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Significance of photosynthesis and the photosynthesis related genes TMP14, FBPase and P700 in Russian wheat aphid resistant wheat

by

C.S. Jackson

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Prof. AM Botha-Oberholster

“Yet one thing secures us what ever betide, the scriptures assures us the Lord will provide.” Isaac Newton

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Declaration

I, the undersigned, hereby declare that the dissertation submitted for the degree *Magister Scientiae* to the University of Pretoria, contains my own independent work and has not been submitted for any degree elsewhere.

Carlo Jackson

2009

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Abbreviations

APX	Ascorbate peroxidase
AOX	Adsorbable organically bound halogens
ATP	Adenosine Triphosphate
Avr	Avirulence
BA	Benzoic acid
BSMV	Barley stripe mosaic virus
CAT	Catalase
CDPK	Calcium dependent protein kinases
CC	Coiled-coil
Chl	Chlorophyll
cpATPase	Chloroplast ATPase
CuZnSOD	Cu/Zn superoxide dismutase
DEPC	Diethyl pyrocarbonate
DDRT-PCR	Differential display reverse transcriptase polymerase chain reaction
dNTPs	Deoxynucleosides
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EPG	Endo polygalacturonases
EST	Expresed sequence tags
FBPase	Fructose-1,6-bisphosphatase
FD	Ferredoxin
FeSOD	Iron superoxide dismutase
GFP	Green Fluorescent protein
GITC	Guanidinium isothiocyanate
GPX	Glutathione peroxidase
GPWG	Grass Phylogeny Working Group
GST	Glutathione S-transferase
HR	Hypersensitive response
JA	Jasmonic acid
LAR	Local acquired resistance

LPS	Lipopolysaccharides
LRR	Leucine-rich repeats
LSU	Large subunit
MAPK	Mitogen activated protein kinase
MeJA	Methyl jasmonate
MeSA	Methyl salicylate
NADPH	Nicotinamide adenine dinucleotide phosphate
NBS-LRR	Nucleotide-binding site leucine-rich repeat
NBS	Nucleotide binding site
NILS	Near-isogenic lines
NO	Nitrous oxide
OD	Optical density
ORF	Open reading frame
PAMPs	Pathogen-associated molecular patterns
PCD	Programmed cell death
PCR	Polymerase chain reaction
PDR	Pathogen-derived resistance
PDS	Phytoene desaturase
PFI	Phloem feeding insects
PI	post infestation
PKC	Protein kinase C
PMIDB	Plant-microbe interaction database
PQ	plastoquinol
PR	Pathogenesis related
PrxR	Peroxiredoxin
PS	Photo system
PSI	Photosystem I
PSII	Photosystem II
PTGS	Post-transcriptional gene silencing
qRT-PCR	Real-Time Quantitative Reverse Transcription PCR
R	Resistance
RISC	RNAi silencing complex
RLK	Receptor-like protein kinases

RT-PCR	Reverse transcriptase polymerase chain reaction
ROs	Reactive oxygen species
RWA	Russian wheat aphid
SA	Salicylic acid
SAR	Systemic acquired resistance
siRNA	Short interfering RNA
SNP	Single-nucleotide polymorphism
SOD	Superoxide dismutase
SSH	Suppression subtractive hybridization
T-DNA	Transfer- DNA
TE	Transposable elements
TILLING	Targeting induced local lesions in genomes
TM	Trans-membrane
TMP14	Thylakoid membrane phosphoprotein 14
Tris	Tris(hydroxymethyl)aminomethane
USA	United States of America
UTR	Untranslated region
VIGS	Virus-induced gene silencing
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Chapter 1

Literature review: Genetic resistance of hexaploid wheat (*Triticum aestivum* L.) against the Russian wheat aphid (*Diuraphis noxia* Kurdjumov)

1.1. Introduction

Wheat (*Triticum aestivum* L.), being a primary source of food in most countries of the world, is one of the most important crop plants produced globally and its production is of important economical value (Payne *et al.*, 2001; Gill *et al.*, 2004). A variety of pests and pathogens, including insects such as the Russian wheat aphid (*Diuraphis noxia* Kurdjumov, RWA) (Walters *et al.*, 1980), viruses (Truol *et al.*, 2004), bacteria (Duveiller *et al.*, 1992) and fungi (Pretorius *et al.*, 1997) cause major problems to wheat production. Wheat is subjected to many other environmental stresses that cause major crop reductions, but biotic stresses are the most destructive. For instance the RWA causes millions of dollars in crop losses annually in the United States of America (USA) (Anderson *et al.*, 2003) and the percentage crop loss experienced in South Africa due to the RWA ranges from 21 to 90% (Basky, 2003).

