* was found to have overcome the *Dn4* resistance gene that had been bred into various cultivars (Haley *et al*., 2004). A possible reason for the development of the new aphid biotypes is the overplanting of cultivars expressing the *Dn4* resistance gene over several planting seasons (Jyoti and Michaud, 2005). Since then several other biotypes have developed (Haley *et al*., 2004; Jyoti and Michaud, 2005; Tolmay *et al*., 2007; Randolph *et al*., 2009).

The differences in *D. noxia* virulence to previously resistant wheat lines are used to differentiate biotypes (Haley *et al*., 2003). In 2003 the first of the new US biotypes was identified as US2 (Haley *et al*., 2004; Jyoti and Michaud, 2005). Results showed that aphid fecundity of the US2 *D. noxia* biotype

from Colorado was unaffected by the presence of the *Dn4* or *Dny* genes (Fig. 2.1), thus a change in the aphid genotype allowed it to feed on cultivars previously deemed resistant.

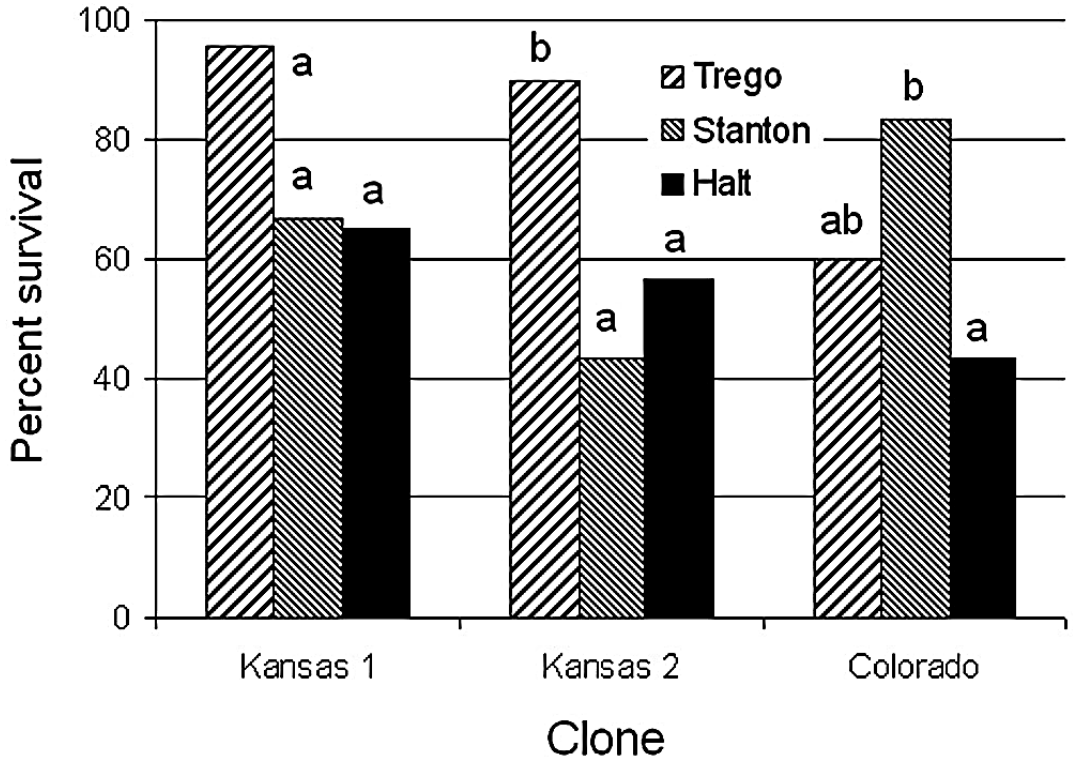

Fig. 2.1. Graph showing the number of nymphs produced by singular *D. noxia* clones from Kansas or Colorado on three wheat varieties; susceptible Trego, *Dny* resistant Stanton and *Dn4* resistant Halt (adapted from Jyoti and Michaud, 2005.

It has been suggested that *D. noxia* and wheat co-evolved in the Fertile Crescent (Botha, 2013). The adaptation of *D. noxia* to plant resistance genes can be attributed to selection pressure put on the aphid population by the *R*-gene. The ETI triggered by the Avr protein of the aphid, will select for variants in the population that can avoid detection in the 'arms-race' (Pieterse and Dicke, 2007). This also forms a part of the cycle described by Jones and Dangl (2006), in which the co-evolution and associated costs of resistance is described for microbes and their plant hosts. The effectors of the pathogen and the *R*-gene from the plant both carry costs, which are balanced by necessity, but may be

maintained by a cycle of frequency (Fig. 2.2) which will maintain both effectors and *R*-genes within the pathogen and plant population respectively.

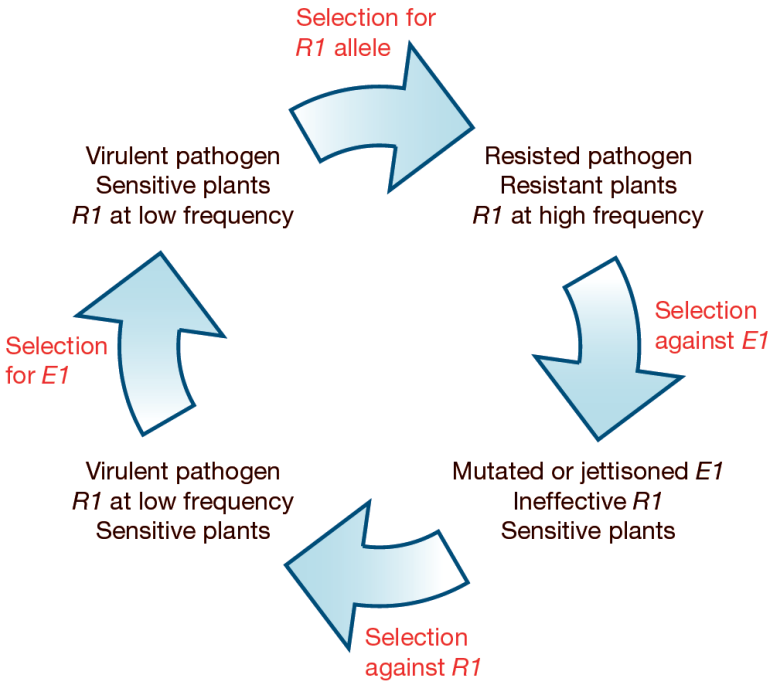

Fig. 2.2. The plant R-gene (*R1*) recognizes the pathogen Avr or effector (*E1*) which results in selection for *R1* within the population (top). Pathogens in which *E1* contains mutations that change the conformation of the Avr-protein and thus avoid detection by *R1* are thus selected for as they can grow on *R1* plants (right). The usefulness of *R1* declines, and because certain *R*-genes may carry a fitness cost, *R1* frequencies will decrease (bottom). The *E1* gene will still exist within the pathogen population, and due to the decrease in frequency of *R1*, *E1* will start to give increased fitness to the population and start rising in frequency (Left), which in turn will resume selection for *R1* (top) (Jones and Dangle, 2006).

2.3.2 Effector proteins: saliva

D. noxia has different feeding habits; probing, tasting and feeding. These can be seen as distinct peaks in electronic penetration graph (EPG) type experiments (Lazzari *et al*., 2009). The feeding behaviors are associated with different types of saliva. The first, which is used to probe, is the hard or gelling saliva which forms part of the sheath that protects the aphid's stylet. The second type of saliva is used

during tasting and feeding and is watery saliva that contains hydrolytic and other enzymes (Miles, 1999).

The gelling saliva from aphids has been found to be comprised of a mixture of proteins with free sulphydryl groups that contribute to polymerization and hardening of the gel sheath (Miles 1965; Tjallingii, 2006; Will *et al*., 2012). The sheath protein candidate identified in several studies on piercing insects to date (Cooper *et al*., 2011; Freeman *et al*., 2001; Brentassi *et al*., 2007) seems to have a high cysteine content and a molecular weight of approximately 130 kDa similar to that found in *D. noxia* (Will *et al*., 2012). Watery saliva constituents from different aphid species include constituents such as phenolic glycosides, glucose dehydrogenase, that hydrolyze sucrose, interact with free calcium in the phloem etc. (*Myzus persicae*, Harmel *et al*., 2008; *Schizaphis graminum*, Ma *et al*., 1990; Miles, 1999; *Megoura viciae*, Will *et al*., 2007). A salivary study has identified alkaline phosphatase (Cooper *et al*., 2010) as a unique component in *D. noxia* saliva.

Initial probing by *D. noxia* has been shown to induce a resistance response; however it is retarded and not maintained in susceptible varieties (Botha *et al*., 2010). Aphids sustain feeding by avoiding detection, rerouting nutrients to them that would otherwise have been blocked by Ca^{2+} flux initiated sieve element (SE) occlusion (King and Zeevaart, 1974). It is thought they accomplish this by plugging wounds with gelling saliva (Tjallingii and Hogen Esch, 1993) preventing the influx of Ca^{2+} necessary for SE occlusion (Will and Van Bel, 2006). The watery saliva is thought to contribute to protein stability and thus the prevention of SE blockage by insoluble protein complexes (Knoblauch and Van Bel, 1998), as watery saliva excretion coincides with penetration events (Will and Van Bel, 2006).

In an interesting experiment by Burd (2002), greenbugs were fed on an artificial diet containing ^{14}C-sucrose and then allowed to feed on wheat plants. The radioactive compound was then monitored

within different sections of the plant. Radioactivity was found in the shoots and in the roots. It was previously found that damage seen in seedling roots was not the result of damage to- or loss of-chloroplast function (Holmes *et al*., 1991) thus they suggested that greenbug salivary toxins were responsible for damage.

Similarly it is thought that the symptoms of *D. noxia* feeding are as a result of the introduction of salivary proteins into the plant that act as effector proteins. In a study by Lapitan *et al*. (2007a), using two US biotypes, it was found that US1 protein extracts, after injected into *D. noxia* susceptible plants, induced leaf rolling. Injections of US2 protein extracts induced chlorosis, head trapping and stunted growth. The observations in this study support the hypothesis that the *D. noxia* elicitor is a protein that is recognized by the plant in a gene-for-gene resistance response (Botha *et al*., 2006). The chlorosis symptom was found to develop only after several injections of *D. noxia* protein, whereas the leaf rolling symptom developed after only one. It was thus suggested that the pathways that activate the symptoms are different, and that chlorosis only develops after continuous exposure to the *D. noxia* elicitor (Lapitan *et al*., 2007a).

2.3.3 Wheat- *D. noxia* interactions

Susceptible wheat lines tested during 2000 to 2002 in a field trial in South Africa displayed yield losses of up to 78% under heavy *D. noxia* infestation conditions (Tolmay, 2002). There is a direct relationship between yield and aphid load in susceptible wheat, with yield decreasing to zero under heavy aphid loads per tiller (Randolph *et al*., 2003, 2009). When feeding on susceptible wheat varieties, the *D. noxia* is able to form secondary sinks, thereby redirecting assimilates toward them. They also induce blockages in the phloem sieve tubes by callose deposition, which occurs upon loss of turgor within the phloem system (Botha and Matsiliza, 2004). These two reactions to aphid feeding cause a rerouting of nutrients that would have naturally gone to budding leaves or seed formation.

Russian wheat aphid feeding can result in the loss of up to 40% chlorophyll in 7-10 days when feeding on a susceptible cultivar (Deol *et al*., 2001). In 1996, Burd and Elliot measured the chlorophyll *a* fluorescence of wheat and barley under infestation conditions in order to gain insight to the physiological mechanism behind chloroplast damage by *D. noxia*. They found a significant decrease in chlorophyll *a* levels in susceptible plants during feeding, concluding that *D. noxia* feeding caused extensive damage to photosystem II. Burd and Elliot's work also suggested that the damage was caused by a photo-inhibitory mechanism, caused by a blockage in electron transport at the acceptor site of photosystem II.

Chlorophyll content may decrease in wheat carrying a resistance gene such as *Dn1*; however the decrease seen is very small in comparison with susceptible isolines. In contrast, chlorophyll concentrations in lines with the tolerance phenotype, such as those carrying the *Dn2* gene, do not lose chlorophyll content but showed an increase in chlorophyll levels, displaying a possible mechanism for compensation in these plants. Analysis of the carotenoid content revealed similar expression patterns and may thus be similarly affected by *D. noxia* feeding (Heng-Moss *et al*., 2003).

D. noxia feeding on susceptible cultivars causes loss of fresh leaf weight and considerable chlorosis in comparison to other aphid species such as *Rhopalosiphum padi* (Ni *et al*., 2001). Resistant wheat leaves (*Dn1*) infested with *D. noxia* show an increase in peroxidase activity (Van der Westhuizen *et al*., 1998; Moloi and Van der Westhuizen, 2006). A strong correlation with systemic acquired resistance (SAR) has also been seen in *Dn1* plants, as a strong induction of H_2O_2 was reported in correlation to an increase in the levels of SA which starts at 72 hours post infestation which then peaks at 96 hours post infestation. These results indicated involvement of SA in the *D. noxia* defence response in the *Dn1* mediated resistance response (Mohase and Van der Westhuizen, 2002).

The involvement of the SA and JA pathways in resistance to aphids varies for each of the *Dn* genes under investigation. A combination of both JA and SA pathways may be elicited by aphid infestation (Ellis *et al*., 2002; Gao *et al*., 2007; Zhu-Salzman *et al*., 2004). In the study of Smith *et al*. (2010), on the *D. noxia* resistance gene *Dnx*, evidence for expression of key components of the octadecanoid pathway were found, indicating that the JA pathway may be involved in *Dnx* resistance.

Wounding a plant triggers a type of resistance response; however, it has been found that although the aphids wound the plant, they do initiate different resistance responses to those of just simple wounding (Botha *et al*., 1998). The reaction of plants in general to aphid feeding activates similar biochemical pathways as against pathogens such as fungi or bacteria rather than that of chewing insects (Moran and Thomson, 2001; Moran *et al*., 2002; Reymond *et al*., 2000).

Pathogenesis-related proteins are known to be induced by SAR. One such protein is the β-1,3 glucanases (Van der Westhuizen *et al*., 1998), which are thought to be involved in *D. noxia* resistance and have been found to accumulate in the apoplast of resistant wheat. The protein has also been found to accumulate in the cell walls of vascular bundle of *Dn1* resistant cultivars (Van der Westhuizen *et al*., 2002). They suggest that β-1,3 glucanases act to remove callose deposits. Callose formation in sieve tubes is thus deterred in resistant cultivars, a mechanism that is absent from susceptible cultivars.

In a study by Valdez (2010) on *Dn7*, it was found that β- (1,3:1,4) glucanases are down-regulated within a 5 hour time period in resistant plants. *D. noxia* susceptible plants became more resistant to aphid infestation after silencing β- (1,3:1,4) glucanases with virus induced gene silencing (VIGS), indicating that this enzyme may act differently depending on the type of resistance, or the timing of expression as was previously seen (Van der Westhuizen *et al*., 1998).

Other PR-proteins that are induced in resistant cultivars are chitinase and peroxidase, which have been found to have similar expression pattern based on the presence or absence of the *Dn1* gene,

although the level of induction of the proteins is background dependent. These proteins were also found to be expressed to a certain level in the susceptible counterparts of the lines studied, however they were expressed much later after infestation (12-14 days), so the resistance response in these lines were considered to be initiated too late to be effective (Van der Westhuizen *et al*., 1998).

2.3.4 Existing resistance in wheat to *D. noxia*

Genes conveying resistance in wheat to *D. noxia* have been given the nomenclature '*Dn'* genes. Eleven resistance genes have been found to date, (Table 2.1) some of which have been mapped, none of which however have been fully sequenced or characterized. The resistance interaction between wheat and the *D. noxia* is species specific. For instance, in an experiment by Ni *et al*. (2001), it was found that *D. noxia* -resistant Halt was not resistant to the bird cherry aphid *R. padi*. Also, the presence of the *Dn* genes did not affect the feeding habits of *R. padi* (Messina and Bloxham, 2004). It can thus be concluded that the *Dn* genes evolved with the wheat during its long relationship with the Russian wheat aphid in an 'arms-race' which resulted in germplasm from the aphid's native habitat that contains sources for resistance.

The *Dn* genes have been characterized phenotypically with three main types of resistance; with some of them conveying antibiosis, some tolerance and others antixenosis or a combination (Table 2.1). Antibiosis is the ability of the plant to adversely affect the biology of the aphid, decreasing fecundity and longevity. Tolerance is the ability of the plant to withstand heavy aphid load without significant yield loss. Antixenosis results in non-preference of the aphid for the plant for food or shelter (Painter, 1958).

Table 2.1: *Dn* genes with their accession numbers and estimated position in the genome

Dn gene	Accession	Chromosome	Resistance mode	References
Dn1	PI 137739	7DS	Antibiosis	(Du Toit 1987, 1989; Liu *et al*., 2001)
Dn2	PI 262660	7DS	Tolerance	(Du Toit 1987, 1989; Liu *et al*., 2001)
dn3	SQ24			(Nkongolo *et al*., 1991a)
Dn4	PI 372129	1DS	Tolerance, Antibiosis	(Nkongolo *et al*., 1991b; Ma *et al*., 1998; Liu *et al*., 2005)
Dn5	PI 294994	7DS	Antixenosis	(Du Toit 1987; Du Toit *et al*., 1995; Marais and Du Toit, 1993; Elsidaig and Zwer, 1993; Liu *et al*., 2001)
Dn6	PI 243781		Antibiosis	(Saidi and Quick, 1996)
Dn7	94M370	Rye translocation 1RS/ 1BL	Antixenosis and antibiosis	(Marais *et al*., 1994, 1998; Lapitan *et al*., 2007b)
Dn8	PI 294994	7DS		(Liu *et al*., 2001)
Dn9	PI 294994	1DL		(Liu *et al*., 2001)
Dnx	PI 220127	7DS	Antixenosis	(Liu *et al*., 2001)
Dny	PI 220350		Tolerance	(Smith *et al*., 2004)
Dn626580	PI 626580	7D		(Valdez *et al*., 2011)

2.4. Functional analysis using endogenous gene silencing

In plants and animals, silencing plays an important regulatory role. Silencing RNA can protect against viruses (Al-kaff *et al*., 1998), regulate gene expression (Aravin *et al*., 2001) and protect the genome against selfish genomic elements (Kalmykova *et al*., 2005; Tabara *et al*., 1999). Silencing is mediated through small RNAs and ATP and no translation machinery is utilized during the process (Zamore *et al*., 2000; Tijsterman *et al*., 2002).

RNA silencing was at first described as posttranscriptional gene silencing (PTGS). Wasseneger and Pélissier (1998) described the model of transcriptional gene silencing (TGS; thought to be due to DNA methylation), and PTGS as a result of an RNA dependant RNA polymerase (RdRp) mediated mechanism (Lipardi *et al*., 2001; Wasseneger and Pélissier, 1998). However, methylation was later found not to be essential for PTGS (Wang and Waterhouse, 2000).

The general processes involved in PTGS are similar across taxa. Double stranded RNA (dsRNA) has been found to be the trigger for silencing and activates a ribonuclease (RNase) III enzyme named DICER, which then cleaves the dsRNA into small interfering RNAs (siRNA). A specific nuclease named RNA-induced silencing complex (RISC) incorporates an siRNA, that then act as a guide to target the nuclease to mRNAs in a sequence specific manner (Hammond *et al*., 2000). The siRNA guides the protein as it pairs with the target mRNA. The Argonaute protein, part of the RISC complex (Rivas *et al*., 2005), then cleaves the mRNA molecule on either side of the target sequence. The siRNAs then act as primers for RNA-dependant RNA polymerase (RdRp), creating short dsRNA fragments. The dsRNA then interacts with DICER which cleaves it into secondary siRNAs of 22-25 bp and the cycle continues (Baulcomb 2007).

The double stranded siRNA molecules direct cleavage of target mRNA molecules and have high homology. Sequence specificity plays an important role in the RNAi mechanism as even one or

two base pairs difference drastically decreases the amount of silencing (Sijen *et al*., 2001, 2007). The maintenance and spread of silencing is mediated by RdRp and involves the synthesis of dsRNA molecules (Vaistij *et al*., 2002). *In vivo* synthesized dsRNA acts as a silencing trigger and can amplify silencing systemically across cell types (Alder *et al*., 2003).

Until recently, many of the tools for functional gene characterization were not transferrable to studies done in monocots, and especially wheat due to its allopolyploid genome and monocot characteristics of the leaves. However, virus induced gene silencing (VIGS) has proven useful in the functional characterization of genes within monocot species.

VIGS is an efficient way of silencing genes; utilizing the plants' own sequence-specific PTGS defence system, the plant does not recognize the dsRNA gene fragment as endogenous, and thus is silenced in conjunction with the virus (Holzberg *et al*., 2002; Scofield *et al*., 2005). This technique utilizes a 'rubbing' technique using mRNA run-off of the virus which allows the virus to enter the plant and replicate there. Once the viral RNA is detected by the plant, PTGS is initiated, and all mRNAs homologous to the viral RNA are silenced, including the mRNAs homologous to the gene fragment of interest that is cloned into the virus.

A study in wheat utilizing VIGS in order to study resistance genes identified an interaction between the rust resistance gene *Lr10* pathway and a resistance gene analogue (*RGA2*). Silencing of *RGA2* resulted in loss of resistance in the presence of *Lr10* (Loutre *et al*., 2009). The VIGS system has also been found to be a useful system in the study of genes involved in resistance against the *D. noxia*. The study by Van Eck *et al*. (2010) was the first to utilize insect fertility in conjunction with VIGS in order to gauge the requirement for targeted genes of interest. They found a WRKY transcription factor to be involved in *D. noxia* resistance, functioning down-stream of the resistance gene.

2.5. Conclusion

The mechanism of *D. noxia* resistance afforded by *Dn1* is not completely understood. What is known is that it acts with an antibiotic mode of resistance via ROS and that the HR and SAR have been implicated; however, we do not know to what extent ROS is involved in this type of resistance nor how this resistance response differs to what is seen in wounding. Therefore, the VIGS technique will be used in order to further investigate the involvement of ROS and candidate resistance genes in the *Dn1*-mediated *D. noxia* resistance response.

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Chapter 3

Phi-class glutathione-*S*-transferase is involved in *Dn1*-mediated resistance

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Phi-class glutathione-*S*-transferase is involved in *Dn1*-mediated resistance

3.1 Introduction

Diuraphis noxia (Kurdjumov) (Homoptera: Aphididae), commonly known as the Russian wheat aphid (RWA) is a phloem feeder that deprives the plant of essential carbohydrates and amino acids during feeding. The aphid's feeding causes chlorosis, leaf rolling and leaf- and head-trapping, resulting in decreased yields and/ or death in susceptible plants (Walters *et al.*, 1980). The *Dn* genes of wheat convey resistance against the *D. noxia*, each providing one or a combination of three categories of plant resistance; antibiosis (the plant reduces the reproductive fitness of aphids feeding on it), antixenosis (non-preference of a cultivar as host) and tolerance (lack of plant height reduction despite feeding; Painter 1951, 1958).

Resistance genes (*R*-genes) that recognize elicitors from various pathogens and insects during incompatible reactions have been identified in numerous plants (Rossi *et al.*, 1998; Shao *et al.*, 2003; Tang *et al.*, 1996), however, the sequences of the *Dn* genes are still elusive and pathways involved in conferring resistance still need to be clarified (Bent and Mackey 2007; Chisholm *et al.*, 2006; Dodds and Rathjen, 2010; Jones and Dangl, 2006). Several insect *R*-genes have been mapped (e.g. Anderson *et al.*, 2003; Jena *et al.*, 2002; Liu *et al.*, 2001, 2002; Tan *et al.*, 2004), however only three have been cloned, sequenced and characterized: *Mi.1.2*, *Vat* and *Bph14* (Du *et al.*, 2009; Pauquet *et al.*, 2004; Rossi *et al.*, 1998).

When investigating the biological pathways activated by *Dn1*-mediated resistance, regulation of genes and proteins associated with the hypersensitive response (HR) and systemic acquired (SAR) have been reported (Botha *et al.*, 1998, 2006; Mohase and Van der Westhuizen 2002; Van der Westhuizen *et al.*, 1998a,b). Wheat plants containing the *Dn1* gene have been phenotyped as antibiotic, affecting aphid reproduction (Budak *et al.*, 1999; Du Toit 1989; Quisenberry and Schotzko 1994; Smith *et al.*,

1992). Rapid changes in transcription have been seen in *Dn1*-plants after *D. noxia* infestation (Botha *et al.*, 2006; Van Eck 2007), followed by induced host resistance, thus a gene-for-gene (Flor 1971) recognition event followed by a resistance response has been suggested for this gene (Botha *et al.*, 2006).

Many transcriptomic approaches utilizing suppression subtractive hybridization (SSH) (Boyko *et al.*, 2006), cDNA-amplified fragment length polymorphism (c-DNA-AFLPs) (Zaayman *et al.*, 2009), and micro arrays (Botha *et al.*, 2006, 2010; Smith *et al.*, 2010) have been employed to unravel the interactions between *D. noxia* and the wheat plants containing *Dn* genes. These studies have built valuable profiles of the pathways involved in plant responses to *D. noxia* feeding; however, they were not able to discern the genes directly responsible for the resistance response.

Due to the nature of the wheat genome, gathering evidence for the clarification of the functional role of candidate genes in the *D. noxia* resistance response has been challenging. Wheat is an allopolyploid, with three sets of seven chromosomes making up 17 Gbp which has only recently been sequenced (Brenchley *et al.*, 2012). Over 80% of the genome consists of repetitive sequences, and 1 out of every 87-184 kbp contains a primary retroelement. For reverse genetics approaches, the size and complexity of this genome poses challenges and result in low transformation efficiencies, although certain laboratories are making inroads using *Agrobacterium*-mediated transformation (e.g. He *et al.*, 2010; Przetakiewicz *et al.*, 2004; Wu *et al.*, 2008). A transient alternative that has proven to be effective in wheat, with the development of vectors from the barley stripe mosaic virus (BSMV), is virus-induced gene silencing (VIGS) (Holzberg *et al.*, 2002). VIGS utilizes the plant's own post-transcriptional gene silencing (PTGS) machinery programmed to recognize double-stranded RNA as foreign or of viral origin. When incorporating a short section of an endogenous gene into the VIGS vectors, the PTGS system does not differentiate it from the viral sequence, and it becomes a target for

degradation (Holzberg *et al.*, 2002). This transient silencing system has been used successfully in rust–wheat interaction studies in wheat (Scofield *et al.*, 2005; Cloutier *et al.*, 2007; Zhou *et al.*, 2007), and also the interaction of wheat with *D. noxia* (Van Eck *et al.*, 2010). In this study, VIGS was selected as the preferred method to investigate candidate HR-associated genes during the aphid feeding response.

With the advent of over five new US and three South African *D. noxia* biotypes since 2003 (Randolph *et al.*, 2009; Jankielsohn 2011), it has become urgent to understand the mechanisms underlying aphid resistance. *Dn1*-mediated resistance is associated with the HR and the oxidative burst (Van der Westhuizen *et al.*, 1998a), and several genes involved in this process have been identified by Affymetrix-Gene Chip analysis (Botha *et al.*, 2010; Table S3.1). Therefore, the objectives of this study were to clarify the roles of superoxide dismutase *Cu/Zn* (*SOD*), thylakoid-associated ascorbate peroxidase (*tAPX*), and a phi-class glutathione-*S*-transferase F6 (*TaGSTF6*), by testing their requirement in the *Dn1*–mediated defence response during *D. noxia* infestation.

3.2 Materials and methods

Plant material and growth conditions

All experiments were conducted with wheat (*Triticum aestivum* L.) near-isogenic lines (NILs) Tugela (susceptible, *Dn0*) and TugelaDN (resistant, containing the *Dn1* gene) that were planted in potting soil under 14 h of light and 10 h of dark at 25°C. A total of fifteen plants were used per treatment, and samples were collected at the two-leaf stage. All gene silencing treatments were conducted on the *D. noxia* resistant TugelaDN (TugDN) plants. Each plant was observed as an independent biological repeat (n=15).

Sequence analyses of gene targets

Since the genes *Cu/Zn* (*SOD*) and thylakoid-associated ascorbate peroxidase (*tAPX*) that was selected for silencing contain several copies within the wheat genome, the sequence identity of each gene target had to be confirmed to ensure that the correct gene and protein target was silenced *in planta*. This was done utilizing BLASTn and BLASTx sequence analysis tools (Altschul *et al.,* 1990; Altschul *et al.,* 1997) and SWISSPROT (O'Donovan *et al.,* 2002) with *E*-values lower than 1e-10 considered as significant. The obtained sequences were then aligned using the ClustalW program (Geneious 6.0.3, Biomatters, New Zealand). After confirmation of gene target identity (Fig. S3.1, S3.2), silencing was conducted.

Since the phi-class glutathione-*S*-transferase F6 (*TaGSTF6*) is part of a complex protein family, sequence identity had to be confirmed to ensure that the correct gene and protein target was silenced *in planta*. Sequence identity was originally established with similarity to an mRNA for *TaGSTF6* in *T. aestivum* (AJ440795, *E*-value 9.98e-104; Cummins *et al.,* 2003). However, after further investigation of *TaGSTF6*, it was found to have been previously reported to have homology to *GSTA1* (Mauch and Dudler 1993), as well as to *ZmGSTF1* and *ZmGSTF2* (Cummins *et al.,* 2003), casting doubt on which homologue had been silenced. Analysis of a translated *TaGSTF6* fragment utilizing BLASTx showed similarity with a rice homologue *Os*GSTF1 (O65857, *E*-value 1.27e-23) as well as *GSTF1* (P30110) and *GSTF2* (P30111) from *T. aestivum*. Further sequence analysis was therefore necessary before assigning a putative identity to the gene under investigation. Protein sequence characterization was done in order to remove ambiguity between wheat and the model organism *Oryza sativa* sequences due to the different naming methods employed. The protein sequence for *TaGSTF6* of *Triticum aestivum* (TaGSTF6) was aligned with the GST-phi family members from *O. sativa* and *Arabidopsis thaliana* (Fig S3.5; Table S3.2, S3.3), and then used to draw an unrooted UPGMA tree (Fig. S3.6). Data

obtained indicated that the closest homologue to TaGSTF6 to be OsGSTF1, followed by OsGSTF8. To further avoid off-target silencing of the *TaGSTF6*, the target sequence was scrutinized for similarity to other GSTs using available software packages [http://bioinfo2.noble.org/RNAiScan.htm (Xu *et al.*, 2006; Senthil-Kumar and Mysore, 2011a); http://plantgrn.noble.org/pssRNAit/ (Dai and Zhao, 2011)]. With no targets other than GSTs found *in silico* (Table S3.4), with the predicted efficient siRNA AS (3'-UGGAGUUGGUGAAGGGGAUGU−5'), silencing *in planta* followed.

Construction of silencing vectors

The barley stripe mosaic virus (BSMV) VIGS system was utilized for this study which consists of three plasmids (BSMVα, BSMVβ, BSMVγ) containing the modified BSMV tripartite genome. The PCR fragments used for silencing the genes of interest were amplified from TugelaDN single-stranded cDNA prepared using the Transcriptor kit (Roche, Penzberg, Germany). VIGS primers were designed using Primer3 (Rozen and Skaletsky, 2000), and based on accessions AF38779, U69632 and AJ441055 encoding *tAPX*, *SOD-Cu/Zn* and *TaGSTF6* respectively (Table S3.5). Primers produced fragments of: *tAPX* (274 bp), *SOD-Cu/Zn* (196 bp) and *TaGSTF6* (248 bp). Products were cloned using the T-A cloning vector pGEM-T Easy (Promega, Madison WI, USA) and sequenced to confirm identity. The recombinant pGEM-T plasmids as well as the BSMVγ plasmid, containing a multiple cloning site, were digested with the *Not*I restriction enzyme and run on 1% agarose gel. The excised fragments of interest from the pGEM-T plasmids were purified using the GenElute gel extraction kit (Sigma-Aldrich, St. Louis MO, USA). These fragments were then cloned into the *Not*I site in the BSMVγ plasmid (Holzberg *et al.*, 2002). To determine anti-sense orientation of each fragment, the BSMVγ forward primer was used with the forward primer for each fragment (Table S3.5) in a PCR reaction.

Virus-induced gene silencing

Following the protocol described by Van Eck *et al,* (2010), the three BSMV plasmids were linearized using *Spe*I for plasmid BSMVβ, and *Mlu*I for plasmids BSMVα and BSMVγ. Capped RNA transcripts were made using the T7 mMESSAGE mMACHINE kit (Ambion, Life technologies, Carlsbad CA, USA) following the manufacturer's protocol. Plants were infected using a modified protocol (Holzberg *et al.,* 2002; Scofield *et al.,* 2005): in brief, one ng of each plasmid (BSMVα, BSMVβ, BSMVγ) was added to 35 µl FES buffer (0.1 M glycine, 0.06 M K_2HPO_4, 1% w⁄ v tetrasodium pyrophosphate, 1% w/v bentonite, 1% w/v celite, pH 8.5) and this mixture was used to inoculate the wheat plants at the 2–3 leaf stage by rub inoculation. The components for each treatment consisted of BSMVα and BSMVβ as well as BSMVγ transcripts containing the fragment of the gene of interest. Before the silencing was conducted with the superoxide dismutase *Cu/Zn* (*SOD*), thylakoid-associated ascorbate peroxidase (*tAPX*), and a phi-class glutathione-*S*-transferase F6 (*TaGSTF6*), the VIGS system was optimized in TugDN by silencing phytoene desaturase (Scofield *et al.,* 2005) to remove any genotype specific responses as previously silencing was conducted in other wheat genotypes (Scofield *et al.,* 2005; Van Eck *et al.,* 2010). TugelaDN plants silenced for each construct were denoted as TugDN+$BSMV_{SOD}$, TugDN+$BSMV_{APX}$, and TugDN+$BSMV_{GST}$. The control consisted of BSMVα, BSMVβ and BSMVγ, without an insert, and labelled TugDN+$BSMV_0$. Uninoculated controls TugDN and Tugela were also included. The six treatments used were therefore: TugDN (n=15), TugDN+$BSMV_0$ (n=15), TugDN+$BSMV_{SOD}$ (n=15), TugDN+$BSMV_{APX}$ (n=15), and TugDN+$BSMV_{GST}$ (n=15) and Tugela (n=15). All 15 biological repeats of each treatment had one caged aphid on an emergent leaf and were mass infested with *D. noxia* (biotype: SA1). Infestation treatments were started on the fifth day after viral inoculation.

RT-qPCR

Silencing of candidate genes was confirmed using RT-qPCR. All RNA extractions were performed using the Trizol reagent (Life technologies, Carlsbad CA, USA) and purified using the RNeasy mini kit (Qiagen, Hilden, Germany). cDNA was constructed using the Transcriptor kit (Roche, Penzberg, Germany). All reactions were run on the CFX96 (BioRad, Hercules CA, USA) using SYBR Green I Master Mix (Roche, Penzberg, Germany) according to the manufacturer's protocols. Cycling protocol: 1 x 95°C for 10 min; 40 x 95°C for 10 sec, 57°C for 15 sec, 72°C for 20 sec; 1 x melt curve 65°C - 95°C: increment 0.5°C per 5 sec. Relative quantification was calculated using the mathematical model by Pfaffl (2001) using TugelaDN as the calibrator and normalized to 16S rRNA (Table S3.5).

Aphid reproduction measurements

One of the measurements that determine an antibiotic resistance type is the decrease in reproduction of the aphids feeding on resistant material (Budak *et al.*, 1999; Du Toit 1989; Quisenberry and Schotzko, 1994; Smith *et al.,* 1992). Aphid reproductive measurements were taken following the protocol described by Van Eck *et al.* (2010). Seven days after virus inoculation (AVI) one aphid (biotype: SA1) was caged on the emerged third leaf of each plant (Fig. S3.4), where each plant was considered one biological repeat, with fifteen biological repeats per treatment (n=15). The next day, the mothers were removed and a newly born nymph was left in each cage, which was considered the foundress, and this was recorded as the date of birth. Aphid nymph numbers were measured daily and the mean total number of nymphs was calculated as a measure of fertility (n=15). The theoretical maximum rate of increase of a population per individual for aphids (r_m) was then quantified using the equation by Wyatt and White (1977): Where M_d is the number of young produced in a period equal to (d) and

$$r_m = \frac{(0.738 \cdot ln(Md))}{d} \qquad \text{(Equation 1).}$$

Enzyme activity assays

The VIGS experiment included biological repeats in order to conduct destructive sampling for the enzyme assays. The GST and peroxidase measurements utilized the same total protein extract. Total protein was extracted and enzyme activity was assayed from whole leaf tissue following the protocol described by Ni *et al.* (2001). Protein concentration was determined according to the method of Bradford (1976) using the BioRad protein assay reagent with bovine serum albumin (BioRad, Hercules CA, USA) as a standard. The Glomax spectrophotometer (Promega, Madison WI, USA) was used for this purpose as described by Rybutt and Parish (1982).

GST assays were conducted using the glutathione-*S*-transferase assay kit (Sigma-Aldrich, St. Louis MO, USA) by measuring the rate of increase in absorbance at 340 nm at 22 °C using the manufacturer's protocol. The formation of conjugate-GSH was a linear function of enzyme concentration and GST activity was expressed as mmol GSH mg^{-1} protein. min^{-1}.

Peroxidase activity was calculated by measuring the rate of increase in absorbance at a 470 nm wavelength at 22 °C using a modified protocol (Hildebrand *et al.*, 1986) and horseradish peroxidase (BioLabs, Inqaba, Pretoria, ZA) as a standard. Hydrogen peroxide (0.06%) was added into a mixture containing 2 µg of leaf extract, 6 mM guaiacol, 25 mM potassium phosphate buffer (pH 6.0) and 24% distilled water. The formation of tetraguaiacol was a linear function of enzyme concentration and peroxidase activity was expressed as µmol tetraguaiacol min^{-1}. mg^{-1} protein.

DAB staining

DAB staining of material collected 14 days after virus inoculation was performed (Thordal-Christensen *et al.*, 1997). Three biological repeats were chosen at random from each treatment and the fourth leaf was sampled into 1 mg mL^{-1} 3,3-diaminobenzidine (DAB)-HCl, pH 3.8, (Sigma-Aldrich, St. Louis MO, USA) and incubated, covered, overnight with shaking at 40 rpm. The tissues were de-stained

using 75% ethanol at 37°C. The ethanol was replaced as needed, until the presence of H_2O_2 could be visualized as brown deposits.

Data analysis

All statistical analyses were conducted by using SigmaStat 3.5 (2007) (SyStat Software, San Jose CA, USA) with significance set at $\alpha = 0.05$. Aphid fertility measurements were calculated as described by Van Eck *et al*. (2010), using the mean total number of nymphs born per day. The intrinsic rate of increase was calculated using equation (1). All data was plotted using Sigma Plot 7.0 (2001) (SPSS Inc., Chicago IL, USA). Outliers were identified using the guidelines of Grubbs (1969).

3.3 Results

The effects of silencing on transcript and protein abundance

HR-associated genes, previously identified during the *D. noxia* resistance response in *Dn1* containing wheat (Botha *et al.*, 2010), were targeted for silencing using the BSMV-VIGS system (Holzberg *et al.*, 2002). Recombinant vectors were produced for silencing *tAPX*, *SODCu/Zn* and *TaGSTF6* in the TugelaDN wheat cultivar; with treatments designated as $BSMV_{APX}$, $BSMV_{SOD}$, or $BSMV_{GST}$, respectively. Sampling was carried out five days post-foundress birth (fourteen days PVI) to ascertain the levels of silencing by measuring transcript abundance with RT-qPCR (Fig. 3.1). Inoculation with recombinant vectors resulted in significant knock-down of transcripts in all three treatments.

The TugDN+$BSMV_{SOD}$ treatment group showed the largest knock-down in transcript abundance with a mean decrease of 80% ($P<0.05$) compared to the empty vector treatment and 40% ($P<0.005$) compared to the TugelaDN control. The TugelaDN and TugDN+$BSMV_0$ controls were not found to be significantly different ($P= 0.08$; Fig. 3.1A).

A mean loss of 45% of transcript abundance was recorded for TugDN+BSMV$_{APX}$ treatments when compared to the TugDN+BSMV$_0$ control (P<0.05) and 70% compared to the TugelaDN control (P<0.05). The TugelaDN and TugDN+BSMV$_0$ controls were not found to be significantly different (P= 0.07; Fig. 3.1B).

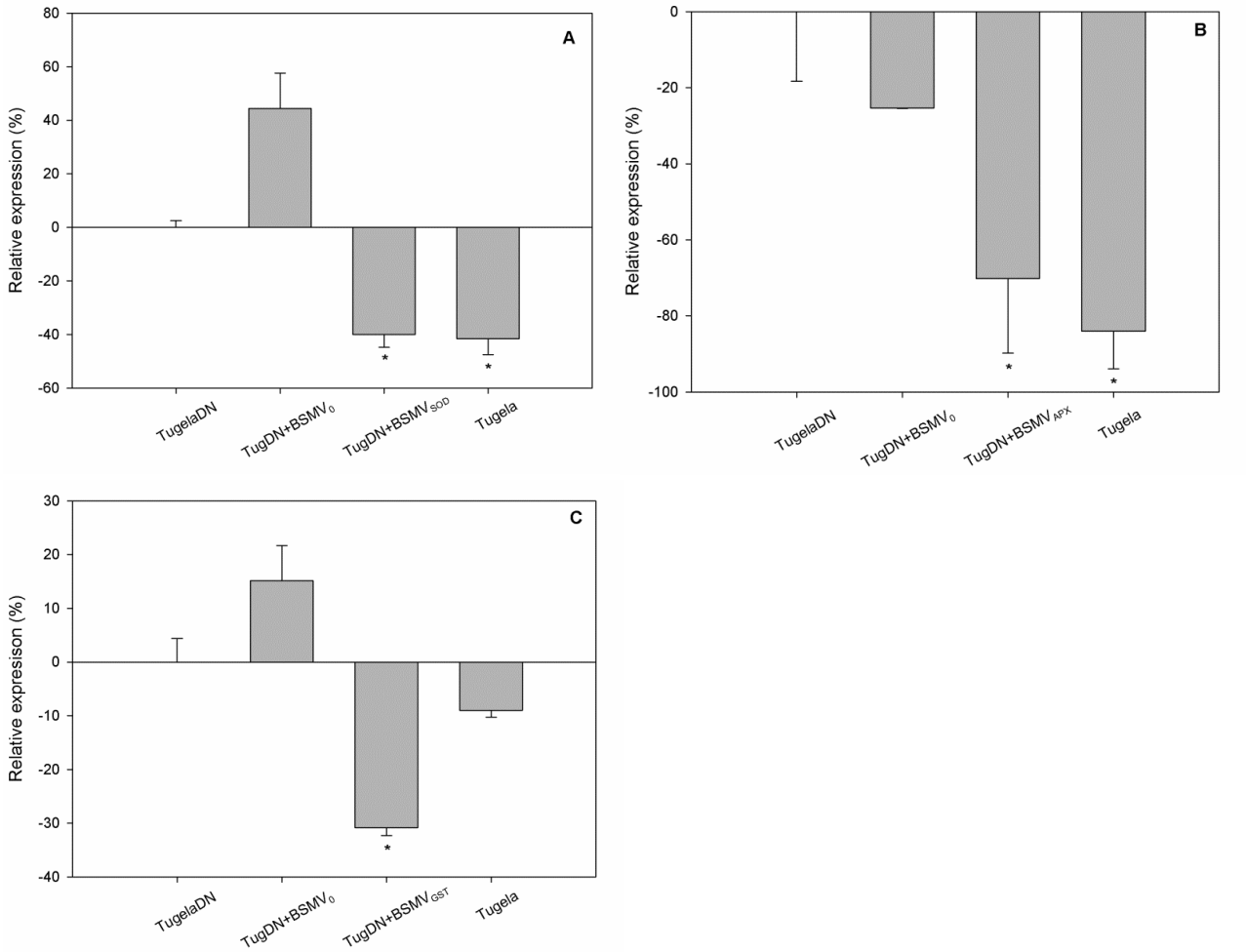

Fig. 3.1. Relative expression levels were measured 14 days after silencing with VIGS in order to confirm knock down of target genes. Expression was determined by using RT-qPCR with levels relative to TugelaDN. Asterisks indicate significant deviation from resistant control (P<0.05). **(A)** Superoxide dismutase *Cu/Zn*, **(B)** Ascorbate peroxidase, **(C)** Glutathione-*S*-transferase.

Relative expression levels of the TugDN+BSMV$_{GST}$ treatment group showed the smallest knockdown effect, with a mean of 45% transcript abundance compared to the TugDN+BSMV$_0$ control

(*P*<0.05) and 30% compared to the TugelaDN control (*P*<0.05). *TaGSTF6* transcript levels in TugelaDN and TugDN+BSMV$_0$ controls were not found to be significantly different (*P*= 0.25; Fig. 3. 1C). And, although Tugela was found to have a mean *TaGSTF6* transcript level lower than that of TugelaDN, the difference was not found to be significant at that time point.

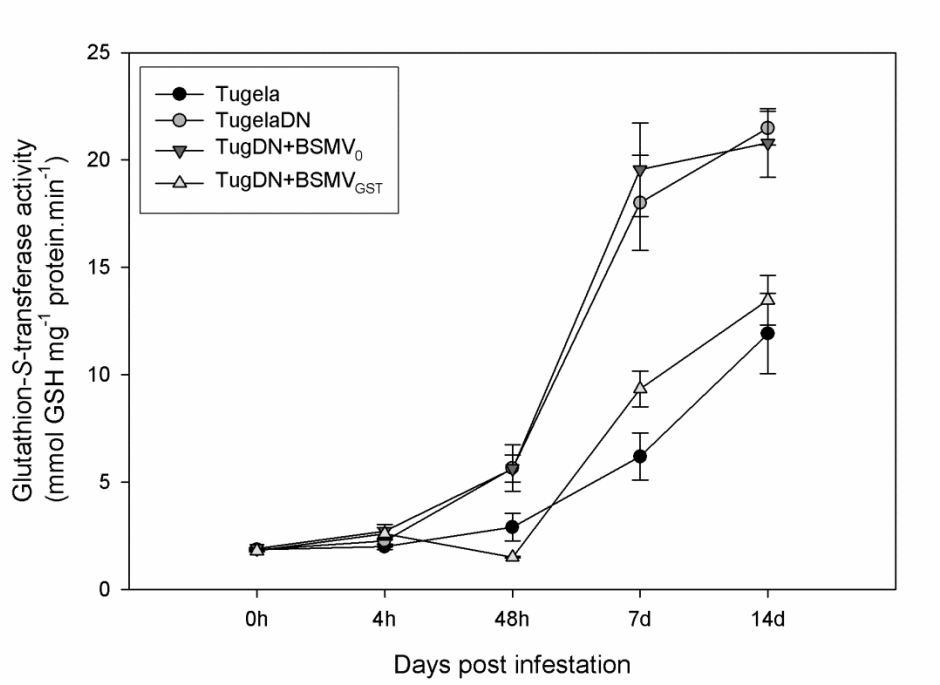

Fig. 3.2. Glutathione-*S*-transferase activity measured across 14 days in infested Tugela, TugelaDN, TugDN+BSMV$_0$ and TugDN+BSMV$_{GST}$ wheat plants.

As an indicator for possible changes in GST activity at the protein level, a GST protein activity assay was conducted (Fig. 3.2). The TugelaDN and TugDN+BSMV$_0$ resistant controls were not significantly different (*P*> 0.65). The TugDN+BSMV$_0$ resistant control had the highest GST activity assay measurements, while the Tugela and TugDN+BSMV$_{GST}$ treatments had the lowest. The assay measurements of Tugela and TugDN+BSMV$_{GST}$ did not differ significantly. By day 14 of infestation, there was 64% less enzyme activity in the TugDN+BSMV$_{GST}$ treatment when compared to TugDN+BSMV$_0$ and 63% less than TugelaDN.

A difference was seen between the GST enzyme assay and qPCR results (Figs. 1 and 2). This was expected, since the GST enzyme assay measured all conjugate-GSH that is formed in the cell and not only GSTF6 which is only one protein within a complex protein family. The qPCR results on the other hand only measured the expression of *GSTF6* and should be a more specific than the enzyme assay which doesn't discriminate between GST isoforms. Also, the small difference seen in TugelaDN and Tugela is recorded at a time point at which an upward trend in GST activity is evident. The Affymetrix study that the gene was chosen from was done at an earlier time point, and shows a significant up-regulation in TugelaDN in comparison to Tugela (Botha *et al.*, 2010).

The effects of silencing on aphid fertility

Antibiosis negatively impacts aphid reproduction (Painter 1951; Smith *et al.*, 1992) and is the mode of action of plants containing *Dn1* (Du Toit 1987, 1989). To ascertain the level of involvement of *tAPX*, *CuZnSOD* and *TaGSTF6* in *Dn1*-mediated antibiosis, *D. noxia* fertility was measured. A newly born *D. noxia* nymph was caged on each plant. The days till- and rate of- reproduction of each aphid was measured for 21 days, and the mean total number of nymphs of each treatment was compared (Fig. 3.3). Using the time point of 12 days post birth (21 days PVI) TugDN+BSMV$_0$ had a mean total number of nymphs of 9.7, and was similar to TugelaDN with 9.6. The mean total number of nymphs observed from foundresses caged on TugDN+BSMV$_{APX}$ (Fig. 3.3A), TugDN+BSMV$_{SOD}$ (Fig. 3.3B) and TugDN+BSMV$_{GST}$ (Fig. 3.3C) were 10.4, 9.3 and 13.3 respectively. The foundresses on the susceptible Tugela control showed a mean of 17.6 nymphs.

The foundresses on TugDN+BSMV$_{APX}$ showed an increase in fertility of between 14% and 34% between days 8 and 11 (Fig. 3.3A). The TugDN+BSMV$_{SOD}$ treatments showed no deviation from the resistant controls (Fig. 3.3B). In contrast, *D. noxia* fed on TugDN+BSMV$_{GST}$ were found to have a

higher fertility than the resistant controls with between 33% and 58% increase in nymphs born to the foundresses on these treatments over two weeks (Fig. 3.3C).

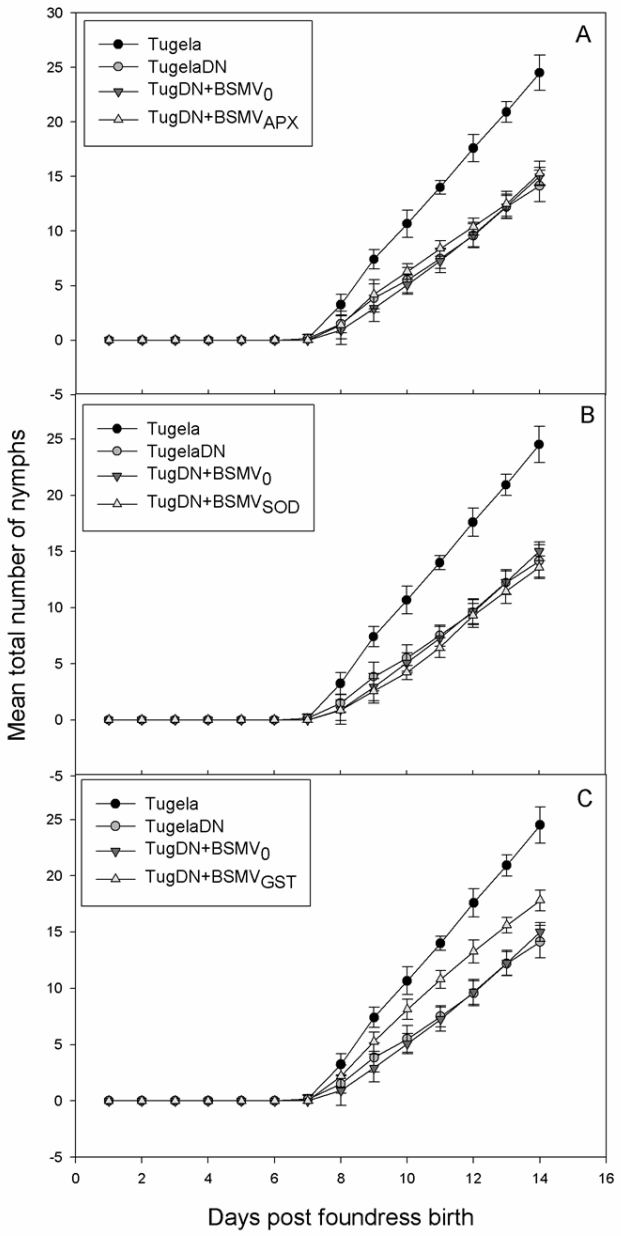

Fig. 3.3. A comparison of mean total number of *D. noxia* nymphs born per day to the foundress over 14 days from her date of birth using infested controls Tugela and TugDN+BSMV$_0$ and **(A)** TugDN+BSMV$_{APX.}$ The highest and lowest number of nymphs was born on wounded Tugela and TugDN+BSMV$_0$ respectively. **(B)** TugDN+BSMV$_{SOD}$. The highest and lowest numbers of nymphs were born on wounded Tugela and TugDN+BSMV$_{SOD}$ respectively. **(C)** TugDN+BSMV$_{GST}$.

The mean intrinsic rate of increase (r_m) was calculated for each treatment group (Table 3.1). No statistical difference was recorded between TugDN+BSMV$_0$ and TugelaDN treatments (P=0.58). TugDN+BSMV$_{APX}$ and TugDN+BSMV$_{SOD}$ r_m measurements were not statistically different from the resistant controls (P<0.01). A significant increase in r_m was observed in TugDN+BSMV$_{GST}$ treatments (r_m= 0.306, *sd*= 0.013) when compared to TugDN+BSMV$_0$ (r_m= 0.256, *sd*= 0.024; P<0.01) and TugelaDN (r_m= 0.264, *sd*= 0.028; P<0.01).

Table 3.1. The *D. noxia* foundress mean intrinsic rates of increase (IR) on the separate treatments with their respective standard deviations (*sd*), where the asterisk indicates a statistically significant difference when compared to TugDN+BSMV$_0$ (P<0.01).

Treatment	**Mean r_m**	***sd***
Tugela	0.344*	0.019
TugDN	0.264	0.027
TugDN+BSMV$_0$	0.256	0.024
TugDN+BSMV$_{APX}$	0.274	0.023
TugDN+BSMV$_{SOD}$	0.265	0.025
TugDN+BSMV$_{GST}$	0.306*	0.013

The production of hydrogen peroxide is affected by silencing TaGSTF6

The silencing of *TaGSTF6* had a positive effect on aphid reproduction. In order to gauge the effects of silencing *TaGSTF6* on H_2O_2 production, peroxidase activity was assayed (Fig. 3.4A). The highest peroxidase activity was observed in the resistant controls which showed no significant difference (P=0.67), and the lowest activity was seen in the TugDN+BSMV$_{GST}$ treatments. At day 7, TugDN+BSMV$_{GST}$ measurements were found to be 70% and 68% lower than the TugDN+BSMV$_0$ and TugelaDN controls respectively (P<0.01), and 58% lower than the Tugela control (P<0.01). The

Tugela control showed a 27% lower level of peroxidase activity when compared to TugelaDN (P<0.05).

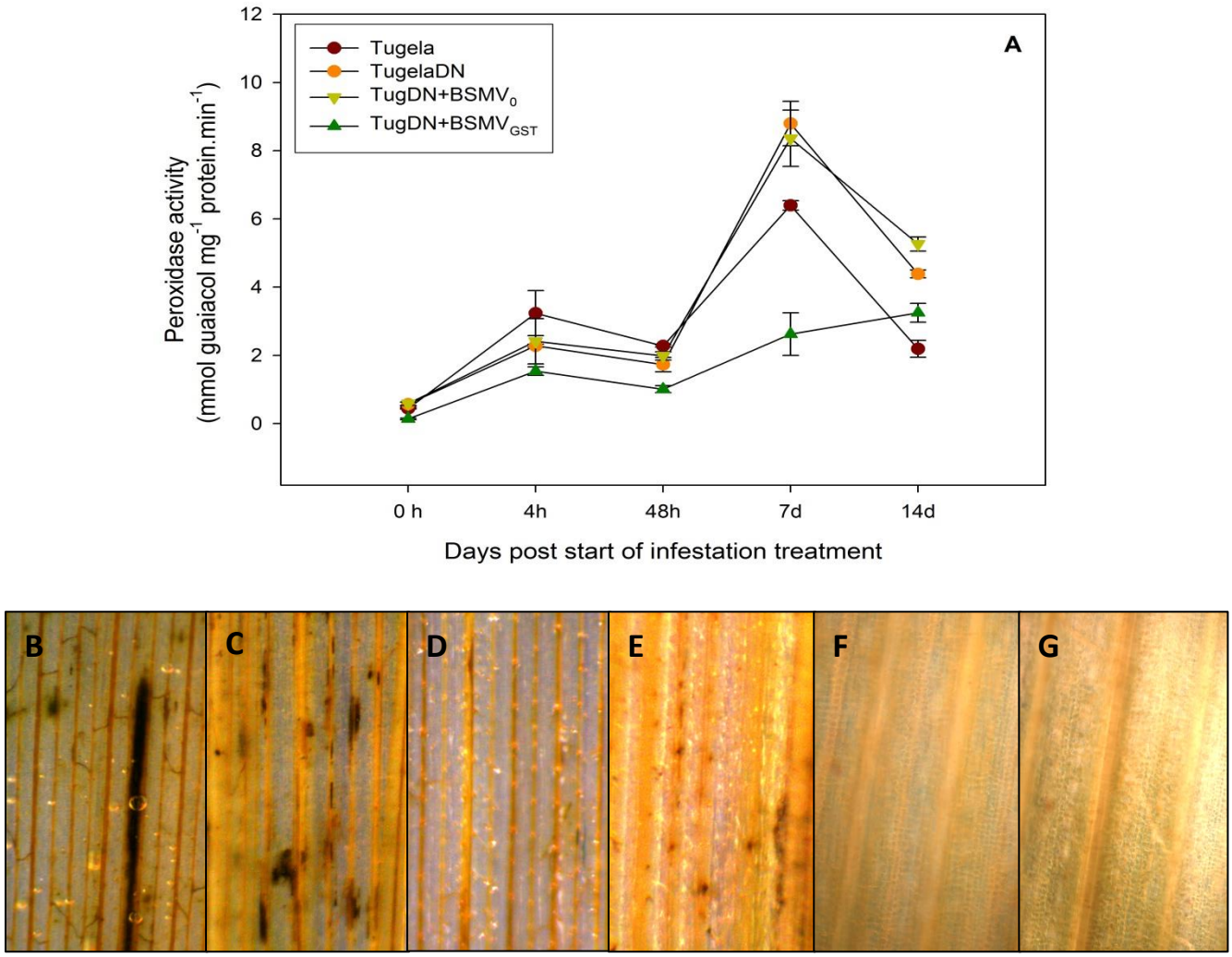

Fig. 3.4. (A) Peroxidase activity measured in Tugela, TugelaDN, TugDN+BSMV$_0$ and TugDN+BSMV$_{GST}$ plants over 14 days from the start of infestation. DAB stained leaves showing dark staining in the presence of H_2O_2 in infested **(B)** TugelaDN, **(C)** TugDN+BSMV$_0$, **(D)** TugDN+BSMV$_{GST}$, **(E)** Tugela leaves, and no H_2O_2 staining in the absence of infestation in **(F)** TugelaDN and **(G)** Tugela.

To test if the difference in peroxidase activity could be seen at a phenotypic level, representative samples from each treatment were DAB-stained for the presence of H_2O_2 (Fig. 3.4B-E).

The resistant infested controls TugelaDN and TugDN+BSMV$_0$ showed dark staining around areas of feeding as well as in the phloem and in the small connecting veins of the leaf (Fig. 3.4B,C), with none evident in the uninfested Tugela and TugelaDN plants (Fig. 3.4F,G). Staining was slight in the Tugela treatment (Fig. 3.4E). The TugDN+BSMV$_{GST}$ samples appeared pale, indicating a reduced amount of staining (Fig. 3.4D), consistent with the reduced peroxidase activity results.

3.4 Discussion

This study investigated the roles of *tAPX*, *CuZnSOD* and *TaGSTF6*, in *Dn1*-mediated resistance response under *D. noxia* infestation conditions. The VIGS recombinant vectors were shown to be effective in knocking-down mRNA transcripts to between 41% and 69% of the controls (Fig. 3.1). Previous studies on wheat have reported silencing of between 45% and 85% which was sufficient for recording differences at both the phenotypic and molecular level (Scofield *et al.*, 2005; Van Eck *et al.*, 2010).

The family of superoxide dismutases is involved in detoxification of superoxide radicals (O_2^-), and in the case of *CuZnSOD*, is found in the cytosol, chloroplast and possibly the extracellular spaces (Bordo *et al.*, 1994). The *CuZnSOD* gene product targeted for silencing is localized to the chloroplast because previously functional chloroplast machinery was suggested important in host feeding tolerance (Ni and Quisenberry, 2003; Botha *et al.*, 2006). Also, overexpression of *CuZnSOD* has been found to help maintain photosynthetic capacity and thus increase plant tolerance to stress (Gupta *et al.*, 1993). However, although there was effective knock-down of *CuZnSOD* transcripts in TugelaDN, silencing had very little effect on aphid fertility. Other isoforms of SOD have been found to be present in chloroplastic fractions in wheat, namely *Fe*SOD (Sairam and Srivastava, 2002). As silencing is sequence specific, this isoenzyme was probably present in the silenced plants, and it compensated for

the lack of *CuZnSOD* in the chloroplast. Also, other thylakoidal mechanisms exist in the chloroplast for reduction of O_2^- and H_2O_2, such as the stromal APX (sAPX) isozyme (Asada 1999).

Ascorbate peroxidase occurs in plant cells as several iso-enzymes, present in the cytosol and chloroplast (Asada, 1999). The thylakoid bound *tAPX* targeted for silencing is found near the photosystem I complex which reduces oxidized ascorbate (Miyake and Asada, 1992). Results show an increase in *D. noxia* number in *tAPX* silenced plants in early measurements only. The effect of silencing *tAPX* may have been mitigated by cross compartmental protection of the chloroplast by cytosolic *APX (cAPX)* as well as other detoxification methods (Davletova *et al.*, 2005). In studies using *Arabidopsis* under photooxidative stress conditions, the chloroplastic *tAPX* and *sAPX* were implicated in the early stages of stress (Maruta *et al.*, 2010).