Unlike abiotic stresses that cannot be easily dealt with, biotic stresses can be controlled with chemical control practices. This type of management is not always successful, is expensive and its environmental impact mostly ignored (Robinson, 1992). It was found that some plants have naturally developed resistance to pathogens due to selection pressure. Thereby selection crossings have been applied earlier to improve resistance of the plants rather than using common management practices including insecticides which is dangerous to the environment (Walters *et al.*, 1980). Cross breeding have two major drawbacks, the process is very time consuming and the new cultivars do not last long due to the development of new pathotypes or insect biotypes (McIntosh *et al.*, 1995; Murray *et al.*, 2005; Weiland *et al.*, 2008). RWA biotypes are defined by their ability to overcome the resistance of previously resistant wheat cultivars as recently reported for the USA and South Africa (Basky, 2003; Smith, 2005; Weiland *et al.*, 2008). This posed a major problem and different integrated management strategies are needed.

The feeding of *Diuraphis noxia* on the wheat plant for the purpose of growth and reproduction diminishes the performance of the plant and can even cause its death. In this parasitic relationship, RWA's presence on the wheat leaves is detected and a signal is generated to activate structural and biochemical defence mechanisms in resistant

plants, in an attempt to deter the aphid. These defence mechanisms that limit the chance of infestation are present in most cultivars but are not always successful due to the plants inability to activate them or being to slow (Bayles *et al.*, 1990). The crucial area of defence lies in the detection of the invading organism. When the plant detects the presence of the RWA, defence mechanisms are activated either non-specifically with general elicitors or specifically with protein elicitors (Johal *et al.*, 1995). The elicitors may originate from the aphid itself or through the action of feeding and stimulate the defence response that renders the aphid incompatible with the wheat cultivar (Yamaguchi *et al.*, 2000). The receptor proteins that perceive the elicitors are mostly enzymes like protein kinases, causing a signal transduction cascade (Suzuki *et al.*, 2004).

During plant-insect interactions these signals include many chemical pathways that are involved in triggering a hypersensitive response (HR) and other reinforcement mechanisms to reduce feeding and the spread of the insects on the plants and between plants (Hammerschmidt and Schultz, 1996). Recognition of the pathogen will also activate defence responses leading to altered gene expression of defence related genes to strengthen the resistance (Yamaguchi *et al.*, 2000). Resistant plants respond quicker to pathogenic attack than susceptible plants, which imply the difference between life and death (Maleck *et al.*, 2000). A similar observation was reported for plant-insect interactions (Van der Westhuizen *et al.*, 1998a).

Genetic engineering techniques developed in the 1990's became a valid alternative for cross breeding. These provide methods to study the biochemical interaction between the aphid and its host, wheat (Perrin and Wigge, 2001). Specific genes involved in resistance of the wheat plant to the RWA can now be identified, cloned and possibly transformed into new wheat cultivars. Understanding the plant-insect interaction is not an easy task as a large number of genes are involved in resistance that have not yet been fully characterized and the pathways involved are poorly investigated. There are many varying interactions depending on the host and the insect meaning that each interaction must be studied in detail (Beetham *et al.*, 1999; Bertioli *et al.*, 2003). As wheat is a major food source and its production has major economic value, large sums of money are spent on developing long-lasting and multi-resistant wheat cultivars. A couple of

monogenic resistant wheat cultivars have been produced but new RWA biotypes have formed that overcame that resistance mechanism (Weiland *et al.*, 2008). Studies done on the specific wheat-RWA interaction revealed possible strategies that could be used pose for providing long lasting resistance (Metraux *et al.*, 1990).