GSTs function to conjugate glutathione (GSH) to a variety of substrates, and are known for their ability to inactivate toxic compounds by using glutathione. The GST family has been divided into eight different classes using genes identified in Arabidopsis: the Phi (GSTF), Tau (GSTU), Theta (GSTT), Zeta (GSTZ), Lambda (GSTL), dehydroascorbate reductases (DHARs) and tetrachlorohydroquinone dehalogenases (TCHQD) (Dixon and Edwards, 2010). Information on the GSTs was garnered after the discovery of their role in herbicide detoxification, and thus the conjugation of xenobiotics (Edwards and Dixon, 2000). The GSTs have discrete substrate preferences (Alfenito *et al.*, 1998) and have been found to target to different parts of the cell, such as the nucleous, cytoplasm and the chloroplast (Dixon *et al.*, 2009; Dixon and Edwards, 2010). The GSTFs are one of the larger classes found in plants and were originally studied because of their herbicide detoxification activity (Edwards and Dixon, 2000). In a functional study of Arabidopsis GSTFs, it was suggested that they play a role in limiting metabolic changes in the plant that arise during oxidative stress (Sappl *et al.*, 2009).

The *TaGSTF6* gene target for silencing falls under the *Phi* class GSTs and its activity has been described to some extent by Cummins *et al.* (2003). It was found to function as a dimer with glutathione peroxidase (GPX) activity, as well as being the only GSTF family member that conjugates ethacrynic acid, an inhibitor of GST activity. The TaGSTF6-6 dimer was found to have similar activity to ZmGSTF1 (Cummins *et al.*, 2003) and is inhibited to some extent by flavonoids. Sequence alignment and phylogenetic analysis revealed the closest rice homologue as OsGSTF1 (Fig. 3.S2 and S3), which has been found to be up-regulated during stress responses (Jain *et al.*, 2010). However, since the GSTF forms part of a large super family, off-target silencing of *TaGSTF6* can't be excluded despite extensive scrutiny and analysis of the target sequence (Senthil-Kumar and Mysore, 2011a; Dai and Zhao, 2011). Previously, it was shown that gene silencing is affected by the gene target position, optimum VIGS insert length and orientation (Burch-Smith *et al.*, 2004; de la Luz Gutierrez-Nava *et al.*, 2008; Ahmed and Zhao, 2011; Senthil-Kumar and Mysore, 2011b). Nonetheless, the silencing of *TaGSTF6* allowed for an increased *D. noxia* reproductive rate and intrinsic rate of increase, indicative of an increase in susceptibility (Fig. 3.3). The results from the mean total number of nymphs as well as the intrinsic rate of increase corresponded, and indicated that silencing of *TaGSTF6* negatively affected the *Dn1*-mediated antibiotic host response (Painter 1958) over the time period of the experiment.

Silencing *TaGSTF6* also decreased hydrogen peroxidase levels to below those of both the resistant and susceptible controls (Fig. 3.4E). The effects of this can be seen in the DAB stained TugDN+BSMV$_{GST}$ treatments, as they show little to no stained deposits. None of the veins in *TaGSTF6* silenced leaves showed staining, similar to the susceptible control (Fig. 3.4C). The resistant controls show the presence of H_2O_2 in the veins of the leaves, indicative of peroxidase activity (Fig. 3.4A,B). This may indicate either the production of- or movement of- H_2O_2 in the veins of the leaf, a feature absent from the susceptible Tugela plants.

H_2O_2 triggers GSH production, and H_2O_2 availability is directly affected by the availability of GSH (Han *et al.*, 2013). The redox status of GSH is thought to act as a rapid signal from the chloroplast to the nucleus (Karpinski *et al.*, 1997). A study by Han *et al.* (2013) utilizing *Arabidopsis* mutants, showed a direct link between the availability of GSH and downstream signalling via H_2O_2. Utilizing a *cat2* mutant which blocked GSH production, they found that H_2O_2 induced salicylic acid (SA) accumulation as well as showing that the SA-mediated resistance responses were negatively affected. The results from our *TaGSTF6*-silencing experiments are consistent with these findings, as there was both a reduction in H_2O_2 production, as well as a prolonged negative effect on the level of resistance. This indicates a role for both *TaGSTF6* and H_2O_2 signalling in *Dn1*-mediated *D. noxia* resistance.

This study utilized VIGS in order to screen HR-associated genes in *Dn1* containing plants. The results of this study indicate that H_2O_2 plays an important role in *Dn1*-mediated resistance and that H_2O_2 and *TaGSTF6* are necessary for the antibiosis-type resistance response. Even though there was down-regulation in H_2O_2 production, the $BSMV_{GST}$ plants were not rendered completely susceptible, therefore, silencing *TaGSTF6* is not sufficient to nullify the *D. noxia* resistance response in TugelaDN but rather acts down-stream of the initial recognition event.

3.5 Acknowledgements

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for BSMV-mediated VIGS.

3.6 References

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3.7 Supplementary data

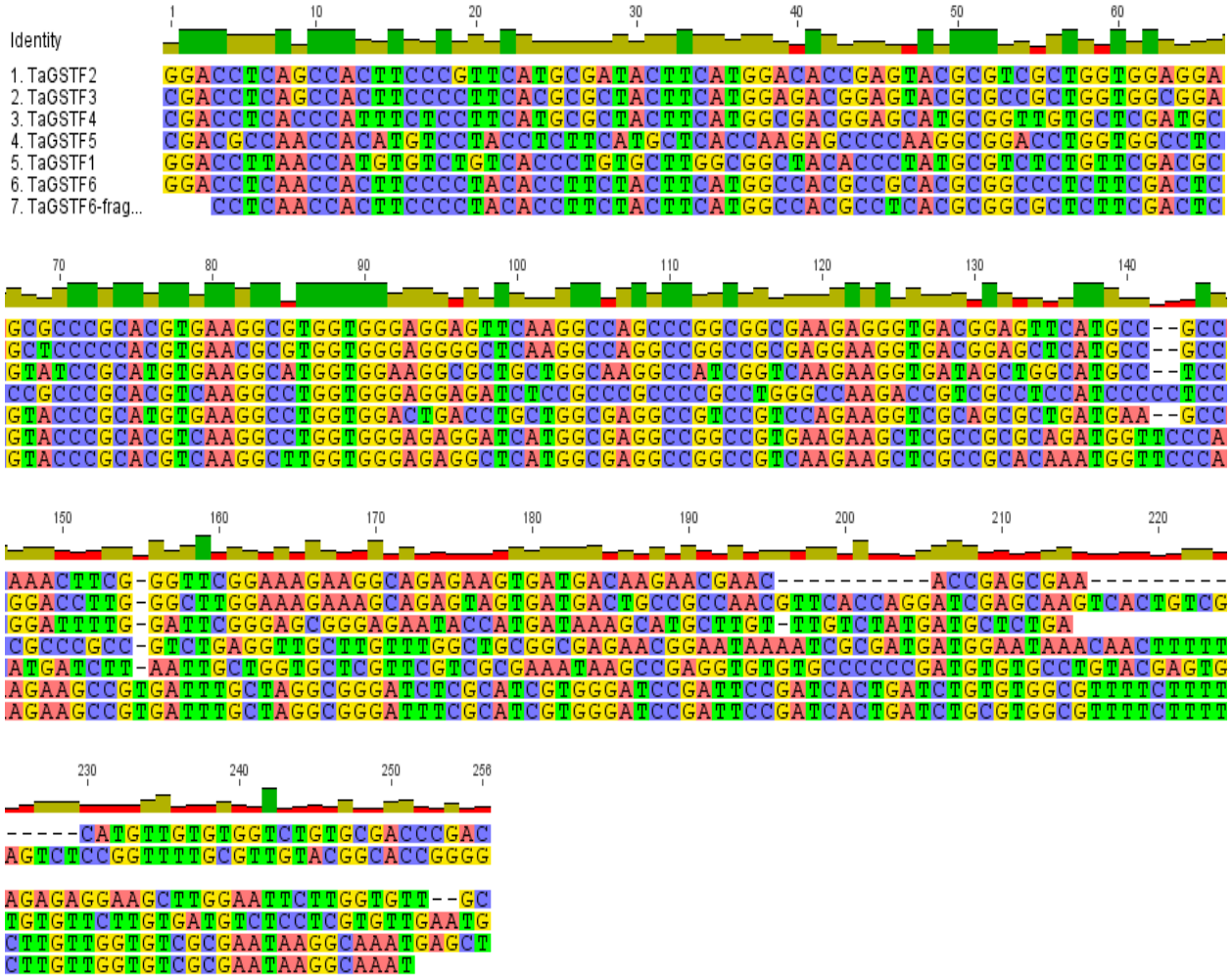
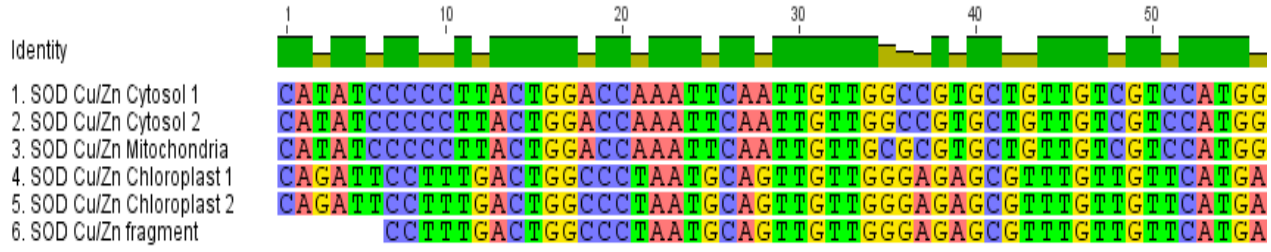


Fig. S3.1. Alignment of *TaGSTF6* gene fragment utilized for VIGS silencing with previously identified *Triticum aestivum* GSTFs (Cummins *et al.,* 2003).



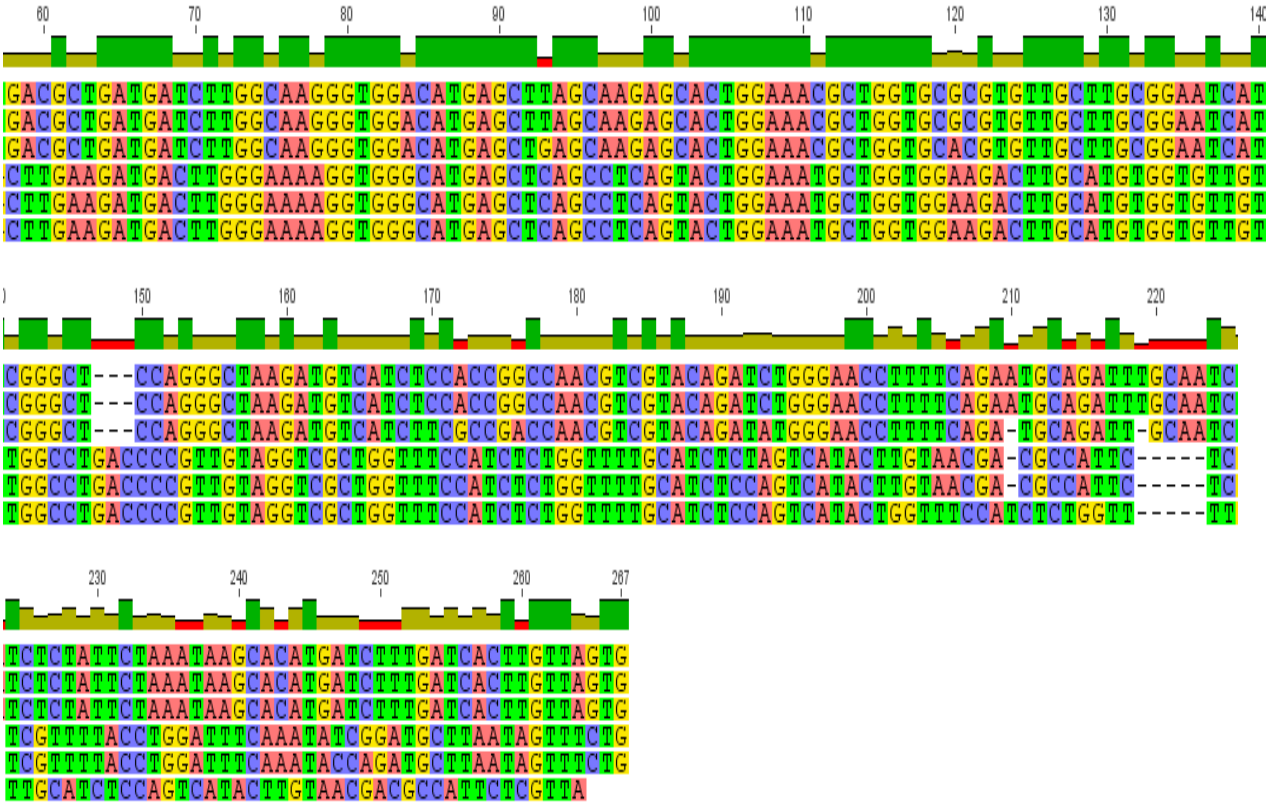
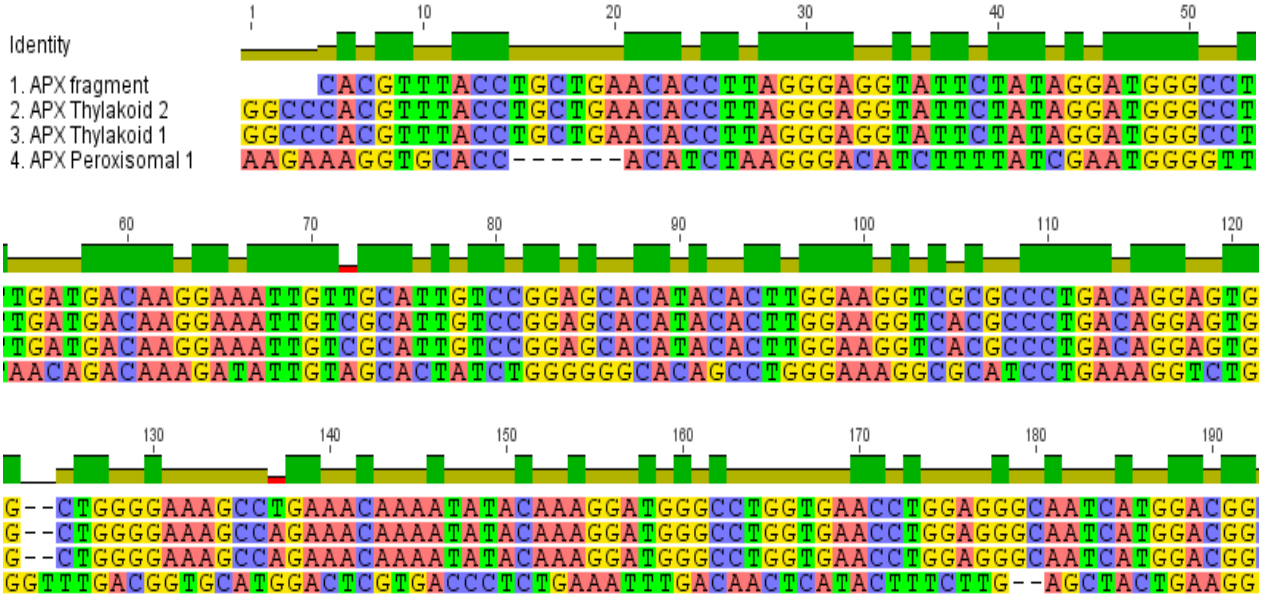


Fig. S3.2. Alignment of *CuZnSOD* gene fragment utilized for VIGS silencing with *Triticum aestivum SOD* orthologues from the chloroplast, cytosol and mitochondria.



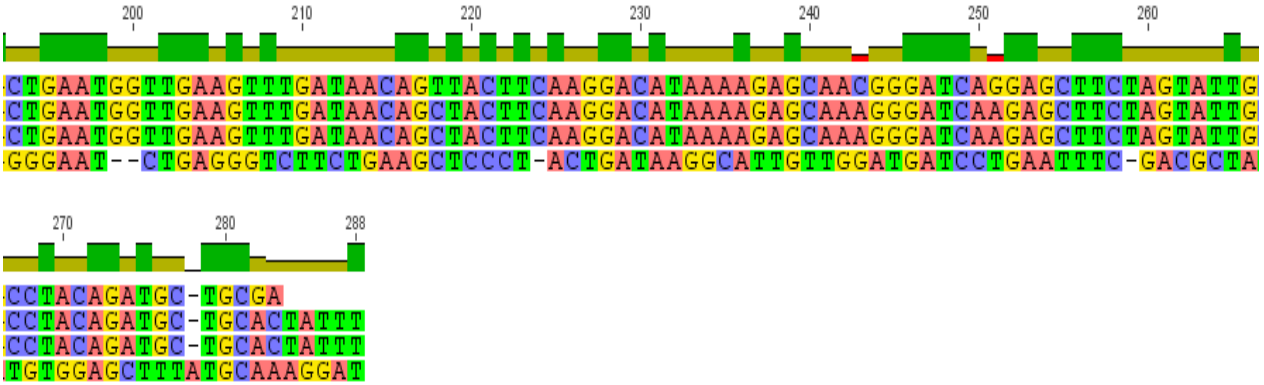


Fig. S3.3. Alignment of *APX* gene fragment utilized for VIGS silencing with available *Triticum aestivum APX* orthologues from the peroxisome and thylakoid.

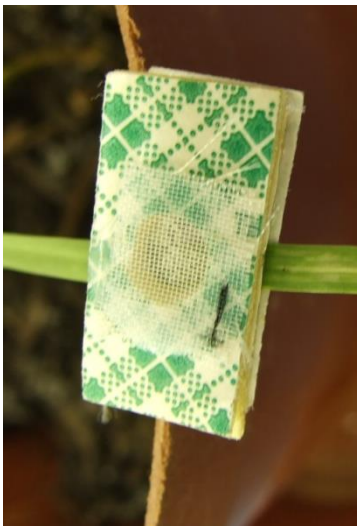


Fig. S3.4. Aphid cage used to cage single aphids on an individual wheat leaf for observation. This allowed for the counting and removal of nymphs born daily.

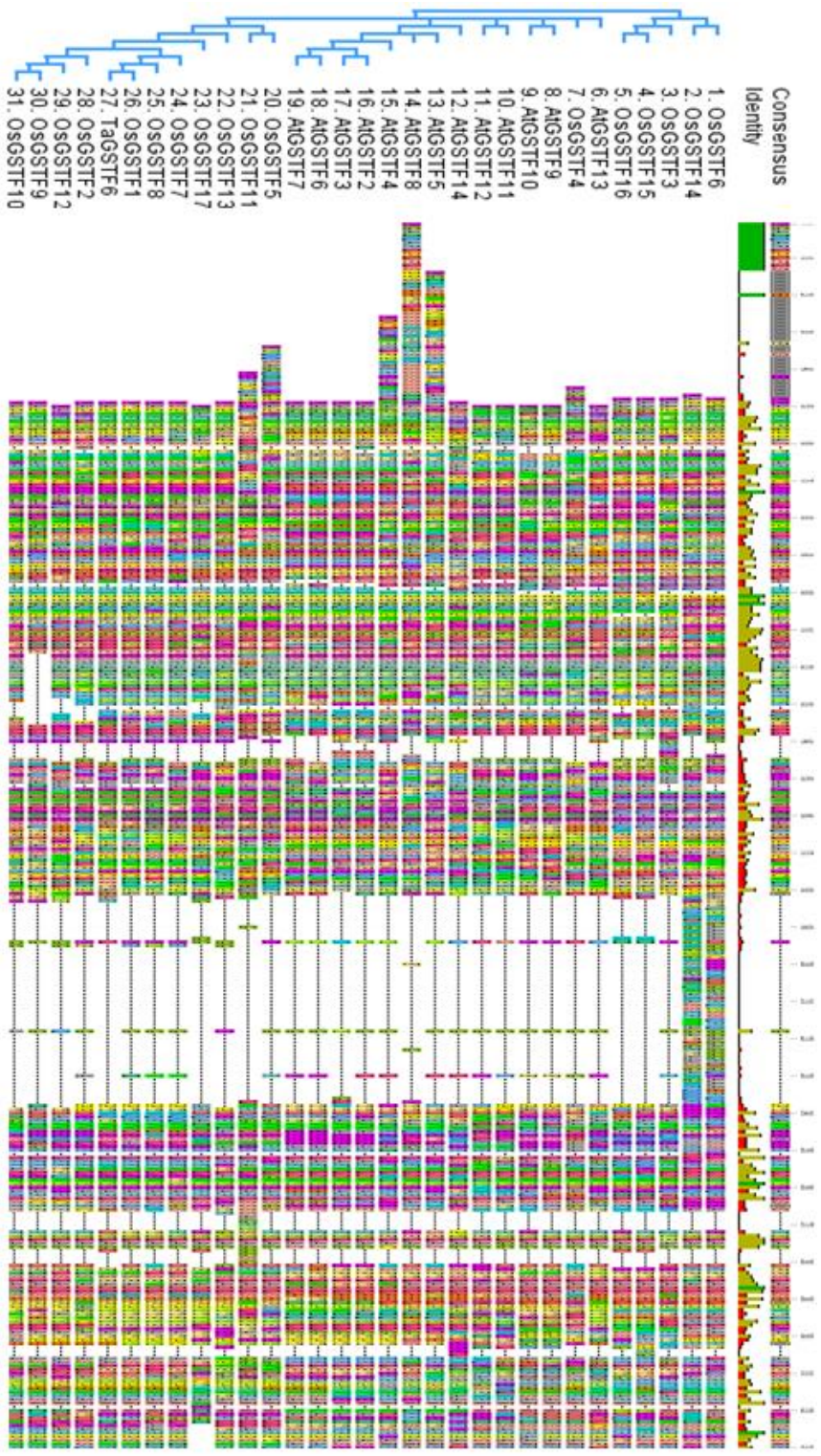


Fig. S3.5. Alignment of GST phi class protein sequences using ClustalW (from *Arabidopsis*, *O. sativa* and wheat) indicating the closest homologue to the protein sequence of interest as OsGSTF1. Figure is coloured to show regions of similarity.

Fig. S3.6. Phylogenetic tree of phi-class GST protein sequences from *Arabidopsis* and *O. sativa* including the translated *TaGSTF6* under investigation (Table S3.3). TaGSTF6 was found to have the most similarity to OsGSTF1 (Orange). Aligned protein sequences using ClustalW program were used to construct the unrooted tree with Geneious Tree Builder (Geneious 6.0.3, Biomatters, New Zealand). The numbers at the nodes were calculated with 1000 bootstrap replicates.

Table S3.1: Table indicating the AFFY chip expression data for Tugela and TugelaDN for the *GSTF3* and *GSTF6* genes

Genbank accession	Gene description	Expression							
		Tugela			TugelaDN			*t*-test	
AJ440792	*Triticum aestivum* mRNA for glutathione transferase F3 (*gstf3* gene).	5.264	4.613	5.312	5.437	5.227	5.716	p=0.2	
AJ440792	*Triticum aestivum* mRNA for glutathione transferase F3 (*gstf3* gene).	5.606	5.316	5.572	6.174	5.628	6.110	p=0.07	
AJ440795	*Triticum aestivum* mRNA for glutathione transferase F6 (*gstf6* gene).	7.410	6.955	7.167	8.570	7.861	8.115	P<0.02	
AJ440795	*Triticum aestivum* mRNA for glutathione transferase F6 (*gstf6* gene).	8.259	7.774	8.154	9.378	8.866	9.083	p<0.01	
AJ440795	*Triticum aestivum* mRNA for glutathione transferase F6 (*gstf6* gene).	8.259	7.774	8.154	9.378	8.866	9.083	p<0.01	

Table S3.2. Protein sequences used for ClustalW alignments and UPGMA tree translated from sequences from Jain *et al.* (2010) and Dixon and Edwards (2010) (Table S3.3).

Locus/ accession	Gene	Protein sequence
AJ440795	TaGSTF6	MAPVKVFGPAMSTNVARVLVCLEEVGAEYEVVDIDFKAMEHKSPEHLVRNPFGQIP AFQDGDLLLFESRAIARYVLRKYKKNEVDLLREGDLKEAAMVDVWTEVDAHTYN PAISPIVYECSSTAHARLPTNQTVVDESLEKLKNVLEVYEARLSKHDYLAGDFVSFA DLNHFPYTFYFMATPHAALFDSYPHVKAWWERIMARPAVKKLAAQMVPKKP
LOC_Os01g27360	OsGSTF1	MAPMKLYGSTLSWNVTRCVAVLEEAGAEYEIVPLDFSKGEHKAPDHLARNPFGQV PALQDGDLFLWESRAICKYVCRKNKPELLKDGDLKESAMVDVWLEVESNQYTPA LNPILFQCLIRPMMFGAPPDEKVVEENLEKLKKVLEVYEARLTKCKYLAGDYISVA DLSHVAGTVCLGATPHASVLDAYPHVKAWWTDLMARPSSQKVASLMKPPA
LOC_Os01g55830	OsGSTF2	MAPMKLYGSTLSWNVTRCVAVLEEAGAEYEIVPLDFSKGEHKAPDHLARNPFGQV PALQDGDLFLWESRAICKYVCRKNKPELLKDGDLKESAMVDVWLEVESNQYTPA LNPILFQCLIRPMMFGAPPDEKVVEENLEKLKKVLEVYEARLTKCKYLAGDYISVA DLSHVAGTVCLGATPHASVLDAYPHVKAWWTDLMARPSSQKVASLMKPPA
LOC_Os03g04260	OsGSTF3	MAAPVTVYGPMISPAVARVAACLLEKDVPFQVEPVDMSKGEHKSPSFLKLQPFGQV PAFKDSLTTVFESRAICRYICDQYADSGNKTLMGRKEDGAVGRAAIEKWIEAEGQS FNPPSLAMAFQLAFAPFMGRATDMAVVEQNEAKLVKVLDVYEQWLGENQYFAGD EFSLADLVHMPNTDLLVRKTNKAGLFTERKNLARWWDEVSARPSWKKVVELQNV PRPS
LOC_Os01g70770	OsGSTF4	MAGEGRKLRVYGMALSANVVRVATVLNEKGLDFDLVPVDLRTAAHKQPHFLALN PFGQIPVLQDGDEVLYESRAINRYIATKYKAEGADLLPAEASPAKLEVWLEVESHHF YPAISGLVFQLLIKPLLGGATDTAAVDEHAAALAQVLDVYDAHLAGSRYLAGNRFS LADANHMSYLLFLSKTPMAELVASRPHVKAWWDDISSRPAWKKTAAAIPFPPAA
LOC_Os01g27210	OsGSTF5	MYQQSAGQIQLQEGPMAPMKVYGWVVSPWMARVLVALEEAGAEYEVVPMSRSG GDHRRPEHLARNPFGEIPVLEDGDLTLYQSRAIARYIFRKYKPEFLGLGEGGSLEES AMVDVWLDVEAHQHEAAVRPILWHCIINKFEGRDRDQGVVDESVRKLEKVLGVY EARLSGSRYLAGDRISLADLSHFSNMRYFMATEYAGVVDAYPHVKAWWEALLAR PTVQKVMAGMPPDFGFGSGNIP
LOC_Os10g39740	OsGSTF6	MPGAVKVFGSPSSAEVARVLACLFEKDVEFQLIRVDSFRGSKRMPQYLKLQPHGEA LTFEDGNVTLVESRKIIRHIADKYKNQGNPDLIGMGALERSSIEQWLQTEAQSFDVP SADVVYSLAYLPAATTQPNKGAAAADGGRCEEEKNDDGGRDRQYSSQRQGGAGA GGGRDGQMAAAHRQKVEEMKQLFEKSSKELSKVLDIYEQRLEEAEYLAGDKFTL ADLSHLPNADRLAADPRTLRMLQSRRNVSRWWADVSGRESWKQVKSLNRPPSAE APF
LOC_Os01g27260	OsGSTF7	MSPVKVFGRAISTNVSRVLVCLEEVGADYELVTVDFLAGEQNSPEHVERNPFGKIP ALQDGDLVLFESRAIAKYILRKYKSSKVDLLRESDIREAALVDVWTEVEAHQYYPA LSPIVFECIIFPIMRGVPTNQQVVHESLEKLKKVLETYEARLSGSRYLAGDFLSFADL NHFPFTFYFMATPCASLFDAYPHVKAWWEGLMSRPSIKKISANMPTKF
LOC_Os01g27340	OsGSTF8	MAPVKVFGPAMSTNVARVLVCLEEVGVEYELVNIDFKAMEHKSPEHLKRNPFGQM PAFQDGDLLLFESRAVGRYILRKYKTSEANLLREGNLTEAAMVDIGIEVEIHQYYPV ISSIVYECLFNPAMYGVPTNQKVVDNSLEKLKKVLEVYEARLSQNTYLAGNFLSFV DLSHFPFTFYFMATPYASLLDKYPHVKAWWDGLAARPSIKKVTAAMVLPLKA
LOC_Os01g27380	OsGSTF9	MAPVKVFGPAKSTAVARVLVCLEEVGAEYELVGIHIPAGEQKSPAHLARNPFGQVPA FQDGDLILFDLLKESNLSQSAIMVDVWLEVESQTFDTAMSAITFQCLTIPTFMGGIA DDKIVEENLGKLKKALEVYEARSCRFRYLAGDFISLADLSHFPMTHYLLATPHASV LDAYPHVKSWINDLMKRPAVKRVRELME

LOC_Os01g27390	OsGSTF10	MAPAKVYGPAMSTNVMRILVCLEEVGAEYEVVPVDMSTGEHKRPPHISRNPFGQV PAFEDGDLTLFESRAISKYILRKHGSDLLRESNLSESAMVDVWLEVESSHFDGAMS PIIFQCFIVPMFMGGATDIGVVNESLEKLKKALEVYEAQLSKSKYLAGDFISLADISH FPTVYYLLASAHASVLEAYPRVKAWIDDVMQRPSVKKVTEALKMPSA
LOC_Os01g25100	OsGSTF11	MEKTCQAYLEQTLCWTSSLLIDFFQLLTPARREAGVEYEVVPLSLTNGDHRRPEHL ARNPFGQIPVLEDGDLTLYQSHAIARYVLGKHKPELLGLGEGGSVEESAMVDMWL EVETHQYEAAVKPIVWHCLVHQHVGLVRDQGVVDESVEKLRAVLEVYEARLSSSS AGRYSYLAGGGSGDRVSLADLSHVPLMHYFTATEYGGVLGEYPRVKAWWEALLA RPSVKKVIAGMPTDFGFGSGNLP
LOC_Os01g27630	OsGSTF12	MAMKVYGLPMSTNVARVLVCLEEAGEQYEVVPIDFSIAEHKSPEHTSRNPFGQVPA LQDGDLILFESRAISKYVLRKNNSELLKEHNLSDAAKVDVWLEAESHHFDEPMSV VIYQCLILPVYFGGQTDAKVVEENLEKLKKTFQVYEERLCKFRYLAGDFLSLADLS HFPTAYYLLATPHAAMLDEFPLVKAWIDGMLARPSVKKVIEMMKATA
LOC_Os01g27480	OsGSTF13	MSPVKVFGSAPFTNVARVLLCLEEVGADYEIVDVDFGDREHKGPDHLARNPFGQV PAFQDGDLMLFESRAICRYILRKHRATDEANLLREGDPSESAVVDAWLDVEALRYE PSVHAVFVQRRVVPALGGEPDERVIAESVARLRETLAVYEARLEATRGYLAGGEVS LADLSHFPYTRYFMEMPYEVPVFGAYPRVTAWWERLLTRPSVRKVAAMMSGGEG
LOC_Os03g04220	OsGSTF14	MAPASVKVFGSPTSAEVARVLMCLFEKDVEFQLVRVDAYRGTQRMPQYLKLQPLG EALTFEDDNLTLSESRGILRHIAHKYARQGNPDLIGTGALERASIEQWLQTEAQSFD VPSAEMVYSLAFLPPNMPKQNDNGNGNGNGYGNSNGREVQVANASSKRVVAGAT DGKTAASGANGNKQQQKEEEMRKVFEKSKKDLEKLLDIYEQRLEEAAYLAGDKF TIADLSHLPNADRLASDPRSRRMFEARKNVSRWWNNISSRESWEYVKSLQRPPSA AHAGNAQQQQQQQSPSAGNNYQHQQGQGQGQQHYRNEQVENYNN
LOC_Os03g04240	OsGSTF15	MAAGLQVFGQPASTDVARVLTCLFEKNLEFELIRIDTFKKEHKLPEFIKLRDPTGQV TFKHGDKTLVDSRAICRYLSTQFPDDGNRTIYGTGSLERASIEQWLQAEAQSFDAPS SELVFHLAFAPQLNIPADEARIAENERKLQQMLNVYDEILAKNKYLAGDEFTLADL SHLPNSHYIVNARSPRGKKLFTSKKHVARWYEEISNRASWKQVVKMQSEHPGAFE
LOC_Os03g04250	OsGSTF16	MAAGLQVFGQPASTDVARVLTCLFEKDLEFELVCIDTFKREHKLPEFIKLRDPNGQV TFKHGDKTIVDSRAICRYVCTQFPEGNKTLYGTGSLERASIEQWLQAEAQNFSPPSS ALVFHLAFAPHLNIPQDHAVIAENEKKLQQVLNVYDEILSKNEYLAGDEFTLADLS HLPNSHYIVSSERGRKLFTGRKNVARWYDQISKRETWKQVVKMQREHPGAFE
LOC_Os05g05620	OsGSTF17	MVAKVYGVAASPYVATVLVCLEEAGASYELVAVDMAAGENRSRHHLARSPFGKIP AFEDGEVTLFESRAIQRYVLRNYNKPDLLREGNLEESAMVDMWMEVEAHHYDPA IFHIIRECVIKPMIGGGARDQAIVDENVEKLRKVLEVYERRLSESEYLAGDFVSVAD LNHFPYTYYLMTTEYATLVESCTNVKAVEIMGI
AT4G02520	AtGSTF2	MAGIKVFGHPASIATRRVLIALHEKNLDFELVHVELKDGEHKKEPFLSRNPFGQVPA FEDGDLKLFESRAITQYIAHRYENQGTNLLQTDSKNISQYAIMAIGMQVEDHQFDP VASKLAFEQIFKSIYGLTTDEAVVAEEEAKLAKVLDVYEARLKEFKYLAGETFTLTD LHHIPAIQYLLGTPTKKLFTERPRVNEWVAEITKRPASEKVQ
At2g02930	AtGSTF3	MAGIKVFGHPASTSTRRVLIALHEKNLDFELVHVELKDGEHKKEPFLSRNPFGQVP AFEDGDLKLFESRAITQYIAHRYENQGTNLLPADSKNIAQYAIMSIGIQVEAHQFDP VASKLAWEQVFKFNYGLNTDQAVVAEEEAKLAKVLDVYEARLKEFKYLAGETFTL TDLHHIPVIQYLLGTPTKKLFTERPRVNEWVAEITKRPASEKVL
At1g02950	AtGSTF4	MDCLQMVFKLFPNWKREAEVKKLVAGYKVHGDPFSTNTRRVLAVLHEKRLSYEPI TVKLQTGEHKTEPFLSLNPFGQVPVFEDGSVKLYESRAITQYIAYVHSSRGTQLLNL RSHETMATLTMWMEIEAHQFDPPASKLTWEQVIKPIYGLETDQTIVKENEAILEKVL NIYEKRLEESRFLACNSFTLVDLHHLPNIQYLLGTPTKKLFEKRSKVRKWVDEITSR EAWKMACDQEKSWFNKPRN

At1g02940	AtGSTF5	MGINASHVPETCYHHCNQTFESSRQCFKWCQELARKDEYKIYGYPYSTNTRRVLA VLHEKGLSYDPITVNLIAGDQKKPSFLAINPFGQVPVFLDGGLKLTESRAISEYIATV HKSRGTQLLNYKSYKTMGTQRMWMAIESFEFDPLTSTLTWEQSIKPMYGLKTDYK VVNETEAKLEKVLDIYEERLKNSSFLASNSFTMADLYHLPNIQYLMDTHTKRMFV NRPSVRRWVAEITARPAWKRACDVKAWYHKKKN
At1g02930	AtGSTF6	MAGIKVFGHPASTATRRVLIALHEKNVDFEFVHVELKDGEHKKEPFILRNPFGKVPA FEDGDFKIFESRAITQYIAHEFSDKGNNLLSTGKDMAIIAMGIEIESHEFDPVGSKLV WEQVLKPLYGMTTDKTVVEEEEAKLAKVLDVYEHRLGESKYLASDHFTLVDLHTI PVIQYLLGTPTKKLFDERPHVSAWVADITSRPSAQKVL
At1g02920	AtGSTF7	MAGIKVFGHPASTATRRVLIALHEKNLDFEFVHIELKDGEHKKEPFIFRNPFGKVPAF EDGDFKLFESRAITQYIAHFYSDKGNQLVSLGSKDIAGIAMGIEIESHEFDPVGSKLV WEQVLKPLYGMTTDKTVVEEEEAKLAKVLDVYEHRLGESKYLASDKFTLVDLHTI PVIQYLLGTPTKKLFDERPHVSAWVADITSRPSAKKVL
At2g47730	AtGSTF8	MGAIQARLPLFLSPPSIKHHTFLHSSSSSNSNFKIRSNKSSSSSSSSIIMASIKVHGVPM STATMRVLATLYEKDLQFELIPVDMRAGAHKQEAHLALNPFGQIPALEDGDLTLFES RAITQYLAEEYSEKGEKLISQDCKKVKATTNVWLQVEGQQFDPNASKLAFERVFK GMFGMTTDPAAVQELEGKLQKVLDVYEARLAKSEFLAGDSFTLADLHHLPAIHYL LGTDSKVLFDSRPKVSEWIKKISARPAWAKVIDLQKQ
At2g30860	AtGSTF9	MVLKVYGPHFASPKRALVTLIEKGVAFETIPVDLMKGEHKQPAYLALQPFGTVPAV VDGDYKIFESRAVMRYVAEKYRSQGPDLLGKTVEDRGQVEQWLDVEATTYHPPLL NLTLHIMFASVMGFPSDEKLIKESEEKLAGVLDVYEAHLSKSKYLAGDFVSLADLA HLPFTDYLVGPIGKAYMIKDRKHVSAWWDDISSRPAWKETVAKYSFPA
At2g30870	AtGSTF10	MVLTIYAPLFASSKRAVVTLVEKGVSFETVNVDLMKGEQRQPEYLAIQPFGKIPVLV DGDYKIFESRAIMRYIAEKYRSQGPDLLGKTIEERGQVEQWLDVEATSYHPPLLAL TLNIVFAPLMGFPADEKVIKESEEKLAEVLDVYEAQLSKNEYLAGDFVSLADLAHL PFTEYLVGPIGKAHLIKDRKHVSAWWDKISSRAAWKEVSAKYSLPV
At3g03190	AtGSTF11	MVVKVYGQIKAANPQRVLLCFLEKDIEFEVIHVDLDKLEQKKPQHLLRQPFGQVP AIEDGYLKLFESRAIARYYATKYADQGTDLLGKTLEGRAIVDQWVEVENNYFYAVA LPLVMNVVFKPKSGKPCDVALVEELKVKFDKVLDVYENRLATNRYLGGDEFTLAD LSHMPGMRYIMNETSLSGLVTSRENLNRWWNEISARPAWKKLMELAAY
At5g17220	AtGSTF12	MVVKLYGQVTAACPQRVLLCFLEKGIEFEIIHIDLDTFEQKKPEHLLRQPFGQVPAIE DGDFKLFESRAIARYYATKFADQGTNLLGKSLEHRAIVDQWADVETYYFNVLAQPL VINLIIKPRLGEKCDVVLVEDLKVKLGVVLDIYNNRLSSNRFLAGEEFTMADLTHM PAMGYLMSITDINQMVKARGSFNRWWEEISDRPSWKKLMVLAGH
At3g62760	AtGSTF13	MAMKLYGDEMSACVARVLLCLHEKNTEFELVPVNLFACHHKLPSFLSMNPFGKVP ALQDDDLTLFESRAITAYIAEKHRDKGTDLTRHEDPKEAAIVKLWSEVEAHHFNPAI SAVIHQLIVVPLQGESPNAAIVEENLENLGKILDVYEERLGKTKYLAGDTYTLADL HHVPYTYYFMKTIHAGLINDRPNVKAWWEDLCSRPAFLKVSPGLTVAPTTN
At1g49860	AtGSTF14	MADSKMKLHCGFIWGNSAALFCINEKGLDFELVFVDWLAGEAKTKTFLSTLNPFG EVPVLEDGDLKLFEPKAITRYLAEQYKDVGTNLLPDDPKKRAIMSMWMEVDSNQ FLPIASTLIKELIINPYQGLATDDTAVQENKEKLSEVLNIYETRLGESPYLAGESFSLA DLHHLAPIDYLLNTDEEELKNLIYSRPNVAAWVEKMKMRPAWLKTVVMKNHIVD LMKQRRLPIKLDSSCHESTVVAQKNAIAIENK

Table S3.3: Nucleotide sequences used for ClustalW alignments and UPGMA tree translated from sequences from Jain *et al.* (2010) and Dixon and Edwards (2010).

Locus/ accession	Gene	Protein sequence
CuZnSOD Chloroplast 1	U69536	AAAGTTCTTCCGCTCCCGACGACCGCCATGGCCGCGCAGAGCCTCCTCTTTG CCGCCGCCGCGCCTCTCTTCCAGGTTCCTGCCTCTGCCCGCCCTTTCCAGTCG CTCCGAATTGTCTCCACTCCAGGAGGCGCCACCGCCGCCGCCAGGGCGCTC GTCGTCGCCGACGCCACCAAGAAGGCAGTCGCGGTGCTCAAGGGCACCTCC CAGGTCGAGGGCGTCGTCACGCTCACCCAGGAAGACGACGGTCCCACGACG GTGAACGTTCGTATCACTGGACTTGCTCCTGGACTTCATGGCTTCCACCTCCA TGAGTTCGGTGACATGACTAATGGGTGCATATCAACAGGTCCACATTTTAACC CAAACGGCCTGACACATGGTGCACCAGAAGATGAAGTCCGTCATGCGGGTG ACCTGGGAAACATTGTTGCCAATGCTGAAGGTGTGGCGGAGACAACCATTGT CGATAGCCAGATTCCTTTGACTGGCCCTAATGCAGTTGTTGGGAGAGCGTTT GTTGTTCATGAGCTTGAAGATGACTTGGGAAAAGGTGGGCATGAGCTCAGCC TCAGTACTGGAAATGCTGGTGGAAGACTTGCATGTGGTGTTGTTGGCCTGAC CCCGTTGTAGGTCGCTGGTTTCCATCTCTGGTTTTGCATCTCTAGTCATACTTG TAACGACGCCATTCTCGTTTTACCTGGATTTCAAATATCGGATGCTTAATAGTT TCTGTTGATCGTTTGTATCAGTGTAAAGTTGAGTTTATCAAATAAATGTTTGCA CACTTTCGTCTTGTTGAGCTATGAAGCGTGAACTTGGGAATTTTGGTGTGCGT AAGCTATAAAATGCTAAGGAATTATATTTGTGGATGTCCTTATTT
CuZnSOD Chloroplast 2	U69632	CCAAAAGTTCTTCCGCTTCCGAAGACAGCCATGGCCGCTCAGAGCCTCCTCT TTGCCGCCGCCGCGCCTCTCTTCCAGGCTCCTGCCTCTGCCCGCCCTTTCCAG TCGCTCCGAATTGTCTCCACCCCAGGAGGCGCCACCGCCGCCGCCAGGGCG CTCGTCGTCGCCGACGCCACCAAGAAGGCAGTCGCGGTGCTCAAGGGCTCC TCCCAGGTCGAGGGCGTCGTCACGCTCACCCAGGAAGACGACGGTCCTACG ACGGTGAACGTTCGTATCACTGGACTTGCTCCTGGACTTCATGGCTTCCACCT CCATGAGTTTGGTGACACGACTAATGGATGCATATCAACAGGTCCACATTTTA ACCCAAACGGCCTGACACATGGTGCACCAGAAGATGAAGTCCGTCATGCGG GTGACCTGGGAAACATTGTTGCCAATGCTGAGGGTGTGGCGGAGACAACCA TTGTCGATAGCCAGATTCCTTTGACTGGCCCTAATGCAGTTGTTGGGAGAGC GTTTGTTGTTCATGAGCTTGAAGATGACTTGGGAAAAGGTGGGCATGAGCTC AGCCTCAGTACTGGAAATGCTGGTGGAAGACTTGCATGTGGTGTTGTTGGCC TGACCCCGTTGTAGGTCGCTGGTTTCCATCTCTGGTTTTGCATCTCCAGTCAT ACTTGTAACGACGCCATTCTCGTTTTACCTGGATTTCAAATACCAGATGCTTA ATAGTTTCTGTTGATCGTTTGTATCAGTGTAAAGTTGAGTTTATCAAATAAAC
CuZnSOD Cytosol 1	JQ269677	CAGAGCATCACCCATTCATCATCCCATCCCCCAAGTCATAAACCCAAGTCGG ACTCGCCTTCCTCCTCCCCAACTACTCCTCTCCTCCGCGCATCGCATCGCACC GGGGGTCACCTGAGATCACATACACAATGGTGAAGGCTGTGGCTGTGCTTAC CGGCAGTGAGGGTGTCAAGGGCACCATCTTCTTCACCCAGGAGGGAGATGG CCCGACCACCGTGACGGGAAGCGTCACTGGACTCAAGGAAGGGCTCCACGG CTTCCACGTGCACGCTCTTGGTGACACCACCAACGGCTGCATGTCAACTGGA CCACACTTCAACCCTGCTGGTCATGTGCATGGGGCACCAGAAGATGAAATCC GCCATGCTGGTGATCTTGGAAATGTGACAGCTGGAGCGGATGGTGTTGCTAA CATCAATGTTACTGACTGCCATATCCCCCTTACTGGACCAAATTCAATTGTTG GCCGTGCTGTTGTCGTCCATGGTGACGCTGATGATCTTGGCAAGGGTGGACA TGAGCTTAGCAAGAGCACTGGAAACGCTGGTGCGCGTGTTGCTTGCGGAAT CATCGGGCTCCAGGGCTAAGATGTCATCTCCACCGGCCAACGTCGTACAGAT CTGGGAACCTTTTCAGAATGCAGATTTGCAATCTCTATTCTAAATAAGCACAT GATCTTTGATCACTTGTTAGTGTGCACCATTCGTGTGAATTCCTATGTGCTACT CTGAACTTTATCACTG
CuZnSOD Cytosol 2	JQ269676	CAGAGCATCACCCATTCATCATCCCATCCCCCAAGTCATAAACCCAAGTCGG ACTCGCCTTCCTCCTCCCCAACTACTCCTCTCCTCCGCGCATCGCATCGCACC GGGGGTCACCTGAGATCACATACACAATGGTGAAGGCTGTGGCTGTGCTTAC CGGCAGTGAGGGTGTCAAGGGCACCATCTTCTTCACCCAGGAGGGAGATGG CCCGACCACCGTGACGGGAAGCGTCACTGGACTCAAGGAAGGGCTCCACGG CTTCCACGTGCACGCTCTTGGTGACACCACCAACGGCTGCATGTCAACTGGA

		CCACACTTCAACCCTGCTGGTCATGTGCATGGGGCACCAGAAGATGAAATCC GCCATGCTGGTGATCTTGGAAATGTGACAGCTGGAGCGGATGGTGTTGCTAA CATCAATGTTACTGACTGCCATATCCCCCTTACTGGACCAAATTCAATTGTTG GCCGTGCTGTTGTCGTCCATGGTGACGCTGATGATCTTGGCAAGGGTGGACA TGAGCTTAGCAAGAGCACTGGAAACGCTGGTGCGCGTGTTGCTTGCGGAAT CATCGGGCTCCAGGGCTAAGATGTCATCTCCACCGGCCAACGTCGTACAGAT CTGGGAACCTTTTCAGAATGCAGATTTGCAATCTCTATTCTAAATAAGCACAT GATCTTTGATCACTTGTTAGTGTGCACCATTCGTGTGAATTCCTATGTGCTACT CTGAACTTTATCACTG
CuZnSOD Mitochondria	FJ890986	CACATAAACAATGGTGAAGGCTGTGGCTGTGCTTACTGGCAGTGAGGGTGTC AAGGGCACCATCTTCTTCACCCAGGAGGGAGAGGGCCCGACCACCGTGACG GGAAGTGTCACCGGACTCAAGGAAGGGCTCCACGGCTTCCATGTGCACGCT CTTGGCGACACCACCAATGGCTGCATGTCAACTGGACCACACTTCAACCCCG CTGGTCATGTGCATGGGGCACCTGAAGATGAAATCCGCCATGCTGGTGATCT TGGAAATGTGACAGCTGGAGTGGATGGTGTTGCTAGCATCAATATTACTGACT GCCATATCCCCCTTACTGGACCAAATTCAATTGTTGCGCGTGCTGTTGTCGTC CATGGTGACGCTGATGATCTTGGCAAGGGTGGACATGAGCTGAGCAAGAGC ACTGGAAACGCTGGTGCACGTGTTGCTTGCGGAATCATCGGGCTCCAGGGCT AAGATGTCATCTTCGCCGACCAACGTCGTACAGATATGGGAACCTTTTCAGAT GCAGATTGCAATCTCTATTCTAAATAAGCACATGATCTTTGATCACTTGTTAGT GTGCACCAGTCGTG
APX thylakoid bound 1	AF387739	GAGGAGTGTCCGGAGGAGGGGAGGCTTCCTGATGCTGGCCCACGTTTACCT GCTGAACACCTTAGGGAGGTATTCTATAGGATGGGCCTTGATGACAAGGAAA TTGTCGCATTGTCCGGAGCACATACACTTGGAAGGTCACGCCCTGACAGGAG TGGCTGGGGAAAGCCAGAAACAAAATATACAAAGGATGGGCCTGGTGAACC TGGAGGGCAATCATGGACGGCTGAATGGTTGAAGTTTGATAACAGCTACTTC AAGGACATAAAAGAGCAAAGGGATCAAGAGCTTCTAGTATTGCCTACAGATG CTGCACTATTTGATGACCCATCATTCAAGGTATATGCAGAGAAGTACGCAGAG GACCAGGAGGCATTCTTCAAGGACTACGCCGAAGCCCACGCA
APX thylakoid bound 2	AF532973	CCGCGGCTACGGGTGGTCCGATGCATGGCGGCGTCGGAGGCGGCGCAGCTC AAGAGCGCGCGGGAGGACATCAAGGAGATCCTCAAAACCACCTACTGCCAC CCTATCCTGGTCCGTTTGGGATGGCATGATTCGGGTACATATGACAAAAATATT GAGGAGTGGCCACAGAGAGGTGGAGCCGACGGAAGCTTAAGATTTGATCCT GAGTTGAGTCATGGAGCCAATGCTGGTCTTACTAGTGCTTTAAAGCTTATTCA ACCAATCAAGGACAAATACCCAGGTATCACCTATGCTGATTTGTTCCAGTTGG CGAGTGCTACAGCAATTGAGGAAGCCGGTGGCCCGAAACTTCCGATGAAATA TGGGCGGGTAGATATCACAGCACCTGAGCAGTGTCCACCTGAGGGGAGGCT TCCTGATGCTGGCCCACGTTTACCTGCTGAACACCTTAGGGAGGTATTCTATA GGATGGGCCTTGATGACAAGGAAATTGTCGCATTGTCCGGAGCACATACACT TGGAAGGTCACGCCCTGACAGGAGTGGCTGGGGAAAGCCAGAAACAAAATA TACAAAGGATGGGCCTGGTGAACCTGGAGGGCAATCATGGACGGCTGAATG GTTGAAGTTTGATAACAGCTACTTCAAGGACATAAAAGAGCAAAGGGATCA AGAGCTTCTAGTATTGCCTACAGATGCTGCACTATTTGATGACCCATCATTCA AGGTATATGCAGAGAAGTACGCAGAGGACCAGGAGGCATTCTTCAAAGACT ACGCCGAAGCCCATGCTAAACTGAGCAACCTTGGTGCAAAGTTTGACCCTCC TGAGGGATTCTCGTTGGACGATGACAAGGGTGCCGTGGCAACTGAAGAGAA GGTGGTTGCTGATCCAGCACCAGCGAGTGATACTAATAGCACAGGACCACAG CCAGAGCCCTTCGTTTCCGCCAAATACTCTTACAAGAAGAGAGAGTTGTCCG ATACGATGAAGCAGAAGATCAGAGCCGAATACGAGGGCCTTGGAGGCAGCC CAAATAAGCCTATGAAGTCCAACTACTTCCTCAACATTATGATCGTGATCGCA GGATTAGCATTCTTGACGTCTCTGACCGGGAACTAAGATCAGTGATCTAATGT TCTTTTTCACATGTTGTAATTAACCATTTTCCAGGGACTGGTCGACATGCAAG ATGACCAGTGCAAATACTTTTGGTCATCTTACGTTGTCTTAAGTTTTCATCATT CAAAGTAAAGTGAGGCAAACCTATTAAATTGCTGGAAACCATGGGATGTTTC TGACT

APX peroxisomal 1	EF555121	TAGGTCGTCCGCGATGGCGGCTCCGGTGGTGGACGCCGAGTACCTGCGCCA GGTCGACAGGGCGCGCCGCGCCTTCCGTGCCCTCATCGCCTCCAAGGGATGC GCCCCCATCATGCTCCGCCTCGCATGGCATGATGCTGGCACCTATGATGTGAA CACAAGAACTGGTGGTGCAAATGGTTCAATTAGATACGAGGAAGAGTACAC CCATGGTTCAAATGCTGGCTTAAAAATTGCTATTGATCTCCTTGAGCCTATTAA AGCGAAGCATCCAAAGATTACATATGCAGACCTTCATCAGCTTGCCGGAGTA GTTGCAGTTGAAGTCACCGGGGGTCCAACCGTTGAGTTCATCCCTGGAAGA CGTGATTCGTCAGTTTGTCCCCGTGAAGGACGCCTTCCTGATGCTAAGAAAG GTGCACCACATCTAAGGGACATCTTTTATCGAATGGGGTTAACAGACAAAGA TATTGTAGCACTATCTGGGGGGCACAGCCTGGGAAAGGCGCATCCTGAAAGG TCTGGGTTTGACGGTGCATGGACTCGTGACCCTCTGAAATTTGACAACTCAT ACTTTCTTGAGCTACTGAAGGGGGAATCTGAGGGTCTTCTGAAGCTCCCTAC TGATAAGGCATTGTTGGATGATCCTGAATTTCGACGCTATGTGGAGCTTTATG CAAAGGATGAGGATGTTTTCTTCAAGGACTACGCTGAATCACACAAAAAACT TTCTGAACTTGGCTTCACACCACGGAGCAGTGGCCCAGCATCTACAAAATCA GATGTTTCAACTGCTGTTGTACTTGCACAGAGTGCAGTCGGGGTAGCAGTTG CTGCAGCTGTAGTTATCGCGGGCTACCTGTACGAAGCTTCCAAGAGGAGCAA GTAAGGGG

Table S3.4. Results from siRNA scan (Appendix CD1).

Table S3.5. Primers used during vector construction and qRT-PCR

Name	Forward Primer	Reverse Primer	Accession
APX-VIGS	5'-CCACGTTTACCTGCTGAACA-3'	5'-CGCAGCATCTGTAGGCAATA-3'	AF387739
SOD-VIGS	5'-TCCTTTGACTGGCCCTAATG-3'	5'-AACGAGAATGGCGTCGTTAC-3'	U69632
GST-VIGS	5'-CCTCAACCACTTCCCCTACA-3'	5'-ATTTGCCTTATTCGCGACAC-3'	AJ440795
APX-qPCR	5'-CGTGGCAACTGAAGAGAAGG-3'	5'-AAGAGTATTTGGCGGAAACG	AF387739
SOD-qPCR	5'- GTGAACGTTCGTATCACTGG -3'	5'- TGACGGACTTCATCTTCTGG -3'	U69632
GST-qPCR	5'-GAAGGTGCTGGAGGTCTACG-3'	5'-TGTAGGGGAAGTGGTTGAGG-3'	AJ440795
16S-qPCR	5'- TCAAGTCCGCCGTCAAATC-3'	5'- TCGCCGTTGGTGTTCTTTC-3'	AJ239003
BSMVγ	5'- TGATGATTCTTCTTCCGTTGC-3'	5'- TGGTTTCCAATTCAGGCATCG-3'	

Chapter 4

Iron homeostasis and *T10RGA2-1D* contribute to *Diuraphis noxia* resistance

This chapter has been submitted for review to *Functional Plant Biology*.

4.1 Introduction

The development of wheat cultivars with durable resistance to biotic and abiotic stressors continues to be an important objective for plant improvement. The Russian wheat aphid, *Diuraphis noxia* Kurdjumov, is a pest of wheat (*Triticum aestivum* L.) that causes yield loss and increases in production costs, and most worryingly, rapidly adapts to resistance gene breeding efforts (Haley *et al*., 2004; Randolph *et al.*, 2009; Jankielsohn, 2011). Several new biotypes of *D. noxia* from both the United States and South Africa (RSA) have been described since breeding for *D. noxia* resistance genes began in the early 1980s (Burd *et al*., 2006; Randolph *et al.*, 2009; Jankielsohn, 2011; Weiland *et al*., 2008).

Although more than ten *Dn (D. noxia)* resistance genes have been described from various wheat accessions (Smith *et al*., 2004), none have been cloned or sequenced. The *Dn* resistance genes of wheat are predicted to function much like classic *R* genes, better-described for plant–pathogen interactions. *R* gene products act as receptors, directly (Flor, 1971) or indirectly (Dangl and Jones, 2001) sensing pathogen attack via the presence of pathogen-derived avirulence (Avr) proteins, and initiate effector-triggered immunity (ETI) upon pathogen recognition (Chisholm *et al.*, 2006). This commonly manifests as a hypersensitive response (HR) which results in cell necrosis at the point of attack and activates systemic acquired resistance (SAR). SAR is activated downstream of the HR by the production of salicylic acid, and in turn induces the acidic pathogenesis-related (PR) proteins (Dodds and Rathjen, 2010; Jones and Dangl, 2006; Niki *et al.*, 1998).

The majority of *R* genes identified to date are characterized as nucleotide binding leucine rich repeat (NB-LRR) proteins (Pan *et al.*, 2000; Tameling and Takken, 2007). The N-terminal domains of these proteins fall into two classes. The first is associated with the toll interleukin receptor (TIR) domain, and the second with a coiled-coil (CC) domain. The TIR-domain has not been found in

monocots and thus divergent evolution has been suggested for *R* genes in monocots and dicots (Cannon *et al.*, 2002; Du Preez *et al.*, 2008; Pan *et al.*, 2000).

The single dominant resistance gene *Dn1* affords aphid resistance phenotypically categorized as antibiosis (Painter, 1958; Botha *et al.*, 2005). This gene has been introgressed from wheat accession PI137739 (Du Toit, 1989) into the cultivar Tugela, to form the near-isogenic line TugelaDN. As aphids produce effector proteins in their saliva capable of eliciting symptoms in susceptible plants (Lapitan *et al.*, 2007), this might be the source of an Avr target for the Dn1 protein to mediate resistance in a gene-for-gene manner (Botha *et al.*, 2006).

Previously identified *R* genes are characterised by LRR domains as well as nuclear binding domains (Eitas and Dangl, 2010). Of the many genes putatively involved in *D. noxia* resistance, two have been putatively linked to the *Dn1* resistance response in Tugela near isogenic lines (NILs): A nucleotide binding protein (*NBP*)-like gene was identified as significantly up-regulated by Affy-chip array analysis (Personal communication A-M Botha) and a wheat resistance gene analogue (*TaRGA2*) gene was identified by suppression subtractive hybridization (SSH) (Lacock and Botha, 2003). In order to investigate the involvement of these genes in *Dn1*-mediated *D. noxia* resistance, a reverse genetics approach was followed using virus induced gene silencing (VIGS).

4.2 Materials and methods

Plant material and growth conditions for VIGS

All experiments were conducted with Tugela (susceptible) and TugelaDN (TugDN; resistant) near-isogenic wheat lines (NILs) grown in potting soil under 14 h of light and 10 h of dark at 25°C. A total of fifteen plants were used per treatment, and samples were collected at the two-leaf stage. All gene-silencing treatments were conducted on the *D. noxia* resistant TugDN plants. Each plant was observed as an independent biological repeat (n=15).

Construction of silencing vectors

The barley stripe mosaic virus (BSMV) VIGS system was utilized for this study which consists of three plasmids (BSMVα, BSMVβ, BSMVγ) containing the modified BSMV tripartite genome. The PCR fragments used for silencing the genes of interest were amplified from TugDN single-stranded cDNA prepared using the Transcriptor kit (Roche, Penzberg, Germany). After sequence analysis, VIGS primers were designed using Primer3 (Rozen and Skaletsky, 2000), and based on accessions: AK333984 and AF458274 encoding *NBP35* and *T10rga2–1D* respectively (Table S4.1). Primers produced fragments of 276 bp for *NBP35,* and (413 bp) for *T10rga2–1D*. Products were cloned using the T-A cloning vector pGEM-T Easy (Promega, Madison WI, USA) and sequenced to confirm identity. The recombinant pGEM-T plasmids as well as the BSMVγ plasmid, containing a multiple cloning site, were digested with the *Not*I restriction enzyme and run on 1% agarose gel. Thereafter, the fragments of interest were excised and purified using the GenElute gel extraction kit (Sigma-Aldrich, St. Louis MO, USA). These fragments were then cloned into the *Not*I site in the BSMVγ plasmid (Holzberg *et al.,* 2002). To determine anti-sense orientation of each fragment, the BSMVγ forward primer was used with the forward primer for each fragment (Table S4.1) in a PCR reaction.

Virus induced gene silencing

Following the protocol described by Van Eck *et al*. (2010), the three BSMV plasmids were linearized using *Spe*I for plasmid BSMVβ, and *Mlu*I for plasmids BSMVα and BSMVγ. Capped RNA transcripts were made using the T7 mMESSAGE mMACHINE kit (Ambion, Life technologies, Carlsbad CA, USA) following the manufacturer's protocol. Plants were infected using a modified protocol (Holzberg *et al.,* 2002; Scofield *et al.,* 2005): in brief, one ng of each plasmid (BSMVα, BSMVβ, BSMVγ) was added to 35 µl FES buffer (0.1 M glycine, 0.06 M K_2HPO_4, 1% w⁄v tetrasodium pyrophosphate, 1% w/v bentonite, 1% w/v celite, pH 8.5) and this mixture was used to inoculate the wheat plants at the 2–3

leaf stage by rub inoculation. The components for each treatment consisted of BSMVα and BSMVβ as well as BSMVγ transcripts containing the fragment of the gene of interest. TugDN plants silenced for each construct were denoted as TugDN+$BSMV_{NBP}$, and TugDN+$BSMV_{RGA}$. The control consisted of BSMVα, BSMVβ, and BSMVγ without an insert, and labelled TugDN+$BSMV_0$. Each plant was considered as a biological repeat (n= 15). All 15 biological repeats of each treatment were mass infested with *D. noxia* (biotype: SA1). Infestation treatments were started on the fifth day after viral inoculation.