Biochemical changes undergone in resistant plants are regulated at transcription level and one can determine which genes play key roles in deterring aphids by comparing susceptible wheat with resistant wheat lines. Through Suppression Subtractive Hybridization (SSH) and micro-array studies many genes have been identified that show an involvement in resistance (Botha *et al.*, 2006a). Some of the identified genes in this study were found to either be defence-related, involved in photosynthesis and even indirectly in cell structure and maintenance-related functions and most shared no homology to any known genes in the GenBank data base. These genes provide an overall picture of the biochemical pathways that are activated by RWA feeding and one can speculate what happens biochemically to provide resistance. The speculation is not that useful in itself until the individual genes are studied in depth. Knowledge gaps that need to be elucidated relate to their functional role, since simply knowing the gene name or whether it is up or down regulated, is not informative on their specific role in a biochemical pathway, metabolic function etc. One way to discover if the regulation changes are crucial or just a reactive effect is to prevent the changes. Gene expression can be silenced and if the changes are significant, specific phenotypic differences will be observable that will provide more information about the involvement of the gene. If a gene that is recognized as being involved in a resistance mechanism was silenced in a resistant cultivar and the plant's resistance decreased, it proves that the gene has functional importance in resistance. This kind of study is called functional proteomics.

The silencing technology termed virus induced gene silencing (VIGS) is used to transiently silence genes of interest and was recently optimized for the use in wheat (Scofield *et al.*, 2005). The technology makes use of the barley stripe mosaic virus (BSMV) vector for the transport of a gene fragment into the plant for post-transcriptional gene silencing (PTGS). Identification of suspect genes partaking in multiple resistance pathways with the use of this system is reliable, highly effective and easy to perform compared to other silencing systems (Cloutier *et al.*, 2007). Incomplete

silencing is achieved with this system, however this creates the opportunity to silence key enzymes in a biochemical pathway without any lethal effects to the plant. Thus the objective of this study was to silence genes that indicate significant regulation changes upon RWA feeding, in resistant cultivars to obtain a better understanding of the role these genes play in resistance.

When RWAs feed on wheat phloem, they inject virulence factors that cause leaf rolling and chlorotic streaking in susceptible plants (Fouché *et al.*, 1984). It is hypothesized that these virulence factors cause a change in metabolism and act upon photosynthesis in the area of infection. This results in energy relocation including carbohydrates from other sources to the created sink. The phloem flux created by the aphid will also include a strong supply of necessary essential amino acids and water for the benefit of the aphid. The chloroplast thioredoxin is affected in the process and the change in its redox state cause it to turn other enzymes off such as ATPase, Fructose-1,6-bisphosphatase (FBPase) and NADP malate dehydrogenase. These enzymes are involved in CO_2 assimilation and photoprotection and by turning them off have a major implication in energy production (Botha *et al.*, 2006b). It is hypothesized that photosynthetic changes play a front line role in defence upon recognition of the aphid in resistant wheat lines.

The study of Botha *et al.* (2006b) indicated that certain photosynthesis genes are involved in enabling the TugelaDN resistant wheat cultivar to cope with RWA feeding associated stress and it is hypothesized to be similar for Tugela *Dn2* and *Dn7*. These include transmembrane phosphoprotein 14 (TMP14), FBPase and a P700- chloroplast gene (P700), which are to be silenced in the resistant plants to diminish resistance and thereby increase susceptibility. Resistant wheat lines deter aphids in different ways such as antixenosis and antibiosis. As photosynthesis seems to play an important role it is of great interest to compare the changes undergone due to aphid feeding. The *Dn* resistance gene provides resistance to wheat plants against *Diuraphis noxia* Kurdjumov by means of antibiosis, *Dn2* by the means of tolerance and *Dn7* through antixenosis (Smith *et al.*, 1992; Anderson *et al.*, 2003).

The aim of the present study was (i) to optimise the VIGS for use in RWA-wheat interaction studies; (ii) to determine if photosynthesis regulation is strongly involved in

resistance, (iii) further if and how it differs between resistant cultivars. Then to elucidate through VIGS what the functional roles the regulation of TMP14, p700 and FBPase may have in the survival of the plant during RWA infestation. The following possibilities will be investigated.