RT-qPCR

Silencing of candidate genes was confirmed using RT-qPCR using the prescribed guidelines (Bustin *et al*., 2009). All RNA extractions were performed using the Trizol reagent (Life Technologies, Carlsbad CA, USA) and purified using the RNeasy mini kit (Qiagen, Hilden, Germany). cDNA was constructed using the Transcriptor kit (Roche, Penzberg, Germany). All reactions were run on the CFX96 (BioRad, Hercules CA, USA) using SYBR Green I Master Mix (Roche, Penzberg, Germany) according to the manufacturer's protocols. Cycling protocol: 1 x 95°C for 10 min; 40 x 95°C for 10 sec, 57°C for 15 sec, 72°C for 20 sec; 1 x melt curve 65°C - 95°C: increment 0.5°C per 5 sec. Relative quantification was calculated using the mathematical model by Pfaffl (2001) using TugDN as the calibrator and normalized to 16S rRNA (Table S4.1).

Aphid reproduction measurements

One of the measurements that determine an antibiotic resistance type is the decrease in reproduction of the aphids feeding on resistant material (Budak *et al.*, 1999; Du Toit, 1989; Quisenberry and Schotzko, 1994; Smith *et al.*, 1992). Aphid reproductive measurements were taken following the protocol described by Van Eck *et al*. (2010). Seven days after virus inoculation, one aphid (biotype: SA1) was

caged on the emerged third leaf of each plant (Fig. S4.1). The next day, the mothers were removed and a newly born nymph was left in each cage, which was considered the foundress, and this was recorded as the date of birth. Aphid nymph numbers were measured daily and the mean total number of nymphs was calculated as a measure of fertility. The theoretical maximum rate of increase of a population per individual for aphids was then quantified using the equation by Wyatt and White (1977): $r_m = (0.738 \cdot ln\ (M_d)) \cdot d^{-1}$, where d is the pre-nymphipositional period in days, and M_d is the number of young produced over a reproductive period equal to d.

Enzyme activity assays

Total protein was extracted and enzyme activity was assayed from whole leaf tissue following the protocol described by Ni *et al*. (2001). Protein concentration was determined according to the method of Bradford (1976) using the BioRad protein assay reagent with bovine serum albumin (BioRad, Hercules CA, USA) as a standard. The Glomax spectrophotometer (Promega, Madison WI, USA) was used for this purpose as described by Rybutt and Parish (1982).

Peroxidase activity was calculated by measuring the rate of increase in absorbance at a 470 nm wavelength at room temperature using a modified protocol (Hildebrand *et al.*, 1986) and horse radish peroxidase (BioLabs, Inqaba, Pretoria, South Africa) as a standard. Hydrogen peroxide (0.06% v/v) was added into a mixture containing 2 µg of leaf extract, 6 mM guaiacol, 25 mM potassium phosphate buffer (pH 6.0) and 24% (v/v) distilled water. The formation of tetraguaiacol was a linear function of enzyme concentration and peroxidase activity was expressed as µmol tetraguaiacol $min^{-1} \cdot mg^{-1}$ protein.

DAB staining

DAB staining of material collected 14 days after virus inoculation was performed (Thordal-Christensen *et al.*, 1997). Three biological repeats were chosen at random from each treatment and the fourth leaf

was sampled into 1 mg · mL^{-1} 3,3-diaminobenzidine (DAB)-HCl, pH 3.8, (Sigma-Aldrich, St. Louis MO, USA) and incubated, covered, overnight with shaking at 40 rpm (n=3). The tissues were de-stained using 75% (v/v) ethanol at 37°C. The ethanol was replaced as needed, until the presence of H_2O_2 could be visualized as dark brown deposits. The relative intensity (*ri*) of DAB staining around the wounds as well as between the wounds (Fig S4.2) was calculated from the leaf samples using an adapted protocol described by Miller *et al*. (2009) using the equation: *ri* = mean · pixels (treatment) / mean · pixels (standard). A histogram was used for each area measurement which gave a mean intensity per area selected. Areas selected were kept standard across the leaves in order to allow for comparison (800 pixels; n=10). A standard of 50% grey was used (800 pixels).

Phenotypic screening

Scoring of senescence was completed using altered guidelines by CIMMYT (Pask *et al*., 2012) with non-destructive visual measurements of third and fourth leaves taken where 0 is equal to 0% senescence and 10 is equal to 100% senescence. Chlorosis was scored using the scale developed for *D. noxia* damage (Webster 1990), where 1 is only small isolated spots and 9 is plant death or beyond recovery (Burd *et al*., 1993).

ICP-MS of iron content

Leaf samples from Tugela and TugDN, controls and infested (n=4), were digested using a MARS Microwave Digester (CEM, M.A.D. Technology, Johannesburg, South Africa) under the following conditions: Power Level: 1600W, 100%, ramp time: 15 min, pressure: 800 psi, temp: 180°C, hold time: 20 min. Digestion was completed in 7 ml supra-pure HNO_3 (Merck, Darmstadt, Germany). Deionised water (20 ml) was added by weight upon cooling. Digested samples were diluted an

additional 5x, then analysed on an Icap 6200 ICP-AES emission spectrometer (Thermo Scientific, Waltham MA, USA).

Data analysis

All sequence analysis was conducted utilizing the BLASTn and BLASTx tools (Altschul *et al.,* 1990; Altschul *et al.,* 1997) and SWISSPROT (O'Donovan *et al.,* 2002) with *E*-values lower than 1e-10 considered as significant. All data was plotted using Sigma Plot 7.0 (2001) (SPSS Inc., Chicago IL, USA). Outliers were identified using the guidelines of Grubbs (1969). All statistical analyses were conducted by using SigmaStat 3.5 (2007) (SyStat Software, San Jose CA, USA) with significance set at $\alpha = 0.05$.

4.3 Results

Sequence Analysis of NBP and TaRGA2

As little information was available on the *NBP* gene function, primers were designed that flanked both the VIGS and RT-qPCR sequences, based on the Chinese spring accession (AK333984). These produced a fragment 690 bp in length that was used for further analysis (Fig. S4.2). Utilizing the NCBI alignment algorithm (nBLAST), a putative identity was obtained. The nBLAST hit showed similarity to *Brachypodium distachyon* predicted cytosolic Fe-S cluster assembly factor nubp1-like (Supplimentary Fig. S4.3; XM_ 003575249; *E*=0.0). BLASTx analysis revealed a similarity to *Zea mays* Cytosolic Fe-S cluster assembly factor NBP35 (EU963318; *E*=5e-136) with potential ATPase and possible Fe-S cluster binding domains (Fig. 4.1).

BLASTn analysis of sequences cloned from *TaRGA2* (Fig. S4.4) shows sequence similarity to *T. aestivum ThLr101D rga2* (T10rga2-1D; AF458274; *E*= 0.0) and with BLASTn against the rice genome the following results were obtained: LOC_Os08g42670.2 a putative resistance gene (*E* = 8.9e-

38), and LOC_Os12g37290 resistance gene T10rga2-1A (*E* = 1.2e-29). BLASTx results for *TaRGA2* showed protein homology to an *Aegilops tauchi* RPM1 (RESISTANCE TO *Pseudomonas maculicula* 1; EMT32707; *E*= 1e-57). Due to the ambiguous results, further analysis was necessary.

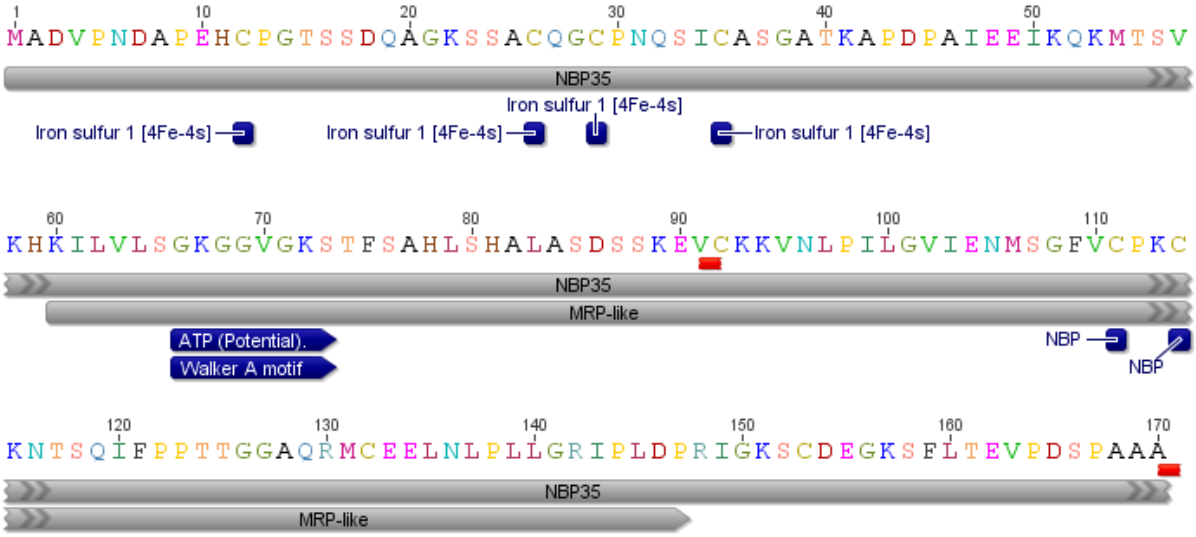


Fig. 4.1. Utilizing the SwissProt tool to analyse the *NBP35* protein sequence, a MRP-like domain with ATPase motif was predicted (*E*= 1.96-e115), cysteine residues involved in the binding of Fe-S clusters (Iron sulphur 1) as well as in dimerization with CFD1 (P-loop NTPase). Fe-S clusters require the binding of both CFD1 and NBP35 for the formation of a working Fe-S scaffold device (Netz *et al*., 2012). Abbreviations: NBP (nucleotide binding protein), Fe-S (iron sulfur). Red annotation represents a portion of sequence that has been removed for ease of visualization (AA 92–160; 170–193).

Both nucleotide and protein sequence alignments were performed in order to remove ambiguity due to the different gene and protein names assigned to the sequences from Genbank. The aligned protein sequences were used to draw an unrooted UPGMA tree (Fig. 4.2). The resultant tree indicated that the closest protein homologues to TaRGA2 are T10rga2-1D and AtaRPM1. AtaRPM1 does not fall within the clade containing the other RPM1 proteins, and thus may have been mistakenly named as such.

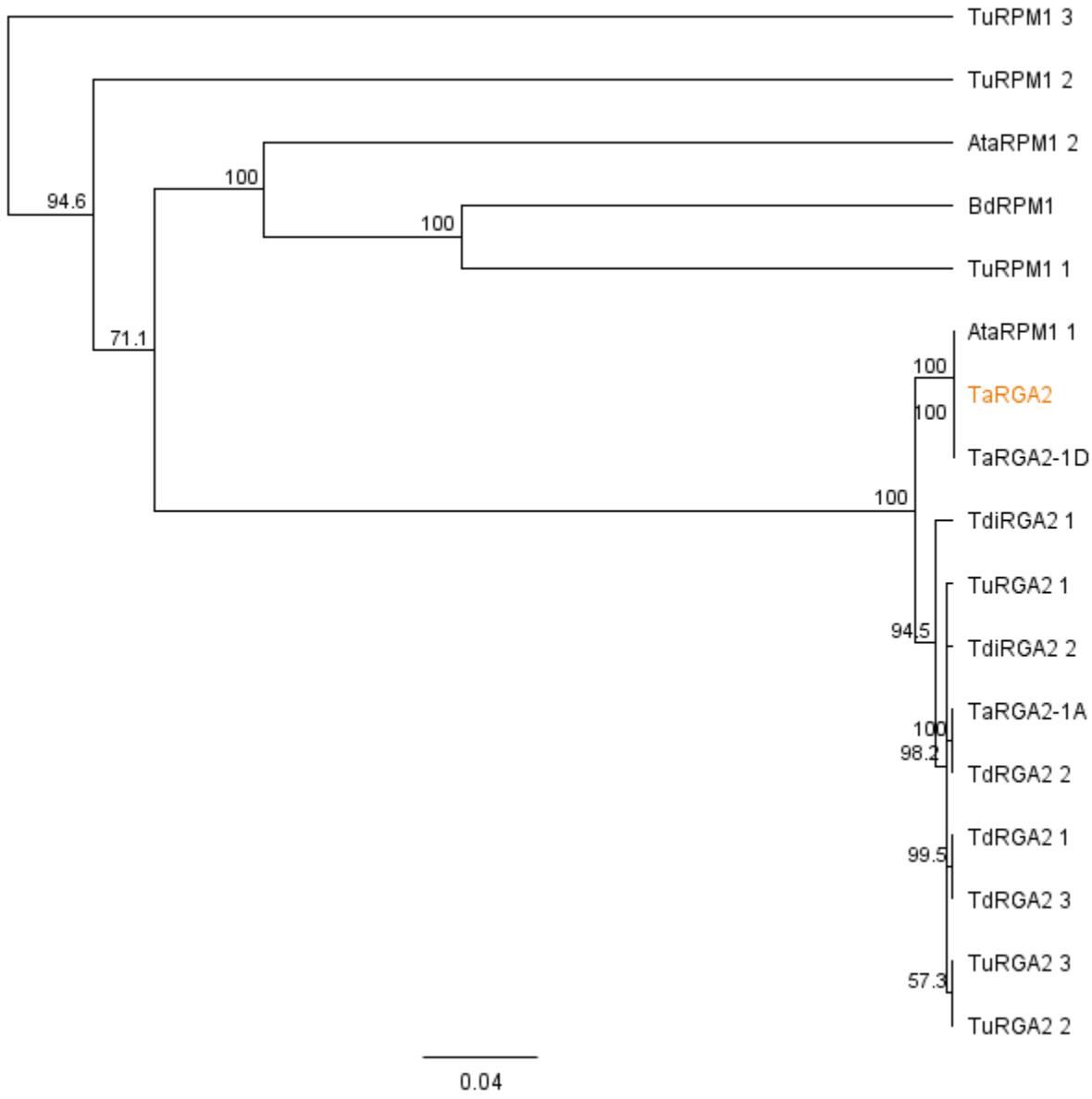


Fig. 4.2. Phylogenetic tree of *RGA2* protein sequences from GenBank (Table S4.2) including the *TaRGA2* sequence from TugDN (indicated in orange). Aligned protein sequences using ClustalW program (Larkin *et al*., 2007) were used to construct the unrooted tree with Geneious Tree Builder (Geneious 6.0.3, Biomatters, New Zealand). The numbers at the nodes were calculated with 1000 bootstrap replicates.

Protein alignment of TaRGA2, AtaRPM1 and T10rga2-1D showed 100% homology (Fig. S4.5). However, alignment of TaRGA2 and AtaRPM1 only showed 54% homology on nucleotide

level, whereas TaRGA2 and T10rga2-1D showed 98.9% homology (Fig. S4.6). Therefore, *TaRGA2* has the closest sequence similarity to *T10rga2-1D*, and due to the sequence similarity is assumed to be this gene. Swiss-prot analysis predicted domains NB-ARC and LRR (*E*= 8.89-e60; Fig. 4.3) The phylogenetic tree (Fig. 4.3) also shows two *RGA2* homologues, consistent with findings from Scherrer *et al*. (2002).

A variable region was chosen from *T10rga2-1D* in order to minimize the chances of silencing both homologues (Fig. S4.7). As NBP35 does not seem to share homology with any family members, a region highly conserved between species was chosen for primer design (Fig. S4.8).

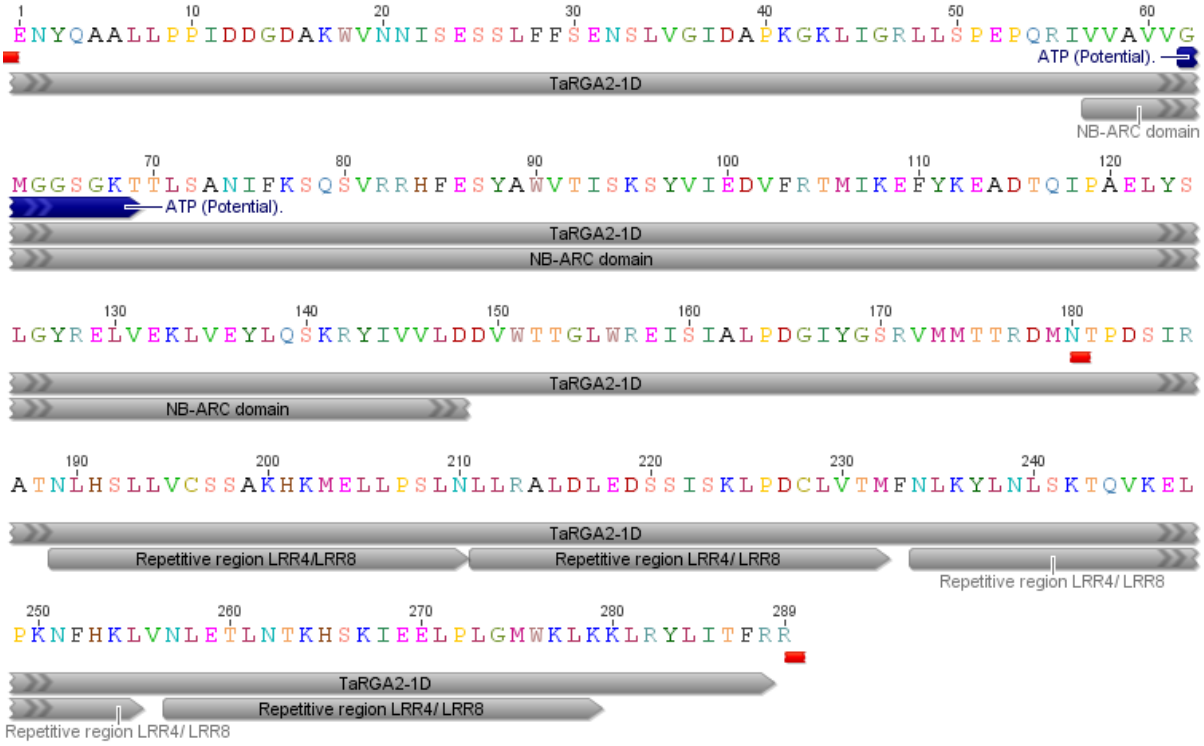


Fig. 4.3. Utilizing the SwissProt tool to analyse the TaRGA2-1D protein, NB-ARC and LRR domains were predicted (*E*= 8.89-e60). Red annotation represents portions of sequence that have been removed for ease of visualization (AA 1–138, 180–413, 289–556 respectively).

Silencing causes susceptibility symptoms

Recombinant BSMV vectors for *NBP35* and *T10rga2–1D* were produced and silencing treatments were named $BSMV_{NBP}$ and $BSMV_{RGA}$ respectively. After silencing with VIGS and infestation, phenotypes of each treatment were recorded.

Fig. 4.4. Representative leaf samples taken during VIGS experiment for infested: A) TugDN, B) TugDN+$BSMV_0$, C) TugDN+$BSMV_{NBP}$, D) TugDN+$BSMV_{RGA}$ E) Tugela showing chlorotic streaking symptoms, F) Tugela showing chlorotic streaking, mass infestation and leaf rolling.

The phenotypes for resistant controls TugDN and TugDN+$BSMV_0$ did not differ (Fig. 4.4A,B) and showed small necrotic lesions indicative of aphid feeding sites. The leaves of the Tugela control showed classical susceptibility symptoms of chorotic streaking, leaf rolling and high numbers of aphids (Fig. 4.4E,F; Burd and Burton, 1992).

TugDN+$BSMV_{NBP}$ treatments showed symptoms of susceptibility such as an increase in number of aphids (62%), however, the most prominent symptom was severe chlorotic streaking score

of 7–9 (Fig. 4.4C). TugDN+BSMV$_{RGA}$ plants showed a senescence phenotype (Fig. 4.4D; Fig. S4.9) resulting in an average senescence score of 10 (Pask *et al*., 2012) which is congruent with plant death.

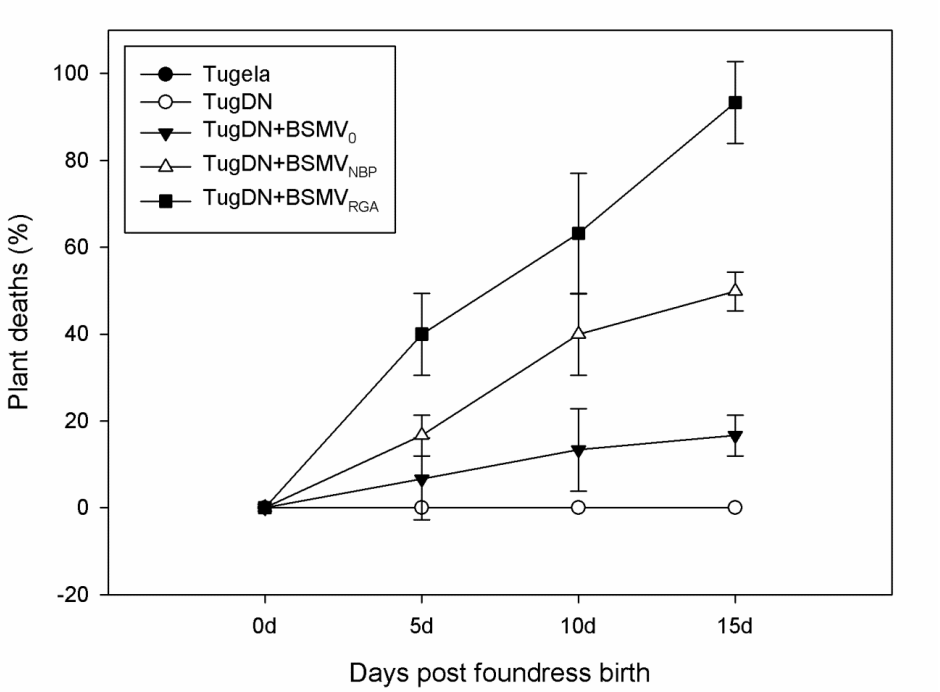


Fig. 4.5. The percentage of plant deaths observed on TugDN+BSMV$_{RGA}$ and TugDN+BSMV$_{NBP}$ treatments over 15 days. The highest percentage of plant death was measured from TugDN+BSMV$_{RGA}$ treatments and occurred between the first and fifth days after *D. noxia* infestation. Only 13% of TugDN+BSMV$_{RGA}$ treatments were alive 15 days after infestation (n=2). There were no plant deaths recorded within Tugela and TugDN control treatments.

Plant death in *RGA* silenced plants was recorded throughout two repeated experiments (n=15) and compared to the other treatments (Fig. 4.5). TugDN+BSMV$_{RGA}$ plants showed the highest number of plant deaths with an average 93% of biological repeats dead within two weeks of aphid infestation. TugDN+BSMV$_{NBP}$ plants showed 49% plant death. Interestingly, TugDN+BSMV$_0$ showed a significant 16% plant death, indicating the VIGS treatment can cause plant stress and plant death in TugDN. However, plant deaths recorded in TugDN+BSMV$_{RGA}$ and TugDN+BSMV$_{NBP}$ were significantly higher than that seen in TugDN+BSMV$_0$ (P<0.05). No deaths were recorded in the Tugela

or in the TugDN control groups indicating that the plant death was due to either the VIGS procedure or the silencing of the respective genes.

Silencing causes increase in aphid fecundity

Dn1 conveys an antibiosis-type resistance which is known to affect the fertility of insects (Smith *et al.*, 1992; Webster 1990). In order to measure the effects of silencing candidate genes on *Dn1*-mediated resistance, aphid fecundity was used as an indicator for antibiosis. A day old nymph was caged on each biological repeat, and the reproductive activity of each nymph was monitored over the duration of the VIGS silencing experiment.

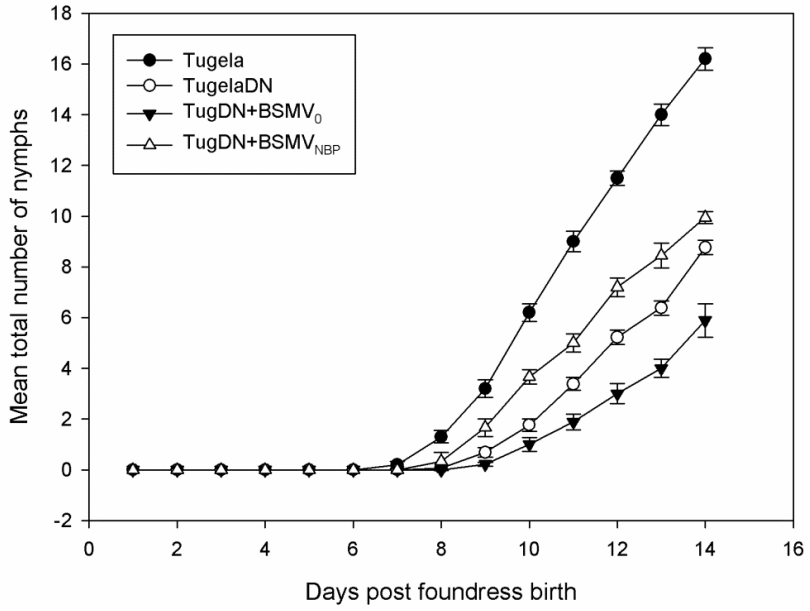


Fig. 4.6. The mean total number of nymphs born to the foundress aphids on Tugela, TugDN, TugDN+$BSMV_0$ and TugDN+$BSMV_{NBP}$ treatments over two weeks after infestation. Error bars represent standard deviation.

The mean total number of nymphs was calculated over this period as an indicator of birth rate (Fig. 4.6). Death of the TugDN+$BSMV_{RGA}$ treatments was pervasive, and thus gathering measurements for aphid fertility was not possible, and is thus not reported.

At 14 days post DOB, the resistant TugDN+BSMV$_0$ control showed the lowest reproductive rate with a mean total of 1.89 nymphs per day, and the susceptible Tugela, the highest with 9 nymphs per day. TugDN had a mean total of 3.4, 63% less than seen on Tugela. The TugDN+BSMV$_{NBP}$ treatment showed a significant increase in reproduction from the TugDN+BSMV$_0$ with a mean total of 5 nymphs per day (P<0.05). This represents a 49% lower rate than Tugela, and 51% higher rate than TugDN+BSMV$_0$.

Table 4.1. The *D. noxia* foundress mean intrinsic rates of increase (IR) on the separate treatments with their respective standard deviations (*sd*), where the (*) indicates treatments statistically different from the TugDN+BSMV$_0$ control (P<0.05).

Treatment	**Mean r_m**	***sd***
Tugela	0.299*	0.027
TugDN	0.196	0.043
TugDN+BSMV$_0$	0.165	0.006
TugDN+BSMV$_{NBP}$	0.240*	0.045
TugDN+BSMV$_{RGA}$	n/a	n/a

The intrinsic rate of increase (r_m) for each treatment was used as an indicator of the potential for the population to increase on each treatment (Table 4.1). Aphids caged on the susceptible Tugela plants showed the highest rate of increase (r_m= 0.299, *sd*= 0.027), 35% higher than that of TugDN (r_m= 0.196, *sd*= 0.043). The lowest rate of increase was measured on TugDN+BSMV$_0$ (r_m= 0.165, *sd*= 0.006). Silencing *NBP35* was found to have a significant effect on intrinsic increase (r_m= 0.240, *sd*= 0.045; P<0.02) with an increase of 31% and 18% in comparison to TugDN+BSMV$_0$ and TugDN respectively

RT-qPCR confirmation of silencing

In order to gauge the effectiveness of the VIGS treatment, relative RT-qPCR was utilized. Silencing in TugDN+$BSMV_{NBP}$ treatments resulted in a lower transcript number, 45% and 49% below the TugDN and TugDN+$BSMV_0$ respectively (Fig. 4.7A). Interestingly, there is a small but significant difference of 7% less *NBP35* transcripts in Tugela in comparison to TugDN. Transcript levels in TugDN+$BSMV_{RGA}$ were 65% and 68% below that of TugDN and TugDN+$BSMV_0$ respectively (Fig. 4.7B). Tugela RGA levels were measured at 45% below that of TugDN.

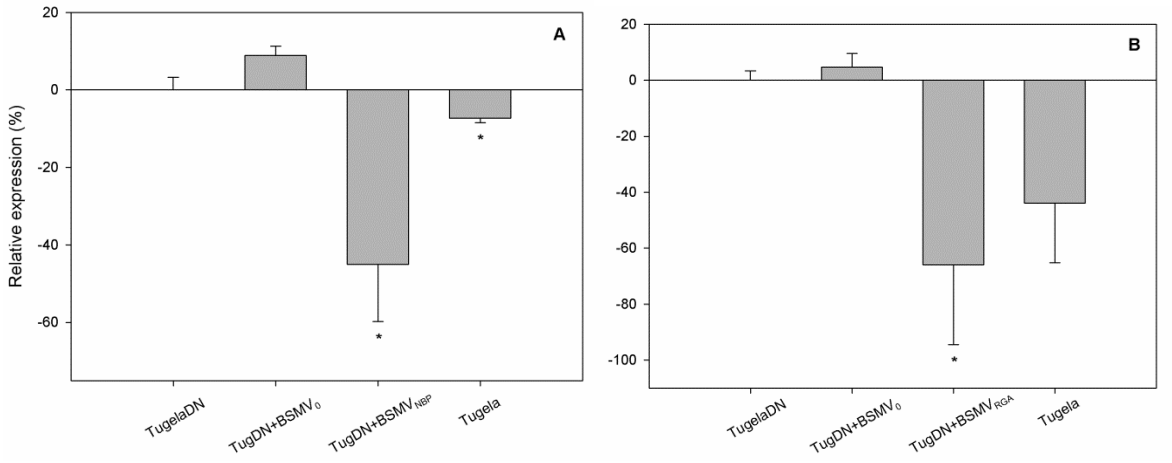


Fig. 4.7. Relative quantification of transcripts using RT-qPCR: (A) *NBP* and (B) *RGA2*. Asterisks indicate a significant deviation ($P<0.05$).

Peroxidase activity affected by silencing NBP35 and T10rga2–1D

As H_2O_2 is involved in the hypersensitive response and has been implicated in TugDN aphid resistance (Van der Westhuizen *et al*., 1998; Moloi and Van der Westhuizen, 2005; Boyko *et al*., 2006; Botha *et al*., 2010; Van Eck *et al*., 2010), representative samples from each treatment were DAB-stained for the presence of H_2O_2 (Fig. 4.8C-G). The resistant controls TugDN and TugDN+$BSMV_0$ showed dark staining along the main veins and in the small connecting veins of the leaf (Fig. 4.8C,D). Staining was slighter in the Tugela treatment (Fig. 4.8G). Staining in the TugDN+$BSMV_{NBP}$ plant was less than in

the resistant controls, but more than the susceptible control. The TugDN+BSMV$_{RGA}$ staining appeared comparable to the Tugela control indicating a reduced amount of hydrogen peroxide (Fig. 4.8F). The leaf samples were analysed for image intensity, in order to indicate the percentage of H_2O_2 staining (Fig. 4.8A). Tugela, TugDN+BSMV$_{NBP}$ and TugDN+BSMV$_{RGA}$ had significantly less staining than the resistant TugDN and TugDN+BSMV$_0$ resistant controls (P<0.05).

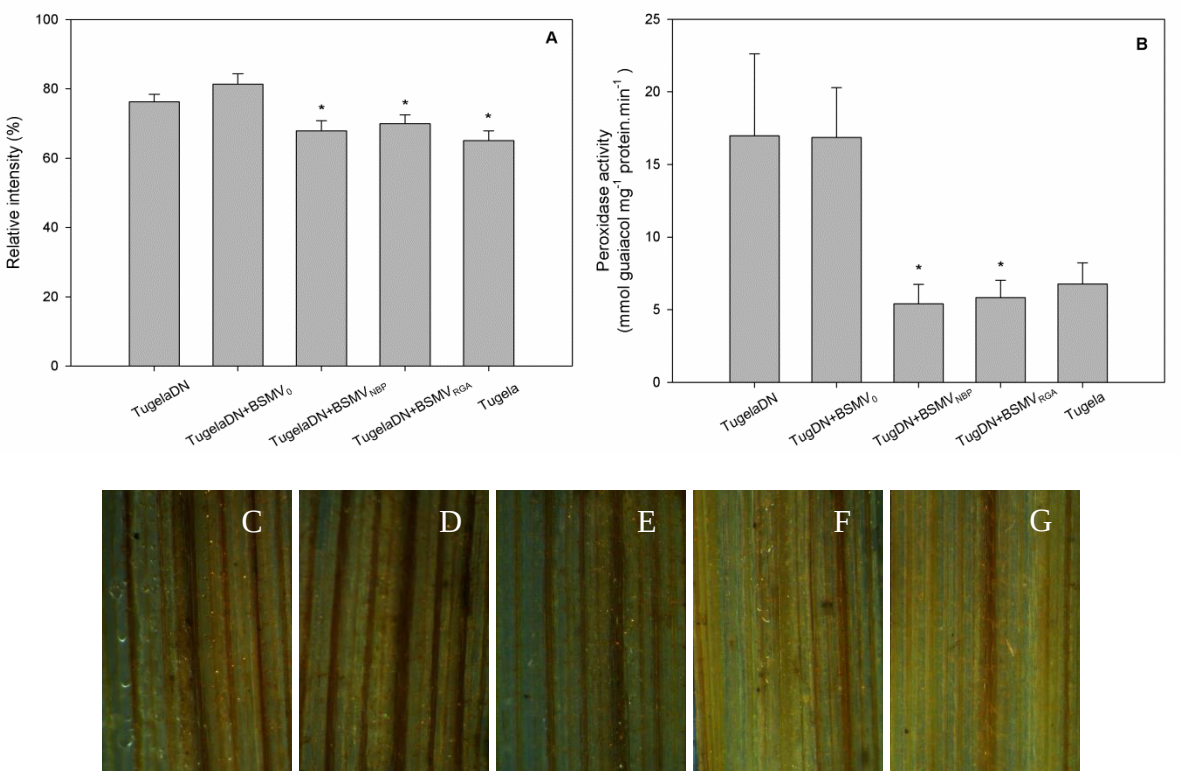


Fig. 4.8. (A) Graph of relative intensity of staining measured from each leaf sample. Asterisks indicate significant deviation (P<0.05). (B) Peroxidase assay from samples taken at 7 days post infestation. Asterisks indicate a significant deviation (P<0.05). (C-G) Representative leaf samples taken during VIGS experiment after DAB staining for infested: C) TugDN, D) TugDN+BSMV$_0$, E) TugDN+BSMV$_{NBP}$, F) TugDN+BSMV$_{RGA}$, G) Tugela.

As the DAB staining results showed decreases in the presence of H_2O_2 in *NBP35* and *RGA* silenced plants, peroxidase activity was assayed (Fig. 4.8B). The highest peroxidase activity was observed in the resistant controls which showed no significant difference from one another (P=0.74).

Peroxidase activity measurements in TugDN+$BSMV_{NBP}$ and TugDN+$BSMV_{RGA}$ were found to be 68% and 65% lower than the TugDN+$BSMV_0$ control (P<0.05) and 64% and 61% lower than TugDN respectively (P<0.05). The Tugela control showed a 51% lower level of peroxidase activity when compared to TugDN, however, due to variation seen in TugDN this was not a significant difference at this time point (P=0.07).

Iron content differs between Tugela and TugDN

ICP-MS analysis revealed differences in the levels of iron content in infested and un-infested wheat leaves (Fig. 4.9).

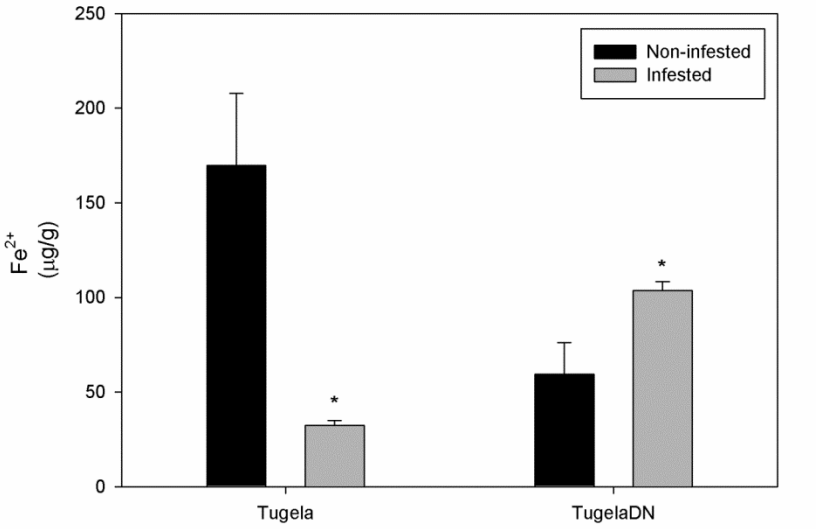


Fig. 4.9. ICP-MS results testing for differences in iron between Tugela and TugDN (P<0.05).

Tugela plants have drastically less iron after infestation (80%), whereas iron levels in TugDN increase by 40% after infestation (P<0.1). Infested TugDN plants have 68% higher iron levels than infested Tugela (P<0.05).

4.4 Discussion

This study investigated the role of two genes previously identified as candidate *R* genes in *Dn1* mediated *D. noxia* resistance. The majority of known *R* genes contain NBS domains (Pan *et al.*, 2000), while RGA (*R* gene analogues) proteins show homology to NBS-LRR proteins (Feuillet *et al.*, 2003; Smith and Clement, 2012). Similar to the NBS-LRR family, *RGA* genes have been found to cluster together and several aphid resistance genes have been found to map within or near clusters of these resistance gene-like sequences (Dubey and Chandel, 2010; Kanazin *et al.*, 1996; Klingler *et al.*, 2005, 2009).

During a study on the *Lr10* rust resistance gene in wheat, two *RGA* gene homologues were found on the A- and D- genomes designated as *T10rga2-1A* and *T10rga2-1D* respectively (Scherrer *et al*., 2002). There are examples of pairs of resistance genes and resistance gene analogues interacting in order to initiate a defence response (Cesari *et al.*, 2013; Kanzaki *et al.*, 2012; Loutre *et al.*, 2009) however, as *T10rga2-1A* was found to be highly conserved, it was suggested to function down-stream of *Lr10* (Feuillet *et al.*, 2003). In experiments by Loutre *et al.* (2009), silencing *T10rga2-1A* in wheat using VIGS resulted in a chlorotic, susceptible phenotype after infection with leaf rust.

Silencing of *T10rga2-1D* in the current study resulted in a senescent phenotype after aphid infestation, rapidly culminating in plant death, indicating that it may play an important role in plant survival under infestation conditions or stress (Fig. 4.1D, S4.1). The plant death as well as the up-regulation of *T10rga2-1D* in TugDN infested plants, implicate that *T10rga2-1D* may be pivotal to TugDN under infestation conditions. Whether *T10rga2-1D* is directly involved in *Dn1* resistance, or whether it is involved down-stream of the resistance response will need to be investigated.

The silencing of *NBP* also caused plant death, however, not to such an extent as *T10RGA2-1D*. Analysis of the *NBP* sequence showed a high degree of homology to a predicted cytosolic Fe-S cluster

assembly factor NBP35-like (Fig. 4.6). The *NBP35* targeted for silencing as well as several other iron-homeostasis genes, such as aconitase, and have been seen to be highly up-regulated during *D. noxia* infestation in previous experiments (Schultz, 2010; Van Eck, 2007) indicating that this pathway plays an important role in *Dn1* resistance. The NBP35 protein is part of the cytosolic Fe-S cluster assembly (CIA) machinery (Hausmann *et al.*, 2005) responsible for the Fe-S cluster maturation in preparation for insertion into various proteins. In Hausmann *et al*. (2005), they found NBP35 to be required for the extra-mitochondrial assembly of cytosolic and nuclear Fe-S proteins downstream of the mitochondria. The NBP35 protein is therefore essential for the assembly of Fe-S clusters and if knocked down will affect the production of multiple Fe-S containing proteins, such as aconitase which plays a role in regulating oxidative stress and cell death (Moeder *et al.*, 2007), as well as electron transport chain proteins in the chloroplast (Briat *et al*., 2007). Fe-S clusters act as regulatory switches which are sensitive to oxidation and reduction (Beinert and Kiley, 1999).

NBP silenced wheat plants showed plant death as well as extensive chlorotic streaking after *D. noxia* infestation (Fig. 4.1C). Chlorotic streaking is one of the measurements of susceptibility of wheat cultivars to *D. noxia* infestation (Botha *et al.*, 2006; Burd *et al.*, 1993; Fouché *et al*., 1984; Webster 1990). Chlorosis is also a known symptom of iron deficiency (Dasgan *et al.*, 2003) and BSMV infection (Alamási *et al.*, 2000), as well as several other pathogens (Betts *et al.*, 2011; Kenyon and Turner, 1990; Oliveira *et al.*, 2012) and heavy metals (Semane *et al.*, 2010). As TugDN inoculated with the empty BSMV vector did not show a comparative level of chlorosis, it is plausible that in TugDN+BSMV$_{NBP}$ silenced plants the combination of stress from the *D. noxia* infestation, as well as the down regulation of a putative Fe-S cluster protein, may have compromised the resistant plants' ability to maintain iron-homeostasis, causing the observed chlorosis (Fig. 4.1C).

But does iron deficiency explain the leaf chlorosis observed in infested Tugela plants? Evidence for this was found in measuring iron in Tugela and TugDN plants. Infested Tugela plants contain less iron than un-infested plants (Fig. 4.9). In contrast, infested TugDN has higher levels of iron that un-infested TugDN. Interestingly, TugDN contained less iron than Tugela plants before infestation, suggesting that the resistant plants may increase iron uptake to cope with the stress associated with feeding, while susceptible plants pool of iron is not replenished. It has been shown that iron levels are depleted in the presence of H_2O_2 (Liu et al., 2006), while up-take is stimulated by salicylic acid (SA) (Gunes *et al.*, 2007). Upon aphid infestation, TugDN expresses significantly higher levels of H_2O_2 (Van der Westhuizen *et al.* 1998; Moloi and van der Westhuizen, 2006) accompanied with increased levels of SA (Mohase and van der Westhuizen, 2002), whereas Tugela may be producing damaging amounts of H_2O_2 with inappropriate timing (Schultz *et al*., 2014). Thus, as *NBP35* is a putative Fe-S cluster assembly protein, it is likely that silencing it will disrupt of iron homeostasis subsequently leading to the chlorotic phenotype observed in the present study, and consequently lead to increased susceptibility.

This study associates iron homeostasis in *Dn1*-mediated resistance, and thus in the antibiotic type resistance response. A closer look at the iron-effected chloroplast constituents may lead to a better understanding of the differences that lead to susceptibility and resistance.

A candidate for up-stream resistance signalling for *Dn1*-mediated resistance has been identified as *T10RGA2-1D,* however, a different course should be followed in order to functionally characterize this gene further.

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4.7 Supplementary data

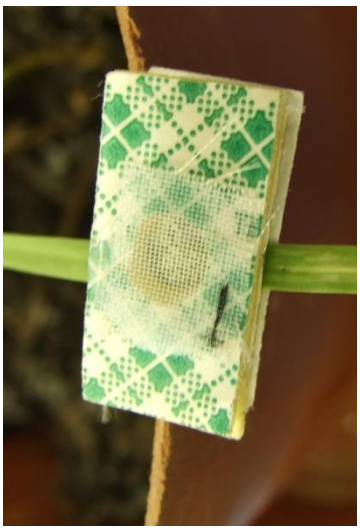


Fig. S4.1. Aphid cage used to cage single aphids on an individual wheat leaf for observation. This allowed for the counting and removal of nymphs born daily.

GATCAACCTTGGTGTCATGTCGATTGGTTTTATGCTGCCTAACCCAGATGATGCTGTCATATGGAGGGGTCCT
CGCAAGAACGGATTGATCAAACAGTTTCTCAAGGATGTTGACTGGGGAGAGATTGACTACCTTGTAGTGGAT
GCACCCCCTGGAACATCAGACGAGCACATCTCAATCGTCCAGTACCTACAAGCCACCGAAGTTGATGGCGCG
ATAATCGTGACGACCCCGCAGCAAGTTTCTCTAATAGACGTGAGGAAGGAGATCAATTTCTGCAAGAAGGTG
GGCGTGCCAGTGTTGGGGGTTGTGGAGAACATGAGTGGCTTGAGGCAGGCGTTCACAGACCTTAAATTCGTA
AAGCCAGGTCTCGCCGGGGAGATAGACGCCACAGAGTGGGCGATGAACTATATCAAGGAGAAGGCTCCGGA
GCTTTTGTCGGTCGTGGCCTGCAGCGAGGTGTTTGACAGCAGCAAGGGCGGCGCGGAGAAGATGTGCCATGA
GATGGGGGTACCTTTCCTGGGTAAGGTGCCGATGGACCCGCAGCTGTGCAAGGCAGCCGAGGAAGGGAGGTC
GTGCTTCGCCGATCAGAGGTGCAGCGCCAGCGCACCGGCTCTGAAAAGCATCATCGACAA

Fig. S4.2. Sequence of *NBP* fragment obtained from TugDN wheat.

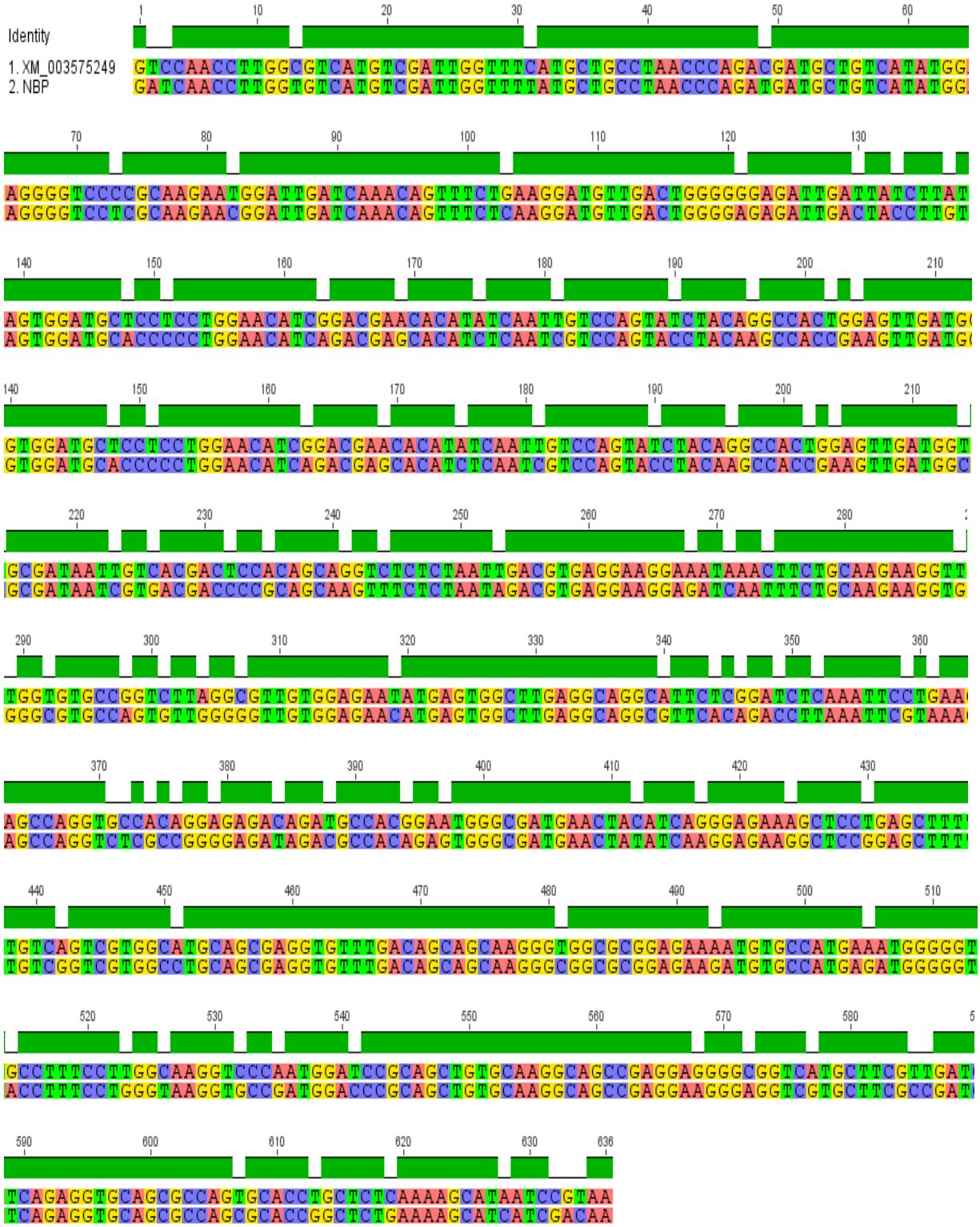


Fig. S4.3. The nucleotide sequence alignment of *NBP* and predicted cytosolic Fe-S cluster assembly factor nubp1-like (*B. distachyon*) showing 86.9% identity.

TCGCGATGCATCTAGATTATGCGCAGGGCCACAGAACGTAGCGCCAGAGCACAAGCAGTTGTCCATTGACCA

GAATACTCTTTATGCTTTCTTTGAGAAGGTAACATTCCAAAGATCAGATTGGATTTCATCCAATCCTGTTGATT

ACTTGTTAGCTTACAGAGGAAGTACAATACAAAGTGCTATGGAGTTAATGGTATATGGTTGTGTAAAATGTTA

ATCCAAGTAACATAGAGTTAACTGTATAGAGTTGTGTAAAATGTTAGTCCAAGTCATAGTAGTACGTTGTGAA

ACTGGTCATAAAAGTGGCAGTTCTGAGACTAACAAAACAGTGCTAGTTCTGCAAAATGAGCTAAACGTAAGC

ACATCAGGGCAACCTTTAAGAAGGCCTTCTTTACACGGGTACTACTAGGGAAGGGAGCCACATCGGAAA

Fig. S4.4. Sequence of *RGA2* fragment obtained from TugDN wheat.

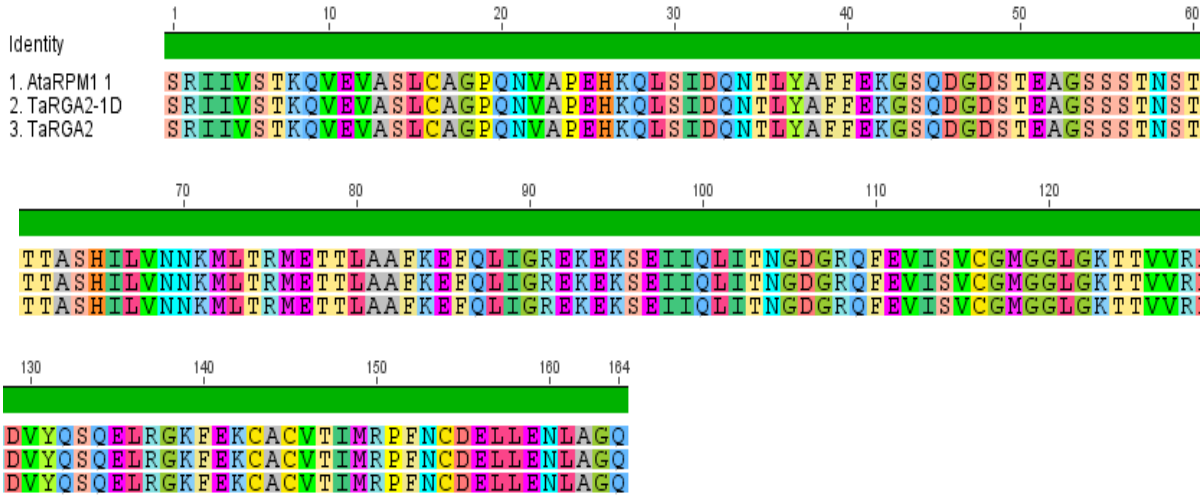
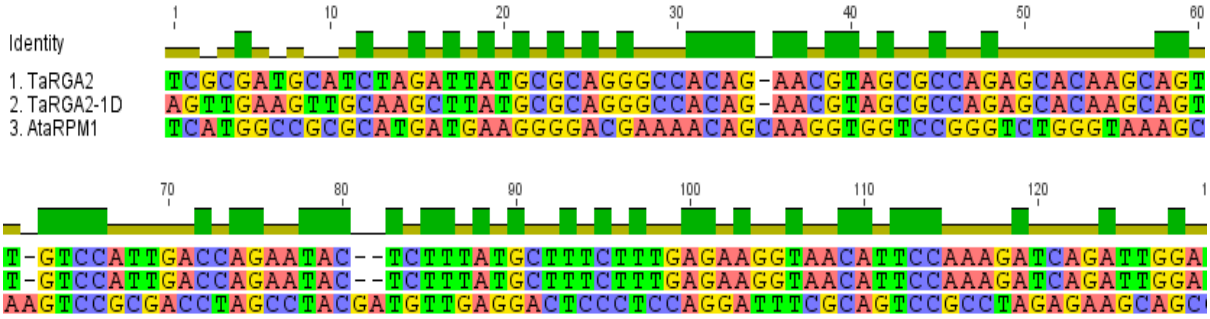


Fig. S4.5. A protein sequence alignment of translated TaRGA2, T10rga2-1D and AtaRPM1 showing 100% identity.



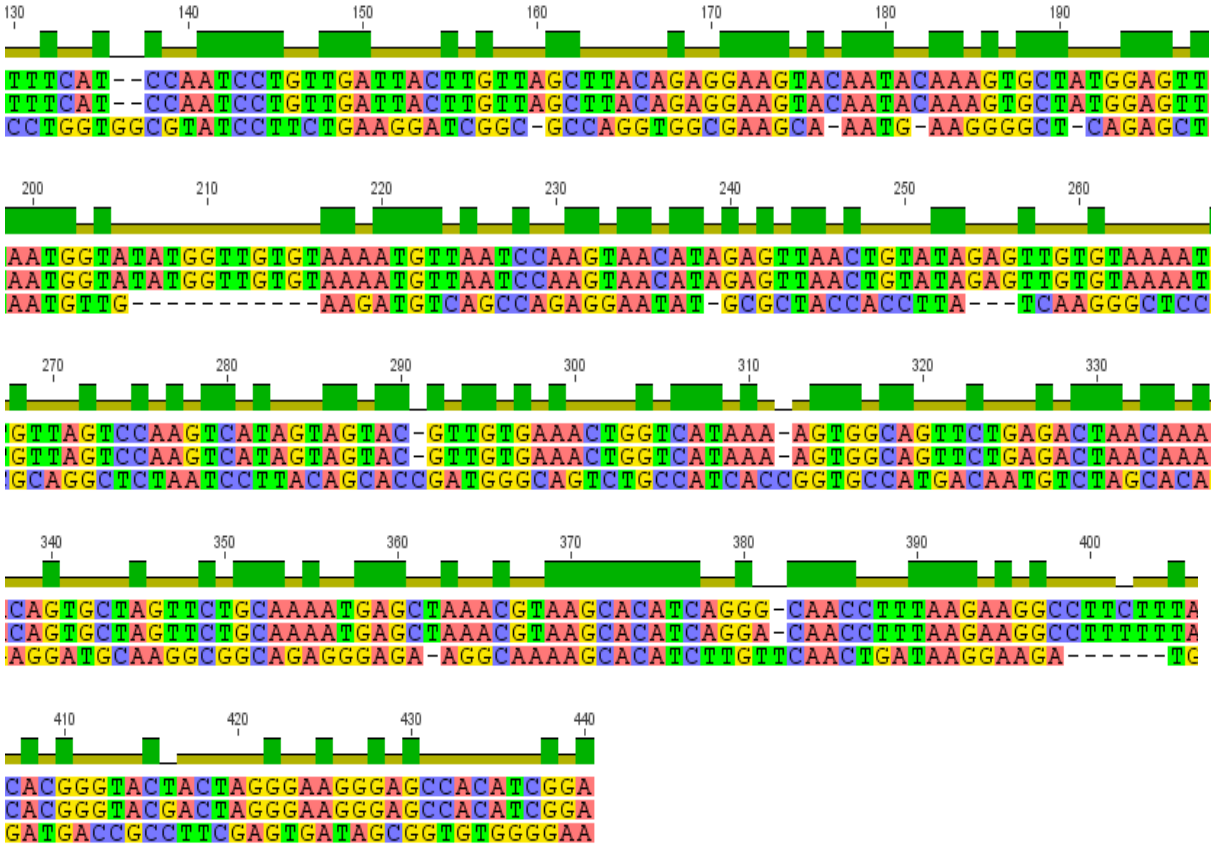


Fig. S4.6. The nucleotide sequence alignment of TaRGA2 are T10rga2-1D and AtaRPM1, showing only 54% identity between TaRGA2 and AtaRPM1.

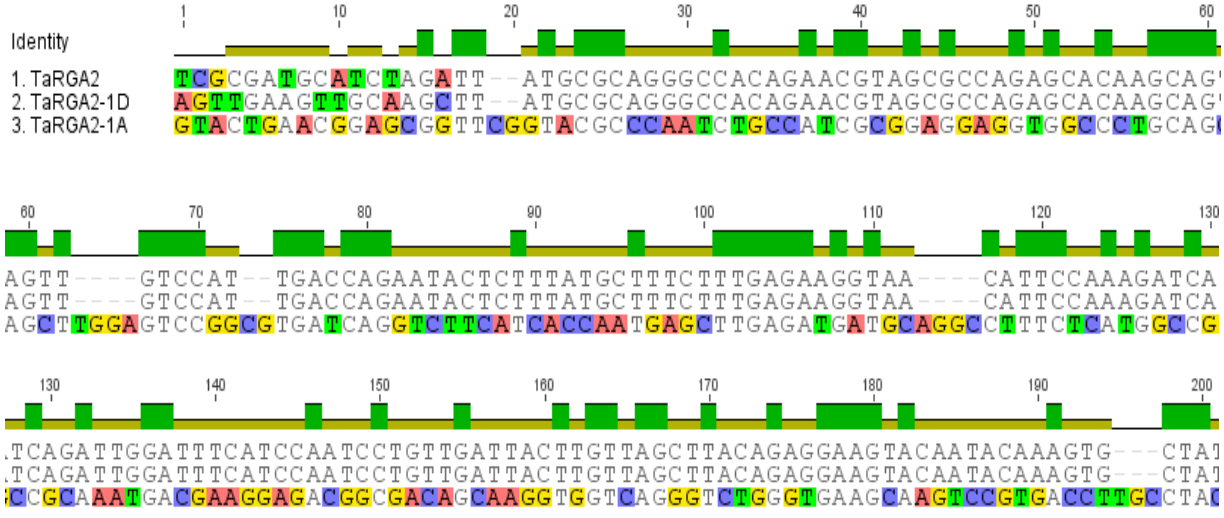
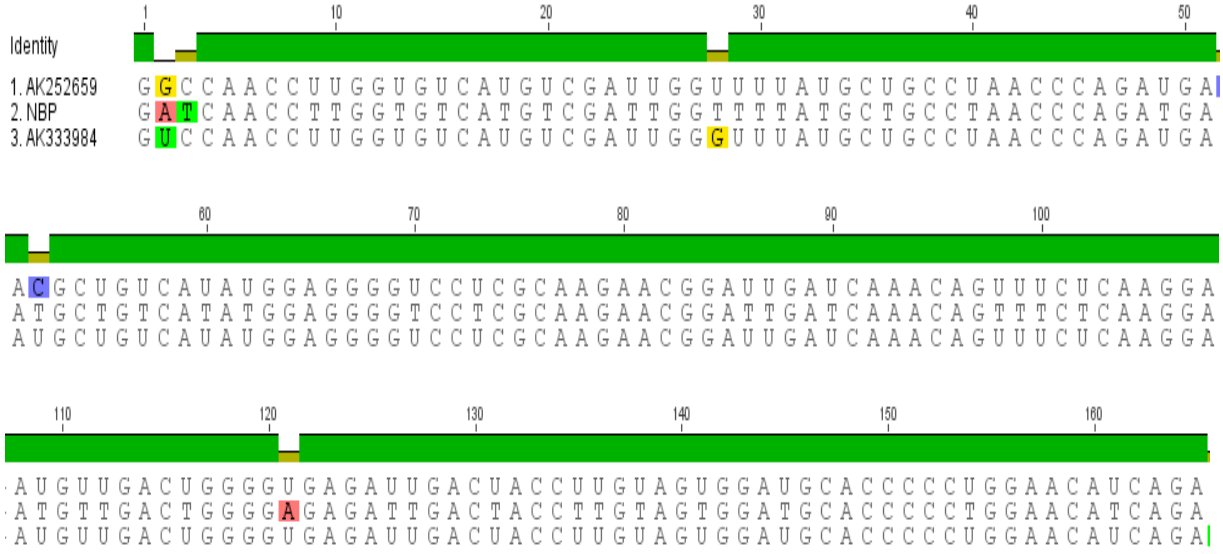


Fig. S4.7. Nucleotide alignment of region identified as variable between the *TaRGA2* homologues. This sequence was utilized for VIGS and RT-qPCR primer design.