1.) Silencing of TMP14, an electron acceptor for the Photosystem I (PSI) system, will result in decreased resistance as less electrons can be passed on to the Calvin cycle and for the production of Reactive oxygen species (ROS).
2.) FBPase plays a role in the Calvin cycle and the silencing thereof will reduce the carbon flux which is hypothesized to be important for the resistance of *Dn2* wheat lines.
3.) When p700 is silenced the PSI system will be affected in that electrons will not be excited and passed on to Ferredoxin (FD) for the activation of other proteins. A possibility is that when this electron flow is hampered electrons may follow an alternative route that might increase ROS production.

1.2. General plant defence

Because plants cannot run away from invading organisms that consume, infect and damage their tissue, plants must protect themselves in various ways (Hatcher, 1995). Plants did not evolve to have a central immune system to elicit a defence response. Rather, each plant cell posses both constitutive and inducible defence capacities (Cervone *et al.*, 1989). These mechanisms allow plants to survive and reproduce in the presence of herbivores, insects and other pathogens such as bacteria, viruses, fungi and nematodes (Jackson and Taylor, 1996).

Each attack is different and therefore the defence response is variable. A clear example is the response to pathogens that differ considerably from that against herbivores, due to pathogen's tendency to colonize and spread rather than to eat and walk away. The continual defence response is successfully implemented by the use of morphological, chemical and indirect adaptations (Marquis, 1990). Plants are thereby rarely susceptible to pathogenic attack especially to bacteria (Johal *et al.*, 1995).

As all animals need to reproduce, plants also tend to fight for their survival. Plants are primary producers in the food chain and are due to be eaten by herbivores such as insects which are next in line (Fritz and Price, 1988). Even the environment may sometimes pose a great threat to plants through stress conditions such as heat, cold, water stress, and mechanical and chemical stresses (Zhang *et al.*, 1998). Plants have general resistance against pathogenic organisms and stress conditions called basic incompatibility and specific resistance mechanisms called host incompatibility (Figure 1.1). Pathogenic organisms have to contend (basic compatibility) with these resistance mechanisms for successful colonization using biotrophic or necrotrophic strategies (Johal *et al.*, 1995). Biotrophs including rusts and powdery mildew are obligate pathogens that try to spread unnoticed and keep host cells alive but recognition by the plant causes cell death that contains the biotrophs (Figure 1.1). Their strategy is to evade perception with the help of compatibility factors that suppress perception, inhibit the cell death pathway and stimulate the production of host cell survival factors (Johal *et al.*, 1995). Necrotrophs including leaf spot and ear mold on the other hand live on dead cells, thus cell death does not prevent spreading. Necrotrophs produce phytotoxic

metabolites that circumvent plant defence by either interfering with its expression or nullifying its effect (Figure 1.1). Resistant plants are capable of counter attacking this by inhibiting the function of the toxins (Figure 1.1) (Glazebrook, 2005).