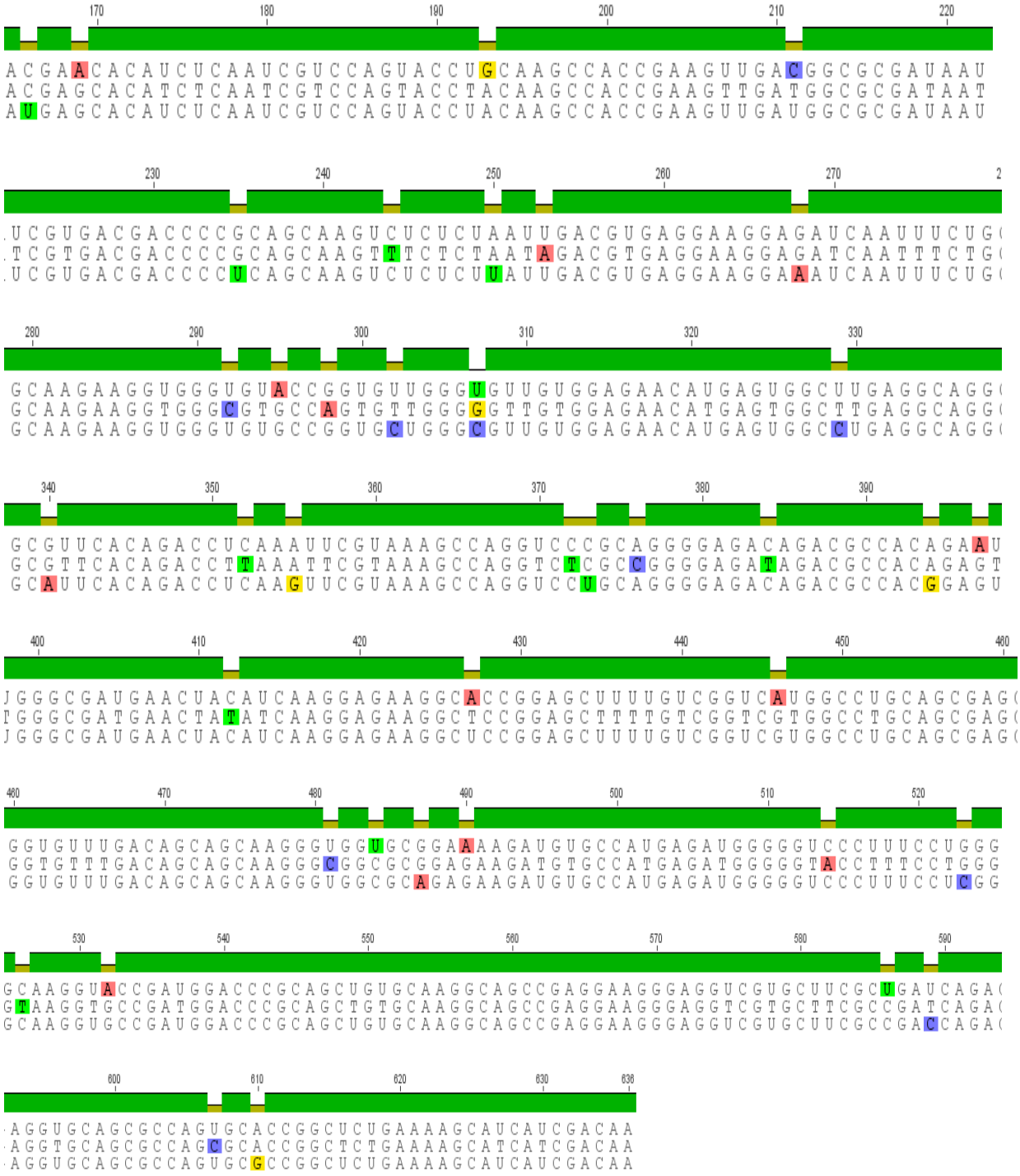


Fig. S4.8. Nucleotide alignment of region identified as conserved between the *NBP* homologues from *Triticum aestivum* (AK333984) and *Hordeum vulgare* (AK252659). This sequence was utilized for VIGS and RT-qPCR primer design.

Fig. S4.9. Photograph showing the plant death phenotype observed in all *RGA2* silenced plants. TugDN+BSMV$_{RGA}$ plants indicated by the blue arrow, flanked by Tugela (Right) and TugDN (Left) plants.

Table S4.1. Primers used during vector construction, qRT-PCR and sequencing.

Name	Forward Primer	Reverse Primer	Accession
NBP-V	5'-ACATTTTCGGGATGCTAGG-3'	5'-TGGAACACAGTCTGATGAGG-3'	AK333984
RGA-V	5'-ATGCGCAGGGCCACAGAACG-3'	5'TCCGATGTGGCTCCCTTCCCT-3'	AF458274
NBP-q	5'-AGGACCCCTCCATATGACC-3'	5'-ATTCATCAGAGCAACCTTGG-3'	AK333984
RGA-q	5'-ACGGGTACGACTAGGGAAGG-3'	5'-ATGCCCTGGAAACAAAAAGG-3'	AF458274
16S-q	5'- TCAAGTCCGCCGTCAAATC-3'	5'- TCGCCGTTGGTGTTCTTTC-3'	AJ239003
NBP-g	5'-CAACCTTGGTGTCATGTCG-3'	5'-GATCAGCTTGTCGATGATGC-3'	AK333984
RGA-g	5'- ATGCGCAGGGCCACAGAACG-3'	5'- TCCGATGTGGCTCCCTTCCCT-3'	AF458274

V= VIGS primers; q= RT-qPCR primers; g= interior of mRNA sequence targeted

Table S4.2. Protein sequences used for phylogenetic analysis. For ease of reference, prefixes have been attached to represent the different species: At = *Arabidopsis thaliana*, Ata = *Aegliops tauchii*, Bd = *Brachypodium distachyon*, Os = *Oryza sativa*, Ta = *Triticum aestivum*, Td = *Triticum durum*, Tdi = *Triticum dicoccoides*, Tm = *Triticum monococcum*, Tu = *Triticum Urartu*

Organism	**Accession**	**Gene label**	**Reference**
Aegilops tauchi	EMT32707	AtaRPM1 1	Jia *et al.* (2013)
Aegilops tauschii	EMT09290	AtaRPM1 2	Loutre *et al.* (2009)
Arabidopsis thaliana	AGC12590	AtRPM1	Ling *et al.* (2013)
Brachypodium distachyon	XP 003569114	BdRPM1	Rose *et al.* (2012)
Oryza sativa Japonica	AAL01163	OsRPM1	Jia *et al.* (2013)
Triticum aestivum	AAM66341	TaRGA2	Feuillet *et al.* (2003)
Triticum aestivum	AAQ01786	TaRGA2-1A	Feuillet *et al.* (2003)
Triticum aestivum	AF458274	TaRGA2-1D	Scherrer *et al.* (2002) Feuillet *et al.* (2003)
Triticum dicoccoides	ACG63519	TdiRGA2 1	Loutre *et al.* (2009)
Triticum dicoccoides	ACG63518	TdiRGA2 2	Loutre *et al.* (2009)
Triticum durum	ACG63517.1	TdRGA2 1	Loutre et al. (2009)
Triticum durum	ACG63515	TdRGA2 2	Loutre *et al.* (2009)
Triticum durum	ACG63517	TdRGA2 3	Loutre *et al.* (2009)
Triticum monococcum	AAK84083	TmRPM1	Wicker *et al.* (2001)
Triticum urartu var. urartu	ACG63522	TuRGA2 1	Loutre *et al.* (2009)
Triticum urartu	ACG63526	TuRGA2 2	Loutre *et al.* (2009)
Triticum urartu	ACG63524	TuRGA2 3	Loutre *et al.* (2009)
Triticum urartu	EMS46571	TuRPM1 1	Jia *et al.* (2013)
Triticum urartu	EMS48513	TuRPM1 2	Jia *et al.* (2013)
Triticum urartu	EMS68902	TuRPM1 3	Ling *et al.* (2013)

Chapter 5

Dissimilar H_2O_2 expression in near isogenic wheat lines after wounding

5.1 Introduction

Plant defence is facilitated by a complex network of pathways. The wounding pathway is characterized by the production of jasmonic acid (JA) and its derivatives which are able to accumulate in areas distal to the site of wounding (Glauser *et al*., 2008). The jasmonic acid pathway involves the recognition of damage associated molecular patterns (DAMPS) which are exposed by tissue damage and sensed by pattern recognition receptors (PRR; Hogenhout and Bos, 2011; Seong and Matzinger, 2004). Herbivores introduce identifiers, in the form of oral contact, saliva and oviposition, into wounds or into the plant tissues that are recognized by PRRs and are called herbivore associated molecular patterns (HAMPs) (Mithöfer and Boland, 2008). Responses triggered by PRRs initiate fast and transient responses from the plant and are refered to as pattern triggered immunity (PTI; Maffei et al., 2007). In order to counteract the plants PTI, insects and herbivores have evolved effectors which act to block the immune response (Hogenhout and Bos, 2011), and in turn, plants have *R*-gene receptors which act to recognise specific effectors in the form of effector triggered immunity (ETI). Pest specific avirulence (*avr*) factors or effectors are usually of proteinaceous origin, and ETI elicited by an effector is characterized by the production of salicylic acid (SA) and its derivatives. Systemic acquired resistance (SAR) is a consequence of a buildup of SA which results in a system wide resistance response. Plants with an active SAR response have been found antagonistic to the JA signaling pathway (Derksen *et al*., 2013; Spoel *et al*., 2003; 2008). In the search for the suppressing agent in the SA pathway, it has been found that acetylsalicylic acid blocks JA synthesis (Pan *et al*., 1998). SA itself does not have this ability (Harms et al., 1998), and has been found to increase the activity of members of the wounding pathway (Laudert and Weiler, 1998; Salzman *et al*., 2005). At certain levels of SA signaling therefore, not all JA production is blocked, and thus a level of cross-talk between the pathways is possible. This

cross-talk potentially provides the plant with a degree of regulation, utilizing the different defence pathways more efficiently depending on the type of attack (Bostock, 2005; Pieterse *et al*., 2009).

Pathogen/pest attack initiates the hypersensitive response (HR), a tightly regulated response observed in plants in response to a recognition event, characterized by the production of reactive oxygen species (ROS), resulting in localized necrosis around the point of attack or cellular damage (Dangl and Jones, 2001; Tenhaken *et al*., 1995). The production of reactive oxygen species (ROS) in plants is associated with tissue damage or recognition of infection (Mittler *et al*., 2004; Orozco-Cárdenas and Ryan, 1999; Suzuki *et al*., 2011) but also occurs in response to abiotic and biotic stressors such as high light conditions and insect attack (Hung *et al*., 2005; Neill *et al*., 2002). ROS species have an oxidative capacity, and are capable of causing damage to proteins, DNA and lipids (Apel and Hirt, 2004). Research into the different ROS species has shown that different signaling pathways are activated by the different ROS intermediates such as hydrogen peroxide, superoxide anion radical and hydroxyl radicals ($H_2O_2/O_2^{\bullet-}/\bullet OH$) as well as singlet oxygen ($^1O_2$). The cross-talk mediated by these pathways may be utilized for the fine control of antioxidants and ROS production and may be essential for adaptation to stress (Laloi *et al*., 2007; Suzuki *et al*., 2011).

Two of the main antioxidants implicated in redox homeostasis and signaling in plants are glutathione (GSSH) and ascorbate (AsA) that form part of a complex antioxidant system with enzymes such as glutathione peroxidase (GPX), glutathione-*S*-transferase (GST), ascorbate peroxidase (APX) and superoxide dismutase (SOD; Foyer and Noctor, 2011; Miller *et al*., 2010). The balance between ROS and the antioxidant systems result in the different physiological responses to stress: from tissue necrosis to signalling (Maruta *et al*., 2010; Mittler *et al*., 1998; Suzuki *et al*., 2012; Tenhaken *et al*., 1995).

Insects such as *Diuraphis noxia* (Kurdjumov, Russian wheat aphid) have also been found to initiate the production of ROS in resistant wheat plants (Van der Westhuizen *et al*., 1998; Moloi and Van der Westhuizen, 2005; Boyko *et al*., 2006; Botha *et al*., 2010; Van Eck *et al*., 2010). The extent to which wounding contributes to the resistance response is yet unknown, as concurrent expression of systemic acquired resistance and wounding pathway components have been shown to be regulated in the presence of *D. noxia* resistance genes (Botha *et al*., 2008, 2010). Distinct enzyme and transcript profiles have been found when comparing aphid infested and wounded material. Wounding a wheat plant initiates a different chitinase iso-enzyme profile in *Dn1* (*Diuraphis noxia resistance gene* 1) containing wheat plants, when compared to infestation with *D. noxia,* indicating that the *D. noxia* resistance response is distinctive, and is not due to a wounding response even though some tissue damage may occur (Botha *et al*., 1998; Schultz, 2010).

In a transcript profiling study using cDNA-AFLPs, comparing wounded and infested *Dn1*-containing wheat samples, transcripts were equated in order to identify those unique to infestation. Results obtained were consistent with the induction of HR during infestation. However, there were several transcripts uniquely regulated in *Dn1* plants after wounding that were not present in the susceptible Betta or Betta *Dn2* near isogenic lines (NILs) indicating possible cross talk between the two pathways (Schultz, 2010). Also, very little information on the activity of the different ROS species in wounded wheat provides plausible answers for this observation. Studies on the function of *Dn1* were mostly conducted during *D. noxia* feeding (Botha *et al*., 1998, 2005, 2006, 2008, 2010; Moloi and Van der Westhuizen, 2005; Van der Westhuizen *et al*., 1998) with little to no information on the gene under abiotic stress. As there is evidence for differential expression during wounding, valuable information on the action of the *Dn1* gene may be collected by studying it under wounding conditions. The *Dn1* gene conveys an antibiosis-type resistance which involves ROS (Van der Westhuizen *et al*., 1998;

Moloi and Van der Westhuizen, 2005; Boyko *et al*., 2006; Botha *et al*., 2010; Van Eck *et al*., 2010), thus, two NILs Tugela and TugelaDN were utilized to ascertain differences in H_2O_2 induction. Also, key detoxification enzymes known to process ROS were targeted for silencing employing virus induced gene silencing (VIGS).

5.2 Materials and methods

Plant material and growth conditions for VIGS

All experiments were conducted with Tugela (susceptible) and TugelaDN near isogenic lines (NILs) (*Triticum aestivum* L.) that were planted in potting soil under 14 h of light and 10 h of dark at 25°C. A total of fifteen plants were used per treatment, and samples were collected at the two-leaf stage. All gene-silencing treatments were conducted on the *D. noxia* resistant TugelaDN (TugDN) plants. Each plant was observed as an independent biological repeat (n=15). Wounding was started 5 days after VIGS inoculation and consisted of 4 pin pricks to the second and third leaves daily.

Construction of silencing vectors

The barley stripe mosaic virus (BSMV) VIGS system was utilized for this study which consists of three plasmids (BSMVα, BSMVβ, BSMVγ) containing the modified BSMV tripartite genome (Schultz et al., 2014). The PCR fragments used for silencing the genes of interest were amplified from TugelaDN single-stranded cDNA prepared using the Transcriptor kit (Roche, Penzberg, Germany). VIGS primers were designed using Primer3 (Rozen and Skaletsky, 2000), and based on accessions AF38779, U69632 and AJ441055 encoding *tAPX*, *SOD-Cu/Zn* and *TaGSTF6* respectively (Table S5.1). Primers produced fragments of: *tAPX* (274 bp), *SOD-Cu/Zn* (196 bp) and *TaGSTF6* (248 bp). Products were cloned using the T-A cloning vector pGEM-T Easy (Promega, Madison WI, USA) and sequenced to confirm identity. The recombinant pGEM-T plasmids as well as the BSMVγ plasmid, containing a multiple

cloning site, were digested with the *Not*I restriction enzyme and run on 1% agarose. The excised fragments of interest from the pGEM-T plasmids were purified using the GenElute gel extraction kit (Sigma-Aldrich, St. Louis MO, USA). These fragments were then cloned into the *Not*I site in the BSMVγ plasmid (Holzberg *et al*., 2002). To determine the anti-sense orientation of each fragment, the BSMVγ forward primer was used with the forward primer for each fragment (Table S5.1) in a PCR reaction.

Virus induced gene silencing

The VIGS system was optimized in TugDN to ensure higher consistency utilizing phytoene desaturase (PDS) silencing (Scofield *et al*., 2005). Following the protocol described by Van Eck *et al*. (2010), the three BSMV plasmids were linearized using *Spe*I for plasmid BSMVβ, and *Mlu*I for plasmids BSMVα and BSMVγ. Capped RNA transcripts were made using the T7 mMESSAGE mMACHINE kit (Ambion, Life technologies, Carlsbad CA, USA) following the manufacturer's protocol. Plants were infected using a modified protocol (Holzberg *et al*., 2002; Scofield *et al*., 2005): in brief, one ng of each plasmid (BSMVα, BSMVβ, BSMVγ) was added to 35 µl FES buffer (0.1 M glycine, 0.06 M K_2HPO_4, 1% w/ v tetrasodium pyrophosphate, 1% w/v bentonite, 1% w/v celite, pH 8.5) and this mixture was used to inoculate the wheat plants at the 2–3 leaf stage by rub inoculation. The components for each treatment consisted of BSMVα and BSMVβ as well as BSMVγ transcripts containing the fragment of the gene of interest. The empty vector control consisted of BSMVα, BSMVβ and BSMVγ and denoted as TugDN+$BSMV_0$. TugelaDN plants silenced for each construct were denoted as TugDN+$BSMV_{SOD}$, TugDN+$BSMV_{APX}$, TugDN+$BSMV_{GST}$.

DAB staining

DAB staining was performed on material collected 14 days after virus inoculation (Thordal-Christensen *et al*., 1997). Three biological repeats were chosen at random from each treatment and were sampled into covered vessels containing 1 mg /ml^{-1} 3,3-diaminobenzidine (DAB)-HCl, pH 3.8, (Sigma-Aldrich, St. Louis, MO) and incubated overnight with shaking at 40 rpm. The tissues were de-stained using 75% ethanol at 37°C. The ethanol was replaced as needed. The relative intensity (*ri*) of DAB staining around the wounds as well as between the wounds (Fig. S5.1) was calculated from the leaf samples using an adapted protocol described by Miller *et al*. (2009b) using the equation:

$$ri = \frac{mean\ x\ pixels\ (treatment)}{mean\ x\ pixels\ (standard)} \quad (1).$$

A histogram was used for each area measurement which gave a mean intensity per area selected. Areas selected were kept standard across the leaves in order to allow for comparison (800 pixels; n=10). A standard of 50% grey was used (800 pixels).

Peroxidase enzyme assays

Total enzyme was extracted and assayed from whole leaf tissue (n=5) per time point, using three technical repeats per biological replicate, following the protocol described by Ni *et al*. (2001). Protein concentration was determined according to the method of Bradford (1976) using the BioRad protein assay reagent with bovine albumin (BioRad, Hercules CA, USA) as standard. The Glomax spectrophotometer (Promega, Madison WI, USA), as described by Rybutt and Parish (1982) was used for this purpose. Peroxidase activity was calculated by measuring the rate of increase in absorbance at 470 nm at room temperature using a modified protocol (Hildebrand *et al*., 1986) and horseradish peroxidase (BioLabs, Inqaba, Pretoria, ZA) as standard. Hydrogen peroxide 0.06% was added into a mixture containing 2 µg of leaf extract, 6 mM guaiacol, 25 mM potassium phosphate buffer (pH 6.0)

and 24% water (v/v). The formation of tetraguaiacol was a linear function of enzyme concentration and peroxidase activity was expressed as µmol tetraguaiacol min^{-1} mg^{-1} protein.

RT-qPCR

Silencing of candidate genes was confirmed using RT-qPCR using the prescribed guidelines (Bustin *et al*., 2009). Sampling was carried out 7 days after VIGS inoculation. All RNA extractions (n=4) were completed using Trizol reagent (Life technologies, Carlsbad CA, USA) per the manufacturer's instructions. Total RNA was *DNase*I (Fermentas, Inqaba Biotec, Pretoria, South Africa) treated and cleaned using the RNeasy mini kit (Qiagen, Hilden, Germany). Purity of the samples (260/280) was confirmed using the NanoDrop ND-1000 spectrophotometer (ThermoScientific, Inqaba Biotec, Pretoria, South Africa). Single stranded cDNA was constructed using the Transcriptor kit (Roche, Penzberg, Germany). All reactions were run on the CFX96 (BioRad, Hercules, CA) using SYBR Green I Master (Roche, Penzberg, Germany) according to the manufacturer's protocols. Cycling protocol: 1 x 95°C for 10 min; 40 x 95°C for 10 sec, 56°C for 15 sec, 72°C for 20 sec; 1 x melt curve 65°C - 95°C: increment 0.5°C per 5 sec. Relative quantification was calculated using the mathematical model by Pfaffl (2001) using TugelaDN as the calibrator and normalized to 16S rRNA (Table S5.1).

Statistical analysis of data

All sequence analysis was conducted utilizing the BLASTn and BLASTx tools (Altschul *et al*., 1990; Altschul *et al*., 1997) and SWISSPROT (O'Donovan *et al*., 2002) with *E*-values lower than 1e-10 considered as significant. All statistical analyses were conducted by using SigmaStat 3.5 (2007) (SyStat Software, San Jose CA, USA) with significance set at $\alpha = 0.05$. Image intensities for the DAB stained leaves (n=10) were tested for significant differences using a student *t*-test with $P<0.01$. All data was

plotted using Sigma Plot 7.0 (2001) (SPSS Inc., Chicago IL, USA). Outliers were identified using the guidelines of Grubbs (1969).

5.3 Results

RT-qPCR

The involvement of antioxidant enzymes in the wounding response was examined using BSMV-mediated VIGS. Silencing was confirmed using RT-qPCR (Fig. 5.1A,B,C), with transcripts knocked-down 48% in TugDN+BSMV$_{APX}$ (Fig. 5.1A), 31% in TugDN+BSMV$_{GST}$ (Fig. 5.1B) and 53% in TugDN+BSMV$_{SOD}$ (Fig. 5.1C; P<0.05).

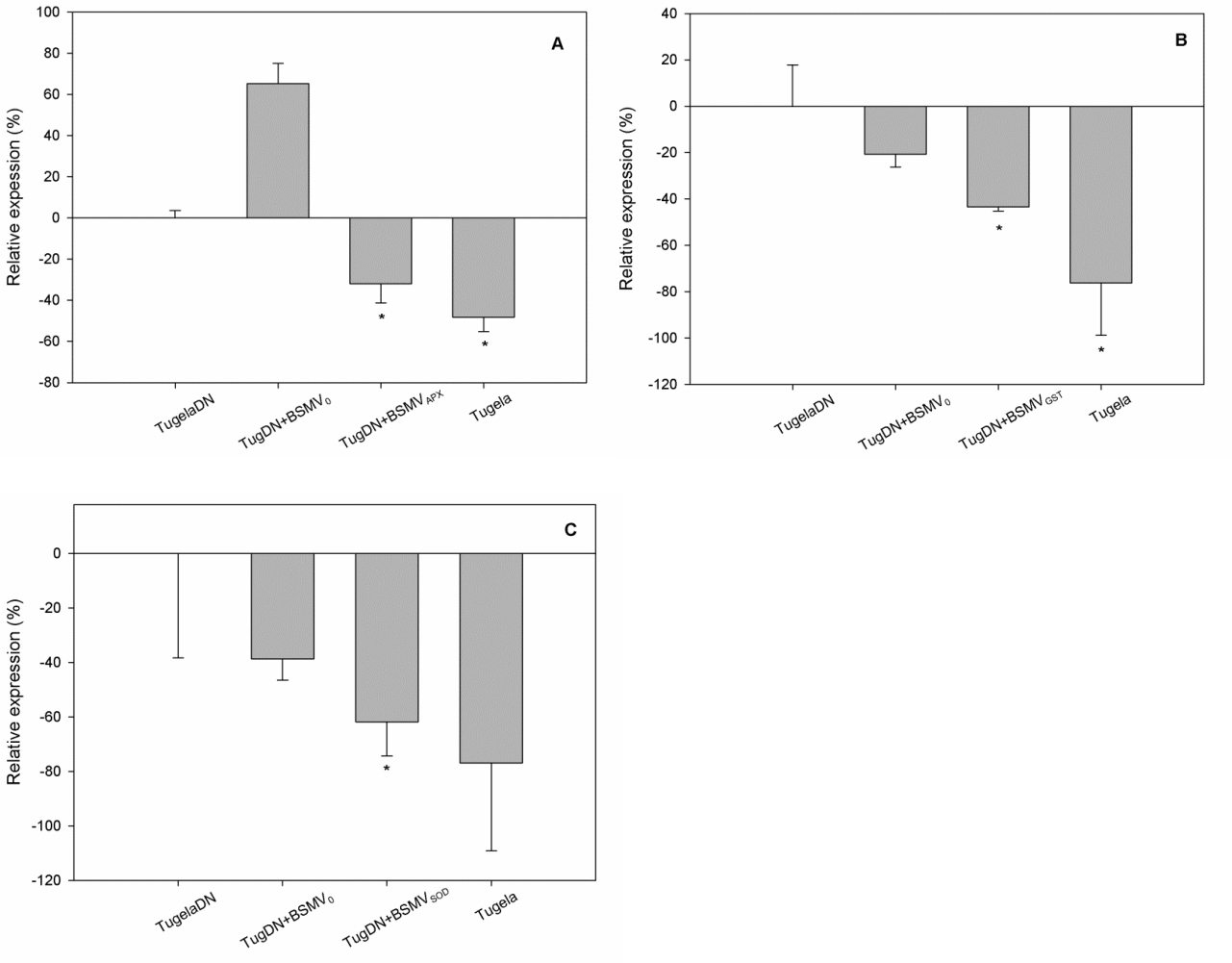


Fig. 5.1. Relative expression levels of wounded **(A)** TugDN+BSMV$_{APX}$ **(B)** TugDN+BSMV$_{GST}$, **(C)** TugDN+BSMV$_{SOD}$ measured with RT-qPCR 14 days after silencing with VIGs with levels relative to TugelaDN. Asterisks indicate significant deviation (P<0.05).

No significant deviation was recorded between the resistant controls TugelaDN and TugDN+BSMV$_0$ (Fig. 5.1A,B,C). The means measured in Tugela were significantly lower than TugelaDN for *GSTF6* (76%) and *tAPX* (90%; Fig. 5.1A,B) but not for *CuZnSOD* (70%; P<0.05; Fig. 5.1C).

Virus induced gene silencing of thylakoid ascorbate peroxidase

In order to ascertain whether silencing of *APX* effected the production of H_2O_2 during wounding, VIGS experiments were performed. Peroxidase activity was measured over a period of a week as a measure of H_2O_2 production. At 0h peroxidase activity was 22% higher in TugelaDN plants than in Tugela. At the 4 h time point peroxidase activity is up-regulated 71% and 46% in BSMV$_0$ and TugelaDN in comparison to Tugela. There was a significant decrease in peroxidase activity in the TugDN+BSMV$_{APX}$ silenced plants in comparison to TugDN+BSMV$_0$ across all time points except for at 48 h where there is a peak in activity (Fig. 5.2A). At 48 h there is a peak in activity in the susceptible Tugela treatment as well, with an increase of 60% in comparison to TugelaDN and 75% in comparison to TugDN+BSMV$_0$. No significant difference in peroxidase activity was observed between the TugelaDN and TugDN+BSMV$_0$ treatments at the 0 h, 48 h and 7 day time points (P<0.05).

As a visual confirmation of a difference in H_2O_2 production, 3,3'-diaminobenzidine (DAB) staining was performed on leaf samples from all VIGS treatments and controls (Fig. 5.2B,C,D,E). The interaction between DAB and H_2O_2 produces reddish-brown deposits visible to the eye. When doing a visual inspection, staining around all wounding sites on leaves from resistant controls TugelaDN and TugDN+BSMV$_0$ appear as indistinct darkly stained coronae. The staining on these leaves extended further than the area around the wounding sites (Fig. 5.2A,B). There are no coronae of staining around

the wounding sites of the susceptible Tugela leaves (Fig. 5.2D). In the *APX* silenced plants, there are distinct darkly stained coronae around the wounds; however, there is no spread of staining as seen in the resistant controls and the areas between the wounds appear clear with no bruising (Fig. 5.2C).

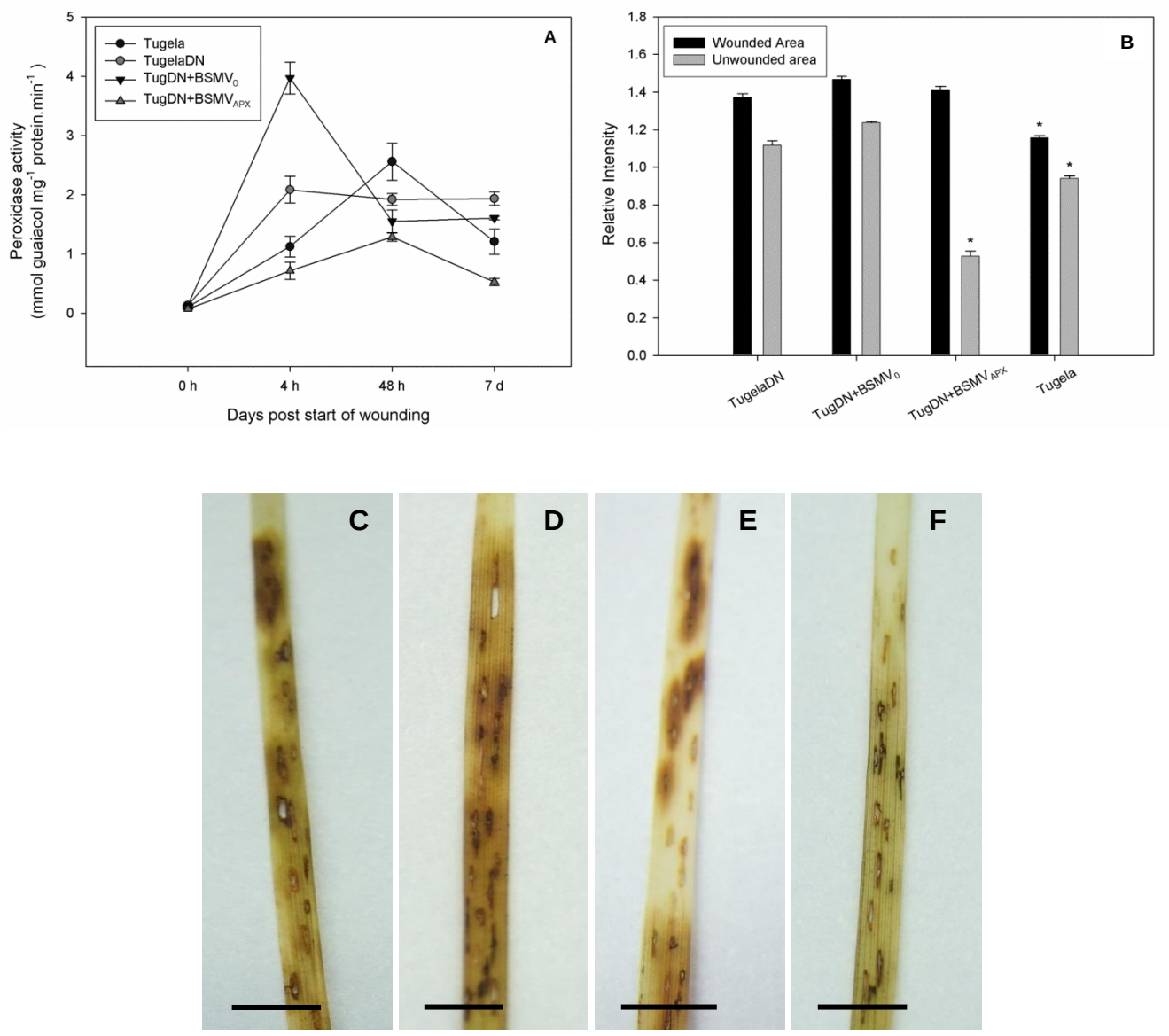


Fig. 5.2. (A) Peroxidase activity measured in TugelaDN, TugDN+BSMV$_0$ and TugDN+BSMV$_{APX}$ plants. **(B)** Graph of relative intensities of areas around wounds and away from wounds of each leaf sample. Asterisks indicate significant deviation from resistant control (P<0.05). **(C-F)** Representative leaf samples taken at 14 days post inoculation, which have been stained for peroxidase activity using

DAB **(C)** TugelaDN wounded, **(D)** TugDN+BSMV$_0$ wounded, **(E)** TugDN+BSMV$_{APX}$ wounded, **(F)** Tugela wounded. Black bar scale represents 1 cm.