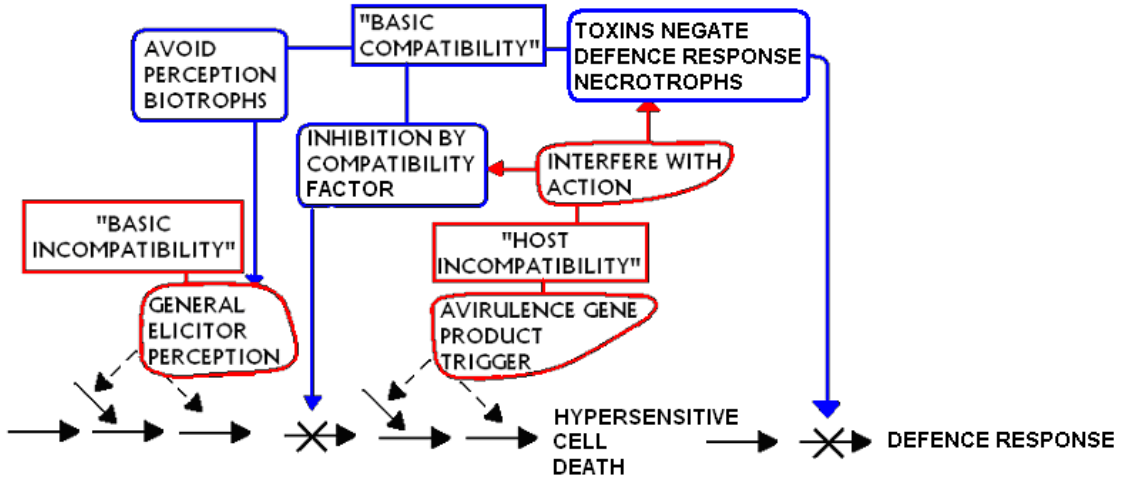

Figure 1.1: Cell death pathway. The pathways in plants that lead to HR and the defence response are indicated. The plant's defence strategies to overcome the pathogens attack is indicated in red and the pathogens offensive strategies are indicated in blue (Johal *et al.*, 1995).

1.2.1. Basic incompatibility

The passive defence of plants is a default resistance against threatening organisms that includes physical and chemical defences. The cell wall and cuticle of the plant forms the first barrier as physical defences that are highly effective against bacteria. The chemical defences include constitutive factors, proteins in seeds and high concentrations of phenolics and alkaloids that are more functional against penetrating fungi (Johal *et al.*, 1995). The best chance for bacteria to enter the plant cell is through wound sites, such as those caused by herbivorous insects (Barthlott and Neinhuis, 1997). As physical defence plant leaves and stems can also be covered with sharp spines or trichomes (Cooper and Owen-Smith, 1986; Bahlman *et al.*, 2003), release wax or have hairs (Tsuba *et al.*, 2002), silica, lignins (indigestible to animals) and contain poisons. They can even mimic other physical structures in nature to ward off animals. In this act to prevent damage caused by herbivores or at least minimizing the damage, the grazer can be deterred, injured or even killed (Williams and Gilbert, 1981).

The active defence of plants respond to invasion by recognizing structural or chemical features of the pathogen or through the general stress and damage associated with specific pathogenesis (Johal *et al.*, 1995). The recognition is mediated by pathogen-associated molecular patterns (PAMPS) such as flagellin and lipopolysaccharides (LPS) (Gomez-Gomez and Boller, 2002). The general response elicitors are recognized by membrane-localized receptors, which is independent of pathogen genotype. Perception results in signal transduction leading to the defence response where oxygen species (oxidative burst), calcium channels, calmodulin, G-protein, protein kinases and phosphatases are involved (Wojtaszek, 1997). H_2O_2, which is formed during the oxidative burst, stimulates cell wall reinforcement by cross-linking and deposition of polysaccharides, proteins, glycoproteins and insoluble phenolics (Wojtaszek, 1997). Together with cell wall reinforcement, cell wall apposition (papilla) made of cellulose by activated β-1-3-Glucan synthase are the first two physical responses before integrity of host cell is threatened (Nakane *et al.*, 2003). If cell walls are breached reactive oxygen species, phenol-oxidizing enzymes, simple salts and heavy metals of pathogens nonspecifically stimulate a HR as biotic and abiotic (general) elicitors. In the pathogen-plant interaction, lytic enzymes from the plant degrade cell walls of fungi and bacteria to components (oligosaccharides) that also act as elicitors in amplifying plant defence responses (Johal *et al.*, 1995).

1.2.2. Host incompatibility

The innate immune response of a plant is very specific and can recognize and respond to multiple biotrophs. This is due to receptors in the plasma membrane, cytosol and nucleus of the plant that is sensitive to specific molecules that indicate the presence of the pathogen (also known as pathogen specific elicitors) (De Wit, 1997; Nurnberger and Scheel, 2001). These resistant plants recognize a specific pathogenic gene product, and the activation of the receptor rapidly generate an internal signal triggering HR into the early stages of the infection process and further down line defence responses (Dangl and Jones, 2001). The HR has been observed as the most common plant resistance response to viruses, bacteria, fungi, nematodes and even insects (De Wit, 1995; Crute and Pink, 1996; Van der Westhuizen *et al.*, 1998b). This plant receptor-pathogen/insect elicitor interaction can be brought forth by non-race-specific/non biotype specific

elicitors which are pathogen/insect or plant cell wall fragments released during the infection/infestation process. Race–specific pathogen recognition are brought forth by molecules that are encoded by genes in the pathogen. This interaction was first proposed by Flor in the gene–for–gene model for the genetic interaction (Flor, 1956; De Wit, 1997).