In order to ascertain the degree to which each leaf was stained, the relative intensities (*ri*) of the areas around the wounds and in between the wounds were compared (Fig. 5.2F). The highest degree of staining around the wounds was measured in the control TugDN+BSMV$_0$ and TugDN+BSMV$_{APX}$ silenced plants (*ri*= 1.467; se= 0.0169 and *ri*= 1.4123; se= 0.0193) respectively. Lowest intensity measurements were found around the wounds on the Tugela wounded control (*ri*= 1.156; se= 0.0129), an average of between 16% and 21% less staining around the wounds when compared to the TugelaDN and TugDN+BSMV$_0$ controls respectively. When comparing the staining in-between the wounds, the TugDN+BSMV$_0$ control had the highest degree of staining (*ri*= 1.237; se= 0.0080), with the lowest measured from the TugDN+BSMV$_{APX}$ silenced plants (*ri*= 0.510; se= 0.0227). The relative intensity of staining in the areas in-between the wounds in the TugDN+BSMV$_{APX}$ silenced plants was found to be 54% and 59% lower than that of the TugelaDN (*ir*= 1.117; se= 0.0247) and TugDN+BSMV$_0$ resistant controls respectively, and 46% lower than the susceptible Tugela control (*ir*= 0.9405; se= 0.0133).

Virus induced gene silencing of glutathione-S-transferase and superoxide dismutase Cu/Zn

In order to ascertain whether silencing of *GSTF6* or *CuZnSOD* effected the production of H_2O_2, VIGs experiments were performed. Peroxidase activity was measured over a period of two weeks as a measure of H_2O_2 production. No significant difference in peroxidase activity was observed between the TugelaDN and TugDN+BSMV$_0$ controls at the 0 h, 48 h and 7 day time points, however, there was a significant decrease in peroxidase activity in the silenced TugDN+BSMV$_{GST}$ plants in comparison to the control TugDN+BSMV$_0$ across all time points (Fig. 5.3A).

Peroxidase activity was on average 22% higher in TugelaDN plants than in Tugela at the 0h time point. At the 4 h time point peroxidase activity in both TugDN+BSMV$_0$ and TugelaDN controls

are up-regulated (32%; 47%) in comparison to the Tugela control (P<0.05). At 48 h there was an up-regulation in activity in the Tugela control, as well as in TugDN+BSMV$_{SOD}$ and TugDN+BSMV$_{GST}$ silenced plants in comparison with the resistant controls.

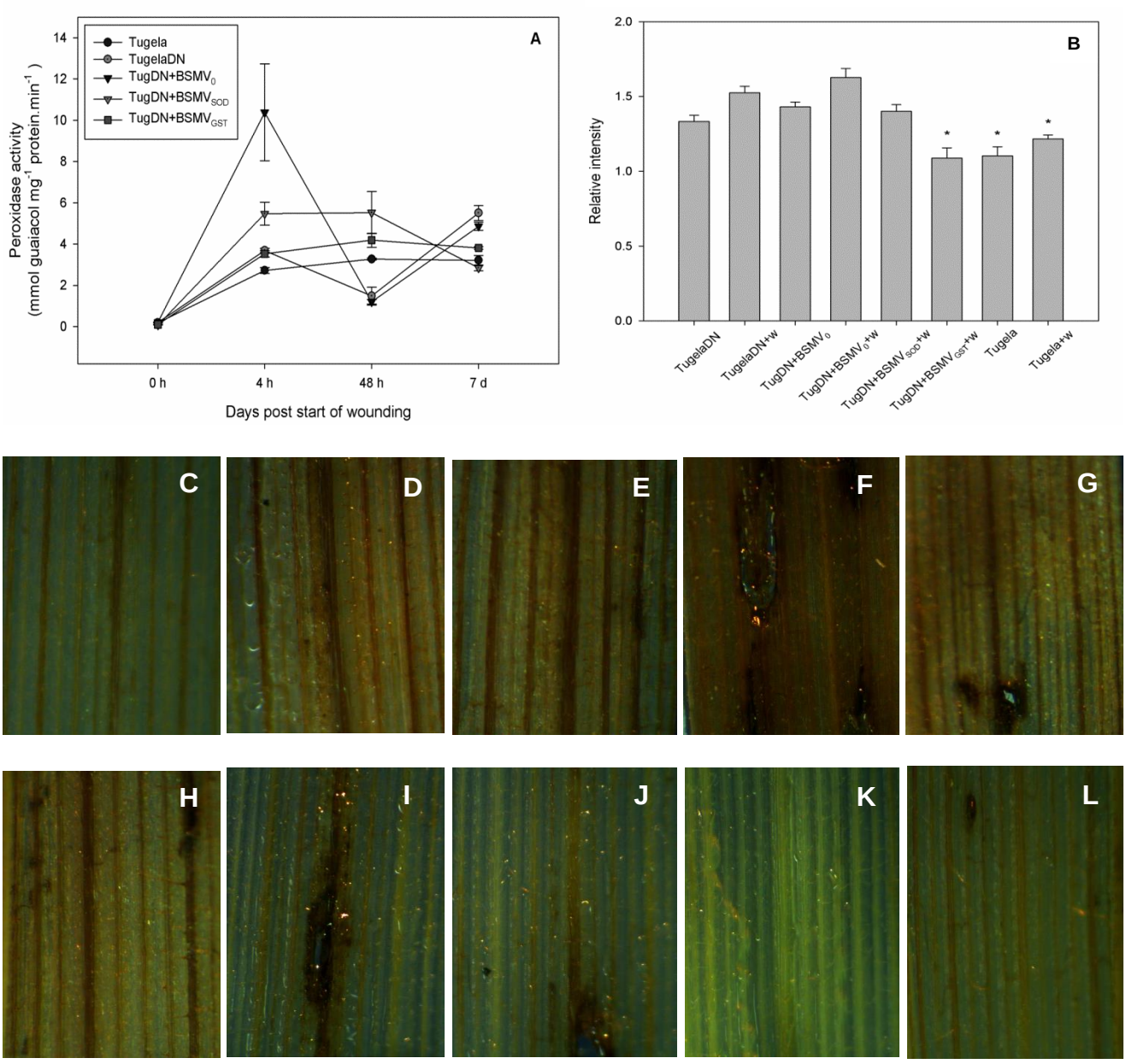


Fig. 5.3. (A) Peroxidase activity measured in Tugela, TugelaDN, TugDN+BSMV$_0$, TugDN+BSMV$_{SOD}$, and TugDN+BSMV$_{GST}$ plants. **(B)** Graph of relative intensities of measured from each leaf sample where (+w) indicates a wounding treatment. Asterisks indicate significant deviation from resistant control (P<0.05). **(C-L)** Microscopy of DAB stained leaf samples **(C)** TugelaDN, **(D)** TugelaDN

wounded, **(E)** TugDN+BSMV$_0$, **(F)** TugDN+BSMV$_0$ wounded, **(G-H)** TugDN+BSMV$_{SOD}$ wounded **(I-J)** TugDN+BSMV$_{GST}$ wounded, **(K)** Tugela, **(L)** Tugela wounded.

The measured peroxidase activity in Tugela was 45% higher than that of TugelaDN and 36% higher than TugDN+BSMV0. TugDN+BSMV$_{SOD}$ and TugDN+BSMV$_{GST}$ silenced plants 28% and 26% more activity respectively to that measured in control TugDN+BSMV$_0$. At the 7 day time point, peroxidase activity was down-regulated in Tugela (41%), TugDN+BSMV$_{SOD}$ (41%) and TugDN+BSMV$_{GST}$ (22%) in comparison to the TugDN+BSMV$_0$ control.

DAB staining was performed as visual confirmation of H_2O_2 on leaf samples from all VIGS silenced plants and controls (Fig. 5.3B–M). The relative intensities (*ri*) of the leaf samples for the TugDN+BSMV$_{APX}$ experiment were compared in both the areas in the immediate vicinity of the wounds and the areas in-between (Fig. 5.3N; Fig. S5.1)

No significant difference was measured in the control plants around the wounds (i.e. TugelaDN and TugDN+BSMV$_0$) nor was any differences observed in the areas in-between the wounds wounded controls TugelaDN and TugDN+BSMV$_0$

The highest degree of staining in-between the wounds was measured in the wounded TugDN+BSMV$_0$ control plants (*ri*=1.627; se= 0.0606), whereas, the lowest intensity measurements were recorded from the TugDN+BSMV$_{APX}$ wounded plants (*ri*= 1.088; se= 0.0692) indicating a significantly lowered presence of H_2O_2 giving the leaves a bleached appearance. In the wounded area there was 17% and 22% less staining when comparing the Tugela control with controls TugelaDN and TugDN+BSMV$_0$ respectively. In the areas in-between there was also 20% and 25% less staining when comparing the Tugela control plants with TugelaDN and TugDN+BSMV$_0$ control plants respectively.

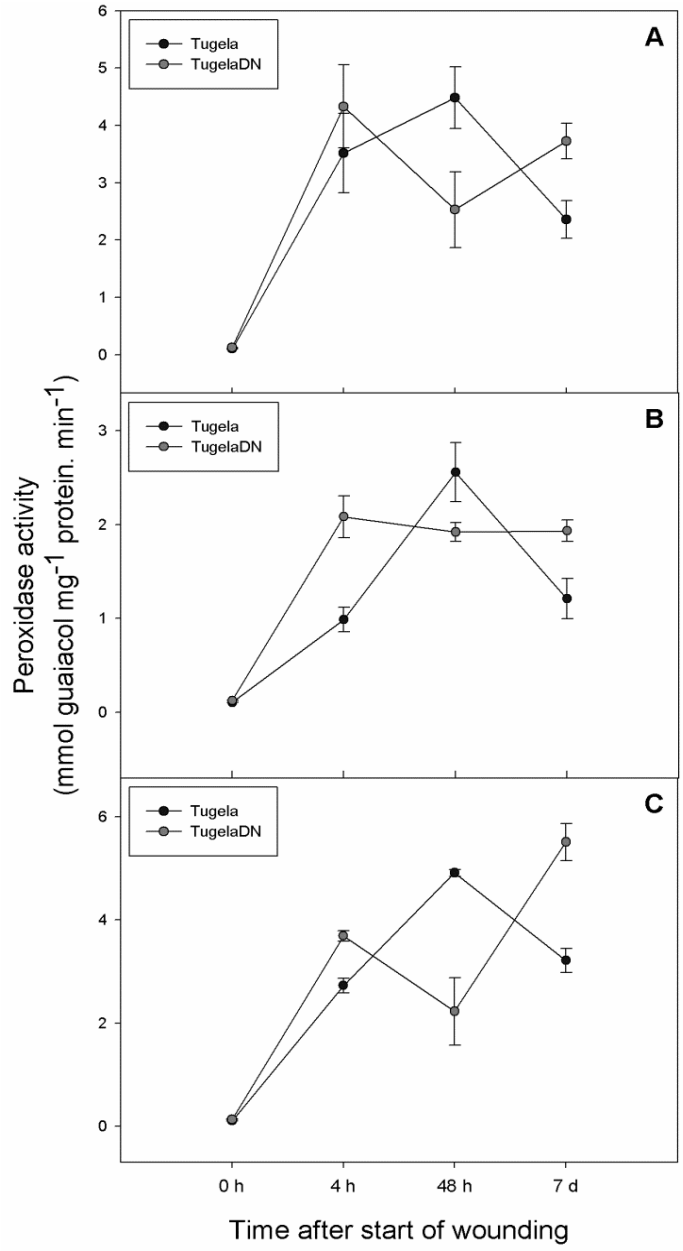


Fig. 5.4. Peroxidase activity measured in the Tugela and TugelaDN NILs after the start of the wounding treatment taken from three different experiments. (A) Tugela vs TugelaDN, (B) Tugela and TugelaDN controls extracted from TugDN+BSMV$_{APX}$ VIGS experiment (Fig. 5.2A), (C) Tugela and TugelaDN controls extracted from TugDN+BSMV$_{SOD}$ and TugDN+BSMV$_{GST}$ VIGS experiment (Fig. 5.3A).

The relative intensities (*ri*) of the leaf samples for the TugDN+BSMV$_{SOD}$ and TugDN+BSMV$_{GST}$ experiment were compared. After wounding, there was no significant difference in

staining between the silenced TugDN+BSMV$_{SOD}$ and TugDN+BSMV$_0$ control plants (*ri*= 1.402; se= 0.0447 and *ri*=1.627; se= 0.0606). There was a significant decrease in staining in the wounded TugDN+BSMV$_{GST}$ plants in comparison to the wounded TugDN+BSMV$_0$ control (*ri*= 1.088; se= 0.0692 and *ri*=1.627; se= 0.0606).

Differences in peroxidase activity in the presence of Dn1 gene

To confirm whether there is a discernable pattern to peroxidase activity in the NILs, a biological repeat of the peroxidase assay was completed on wounded Tugela and TugelaDN control plants (Fig. 5.4A). All wounded Tugela and TugelaDN control data was extracted from the VIGS experiments in order to do a comparison (Fig. 5.4B,C). The results show that the relative activity of peroxidase is similar between the different graphs at the same time points. At 0h, TugelaDN plants show higher levels of peroxidase level than Tugela.

At 4 h peroxidase activity is up-regulated in TugelaDN, as well as in Tugela plants. At 48h, peroxidase activity is further up-regulated only in the Tugela plants. This is followed by down-regulation of peroxidase activity in Tugela at later time points, while peroxidase activity in TugelaDN either stays constantly high or is up-regulated by the seven day time point.

5.4 Discussion

H_2O_2 plays an important role in signal transduction (Apel and Hirt, 2004; Laloi *et al*., 2007; Li *et al*., 2009), and alters the expression of one-third of the known *Arabidopsis* transcription factors with 3-or more fold change after application (Gadjev *et al*., 2006), also, transcripts up-regulated by wounding show a high degree of similarity to those up-regulated by H_2O_2 application (Miller *et al*., 2009a). In this study, as *Dn1* is associated with antibiosis, the production of antioxidants and H_2O_2 was used to draw a

comparison between Tugela and TugelaDN NILs under wounding conditions. The involvement of key detoxification enzymes was also measured utilizing VIGS.

In plant cells, the chloroplast is one of the most important ROS manufacturers, producing both $^1O_2$ and H_2O_2 (Foyer and Shigeoka, 2011). Thylakoid bound ascorbate peroxidase is involved in the detoxification of hydrogen peroxide, and has been involved in signaling and gene-regulation (Maruta *et al*., 2010). In a study utilizing high light treatments and Arabidopsis knock-out mutants it was found that tAPX is involved in the regulation of H_2O_2 responsive genes (Maruta *et al*., 2010). Silencing of thylakoid bound ascorbate peroxidase in TugDN resulted in a down regulation of hydrogen peroxidase activity as well as a lack of H_2O_2 in the spaces between wounds, indicating a lack of perpetuation of H_2O_2 production in the distal tissues of the leaves. Previous studies silencing *tAPX* did so over a shorter period, i.e. two days (Maruta *et al*., 2010), therefore a more direct comparison could not be completed. However, these results suggest tAPX plays an important role in not only chloroplastic H_2O_2 signalling, but also that disruption of this antioxidant also effects signal perpetuation.

Silencing of *GSTF6* resulted in significantly lower peroxidase activity across the leaf, resulting in lighter staining in silenced plants similar to results from preceding studies involving Russian wheat aphid infestation (Schultz *et al*., 2014). This indicates that wheat *GSTF6* is closely linked to H_2O_2 production under both biotic and abiotic stress conditions.

Superoxide dismutase is involved in the detoxification of H_2O_2 under stress conditions. Silencing of superoxide dismutase initially resulted in peroxidase activity levels higher than that of the TugDN control, but lower than the empty vector control TugDN+BSMV$_0$. The buildup of H_2O_2 resulted in heavily stained leaves in TugDN+BSMV$_{SOD}$ silenced plants, similar to that seen in the empty vector control TugDN+BSMV$_0$. However, by the 7 day time point, peroxidase activity in wounded TugDN+BSMV$_{SOD}$ plants was lower than all of the other treatments. The dark staining and

lowered peroxidase activity indicate possible tissue damage due to overexposure to H_2O_2 as has been seen in previous H_2O_2 overexpression studies (Hu *et al*., 2003). These results indicate that superoxide dismutase is vital for plant homeostasis under wounding conditions.

After comparing the Tugela and TugelaDN peroxidase activity assays a pattern was discerned, that showed distinct differences between the two NILs. The low level of genetic difference, suggests that differences in expression or protein activity may be mediated by the presence of the *Dn1* gene insert. Also, significantly lower levels of *tAPX* and *GSTF6* transcripts were measured in Tugela plants in comparison to TugDN under wounding conditions, indicating dissimilar regulation under the same treatment. These results indicate cross-talk between the wounding and *Dn1* pathways (Schultz 2010). A recent study by Van Eck *et al*. (2014) indicates that *WRKY53* may regulate oxidative responses to both abiotic and biotic stresses in wheat, providing a pathway for cross-talk to occur.

This study demonstrates a difference in production of H_2O_2 during a wounding response in the presence of an insect R-gene, *Dn1*. As the DAMP-associated wounding response is common to all plants, it is expected that plants with similar genetic backgrounds will have comparable responses to wounding. However, differences were observed in both the levels of antioxidants and H_2O_2 when comparing wounded Tugela and TugDN plants. Evidence for the involvement of *tAPX* in H_2O_2 mediated signaling was observed. Silencing of *tAPX* resulted in decreased systemic H_2O_2 production, adding impetus to the role of H_2O_2 in signaling (Laloi *et al*., 2007; Morita *et al*., 1999). Silencing of *GSTF6* decreased H_2O_2 production and peroxidase activity, indicating *GSTF6* may be necessary for H_2O_2 production under abiotic conditions. Results from peroxidase and DAB studies indicate an up-regulation of H_2O_2 after wounding over all time points as well as the up-regulation of detoxification enzymes in TugDN in comparison to Tugela. The reaction of the Tugela and TugDN plants to repetitive wounding were dissimilar, indicating cross-talk between the *Dn1* and wounding pathways.

WRKY53 is a candidate for cross-talk between biotic and abiotic stress in GamtoosR that contains the *Dn7* gene and should be investigated further within the *Dn1* cultivar under investigation.

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5.7 Supplementary data

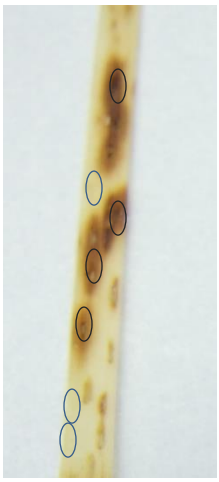

Fig. S5.1. Example of areas sampled in-between wounded areas (blue) and wounded areas (black) for image intensity measurements.

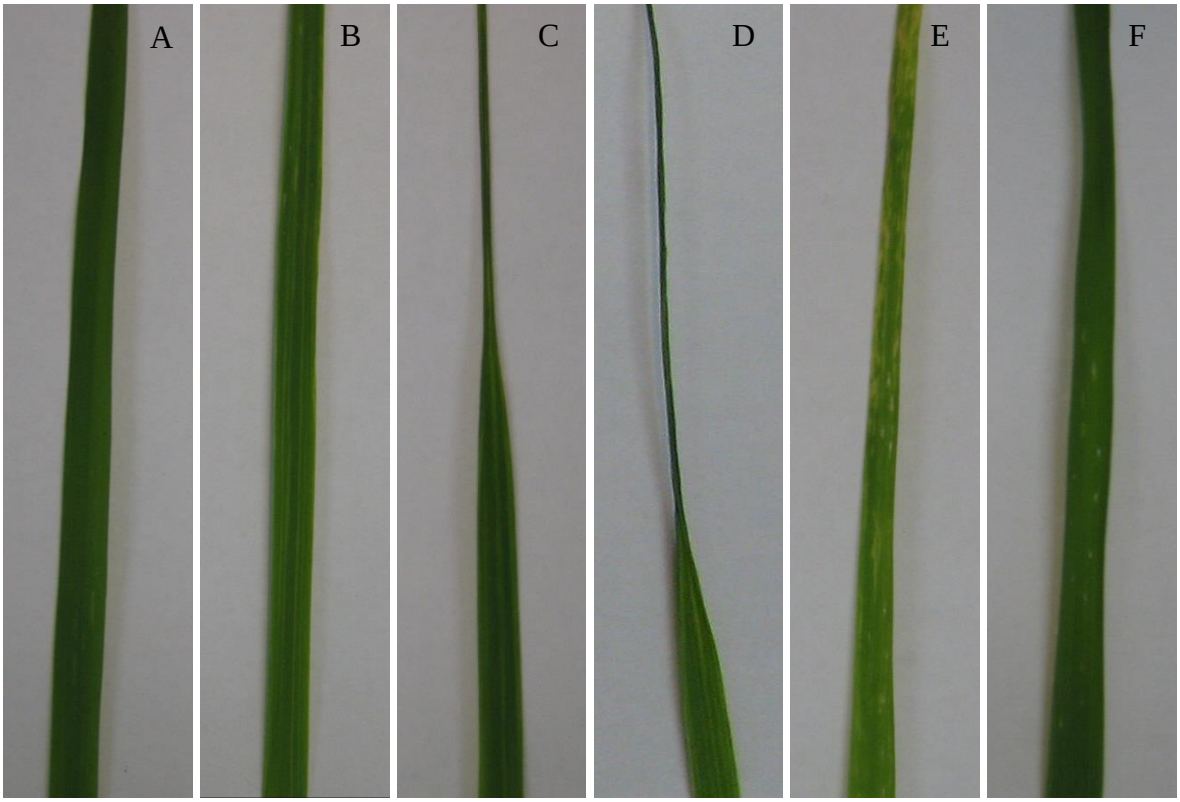

Fig. S5.2. Pictures of sample leaves gathered at 14 days post infection for each treatment. A= TugelaDN, B= TugDN+BSMV$_0$, C= TugDN+BSMV$_{APX}$, D= TugDN+BSMV$_{GST}$, E= TugDN+BSMV$_{SOD}$, F= Tugela

Table S5.1. Primers used during vector construction and qRT-PCR

Name	Forward Primer	Reverse Primer	Accession
APX-VIGS	5'-CCACGTTTACCTGCTGAACA-3'	5'-CGCAGCATCTGTAGGCAATA-3'	AF387739
SOD-VIGS	5'-TCCTTTGACTGGCCCTAATG-3'	5'-AACGAGAATGGCGTCGTTAC-3'	U69632
GST-VIGS	5'-CCTCAACCACTTCCCCTACA-3'	5'-ATTTGCCTTATTCGCGACAC-3'	AJ441055
APX-qPCR	5'-CGTGGCAACTGAAGAGAAGG-3'	5'-AAGAGTATTTGGCGGAAACG	EF184291
SOD-qPCR	5'- GTGAACGTTCGTATCACTGG -3'	5'- TGACGGACTTCATCTTCTGG -3'	U69632
GST-qPCR	5'-GAAGGTGCTGGAGGTCTACG-3'	5'-TGTAGGGGAAGTGGTTGAGG-3'	AJ441055
16S-qPCR	5'- TCAAGTCCGCCGTCAAATC-3'	5'- TCGCCGTTGGTGTTCTTTC-3'	AJ239003
pGamma	5'-TGATGATTCTTCTTCCGTTGC-3'	5'- TGGTTTCCAATTCAGGCATCG3'	

Chapter 6
Conclusions

Wheat *Dn* genes afford resistance to the economically important pest, *Diuraphis noxia* (Kurdjumov, Russian wheat aphid, RWA) and have been the topic of transcriptomic and proteomic studies aimed at unravelling the pathways involved in resistance. The antibiosis resistance conveyed by *Dn1* is characterized by a hypersensitive response (HR) followed by systemic acquired resistance (SAR). Although many candidate genes differentially expressed during the *Dn1*-mediated resistance response have been identified, few have been functionally verified. The aim of this study was to silence three HR-associated candidate genes in *Dn1* containing wheat using virus-induced gene silencing (VIGS): thylakoid-associated ascorbate peroxidase (*tAPX*), phi-class glutathione-*S*-transferase (*TaGSTF6*), and superoxide dismutase *Cu/Zn* (*SOD*). *D. noxia* fertility was used as a measure of antibiosis resistance. Silencing of *CuZnSOD* had little effect on *D. noxia* fertility, while increased aphid reproduction was recorded on *tAPX*- and *TaGSTF6*-silenced plants. However, *tAPX*-silencing only affected early measurements and did not have a prolonged effect on resistance. *TaGSTF6*-silenced plants expressed lowered H_2O_2 production in resistant wheat under infestation conditions, suggesting that *TaGSTF6* and H_2O_2 play an integral role in *Dn1*-mediated *D. noxia* resistance in wheat plants.

During previously reported expression studies, several *Diuraphis noxia* feeding induced genes have been identified in *Triticum aestivum* (wheat). Using VIGS a *resistance gene analogue 2* (*RGA2*) and *nucleotide binding protein* (*NBP*) were silenced in TugelaDN to asses *Dn1*-mediated *Diuraphis noxia* resistance in wheat. Silencing of *resistance gene analogue 2* (*RGA2*), identifies as *T10RGA2-1D* resulted in highly susceptible plants ensuing in death after infestation, suggesting that *RGA2* may be a good up-stream candidate in *Dn1*-resistance. Silencing of the *NBP* gene caused chlorosis, susceptibility and plant death after *D. noxia* infestation. NBP was identified as a Fe-S cluster assembly protein and

thus crucial to iron homeostasis in the plant. It seems likely that *Dn1*-mediated resistance is dependent on iron homeostasis and a fully functional *T10RGA2-1D* gene.

Plants defend themselves with a complex set of both innate and specific resistance interactions, and in crop plants this is a topic of intense study. The TugelaDN cultivar has been phenotyped as a fast grower, as well as having an antibiosis-type resistance to aphids. Two *Triticum aestivum* (bread wheat) near isogenic lines (NILs), Tugela and TugelaDN were studied under wounding conditions in order to further characterize the TugelaDN cultivar. In order to ascertain involvement of key detoxification enzymes in TugelaDN plants under wounding conditions, thylakoid-associated ascorbate peroxidase (*tAPX*), glutathione-*S*-transferase (*GSTF6*), and superoxide dismutase (*SOD*) were cloned into barley stripe mosaic virus (BSMV) to trigger silencing. Silencing of *CuZnSOD* in plants incurred tissue damage and chlorosis. H_2O_2 production was adversely affected by silencing of *GSTF6*. The H_2O_2 staining results from *tAPX* silenced plants show H_2O_2 production is limited to the areas around the wound sites. Results from peroxidase assays show a higher basal level of peroxidase, as well as an early up-regulation in peroxidase activity after wounding in TugelaDN when compared to Tugela. Results from peroxidase activity assays showed up-regulation in Tugela at 48 hours after the commencement of the wounding treatment; however this was followed by a decrease in activity to lower activity levels than was observed in TugelaDN. DAB staining was darker in TugelaDN plants in comparison to Tugela plants indicating the presence of higher H_2O_2 levels. The presence of the *Dn1* gene alters peroxidase and antioxidant activity. Peroxidase activity measurements, staining and up-regulated antioxidants indicate an increased ability in TugelaDN plants to maintain H_2O_2 concentrations over time without sustaining damage.

A variety of regulatory levels may be involved in the initiation of defensive levels of H_2O_2 production in TugDN, and thus the following models are suggested (Fig. 6.1).

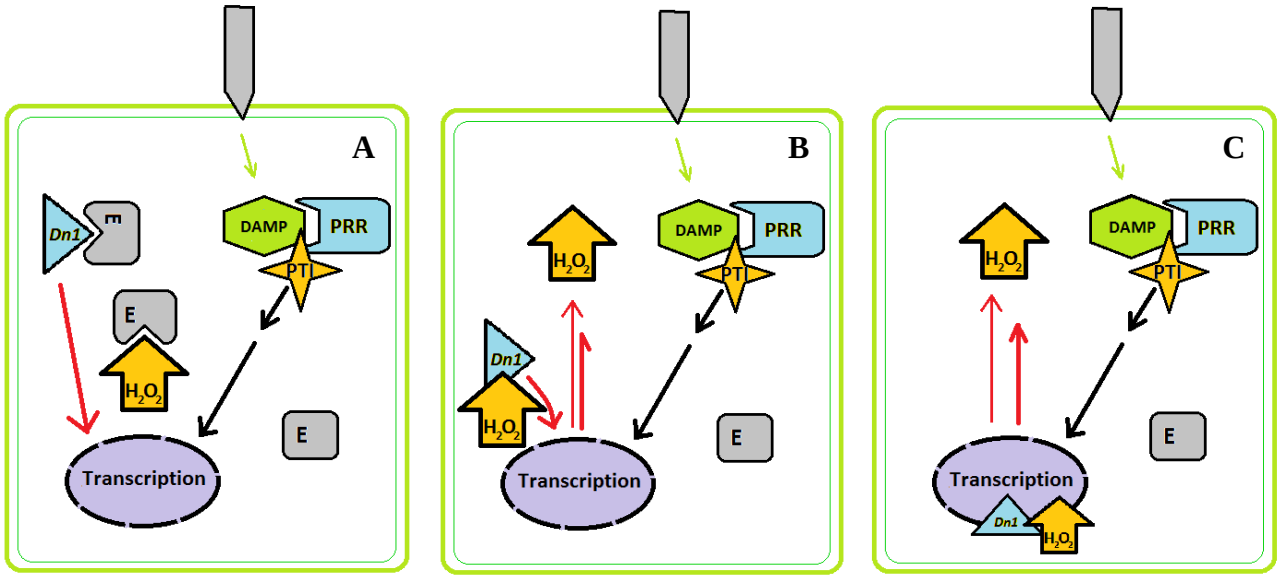

Fig. 6.1. Models of possible crosstalk points between H_2O_2 and *Dn1*. **(A)** Model for the guard hypothesis. H_2O_2 produced is known to alter the structure of certain proteins. The alteration in these enzymes is monitored by the Dn1 protein, which initiates a resistance response after activation. **(B)** Model for activation of the Dn1 protein as a signaling protein. *Dn1* is present in cytosol in an inactive. H_2O_2 produced causes an alteration to the Dn1 causing activation, which in turn initiates a resistance response. **(C)** Model for Dn1 as nuclear element/ affected by transcription factor. After activation by H_2O_2, Dn1 initiation increases and prolongs H_2O_2 and antioxidant expression. DAMP= damage associated molecular pattern, E= enzyme, PRR= pattern recognition receptor, PTI= pattern triggered immunity

H_2O_2 is known to affect the structure and expression of a variety of enzymes (Maruta *et al*., 2012), thus the first model is that the Dn1 protein actions are comparable to resistance proteins that 'guard' cell or protein integrity (Dangl and Jones, 2001; Van der Biezen and Jones, 1998; Van der Hoorn *et al*., 2002). The guard hypothesis states that the plant utilizes R-proteins indirectly, in order to sense a change or alteration to host cellular targets caused by an effector (Jones and Dangl, 2006).

In this model an alteration to either a cell or an important component is monitored by the Dn1 surveillance protein, which initiates a resistance response after activation (Fig. 6.1A). Evidence for this model would be the high number of transcripts for genes involved in redox homeostasis regulated during RWA infestation (Matsioloko, 2011; Schultz, 2010).

The second model, is one in which the Dn1 protein is a signaling protein activated in the presence of H_2O_2 in the cytoplasm (Fig. 6.1B). After activation by H_2O_2, Dn1 protein activation increases and prolongs H_2O_2 and antioxidant generation. Similarly, the third model involves direct alteration of *Dn1* or a transcription factor by H_2O_2 within the nucleus (Fig. 6.1C).

An increase in H_2O_2 concentration causes an alteration in either a cytoplasmic or a nuclear protein resulting in activation, which in turn initiates a resistance response by activation of transcription. Evidence for this hypothesis is recent findings that a *WRKY53* transcription factor is both affected by abiotic and biotic stresses, as well as the elevated peroxidase activity found in TugelaDN plants before treatment, indicating that a level of up-regulation is constitutive in *Dn1* containing plants.

Further investigation and utilization of protein-protein interaction technologies is necessary to further elucidate the functions and interactions of the possible *Dn1* genes and *Dn1* pathway constituents.