The defence strategy against biotrophs was thoroughly studied over the years and new concepts are still being added. The plant-biotroph interactions are governed by interactions between the plant disease resistance (*R*) locus and the pathogen avirulence (*avr*) loci (Dangl and Jones, 2001). Disease resistance genes encode receptor proteins that have either or both recognition and signal transduction capabilities to combat the pathogen (Johal *et al.*, 1995). The *R* gene products recognize specific avirulence gene products or race-specific elicitors found on the pathogen. This triggers the chain of signal-transduction events that initiate HR and the activation of a defence mechanism and the arrest of pathogen growth. Plant resistance occurs when host and pathogen possess a matching pair of *R* gene and *Avr* gene products (Boyes *et al.*, 1998). The pathogen is rendered incompatible and no disease will occur where necrotic lesions will be mostly evidence of successful resistance (Figure 1.2). When the pathogen possesses only virulence gene products, no recognition will take place and it will be free to cause a disease rendering the pathogen compatible (Salmeron *et al.*, 1996). The avirulence genes normally do not play any meaningful role in plant-pathogen systems and the gene can thus undergo mutation and the protein structure be changed without directly affecting the pathogen. The pathogen sometime does exactly this to avoid recognition by the host in order to be compatible with the plant. The plant then has to recruit another *R* gene to acquire resistance. This demand is called the arms race and the cycle gene-for-gene interaction (Flor, 1956). These *R* and *Avr* genes behave as dominant genes for their products has a direct influence on the interaction (Johal *et al.*, 1995) and the bigger the variety, the broader the pathogen range detected by the plant (Barthlott and Neinhuis, 1997).

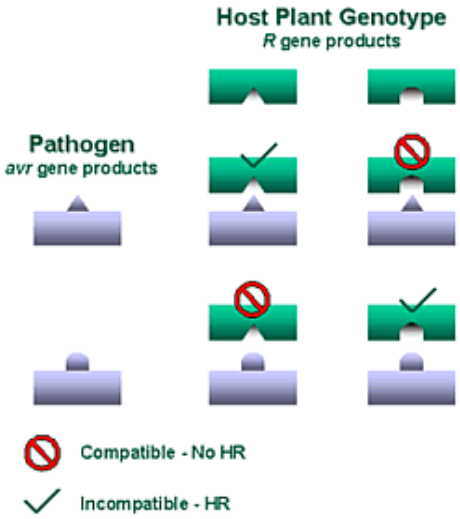

Figure 1.2: Specific host pathogen interaction. The host's *R* gene products interact with the *Avr* gene products of pathogens. Plant resistance occurs when host and pathogen possess a matching pair of *R* gene and Avr gene products, respectively (De Wit, 1995).

1.2.3. Guard Hypothesis

Experimental data supports that this R-and Avr protein combination that activates resistance are rare (Deslandes *et al.*, 2003). Recognition of biotic interaction is proposed to rather function on an indirect manner by 'the guard hypothesis' (Van der Biezen and Jones, 1998; Dangl and Jones, 2001). The hypothesis suggests that the R proteins do not have direct interaction with the Avr proteins but rather screen cellular proteins that are affected by the Avr proteins (Figure 1.3). The R proteins thus fulfill a surveillance role in cellular homeostasis. It is likely that R proteins are large protein complexes that include the *R* gene product. Avirulence proteins are presumed to act as virulence factors that specifically target one or more host proteins in their quest to create a favorable environment (McDowell and Woffenden, 2003). The perturbation of these cellular factors may or may not be required for virulence, but will lead to R protein activation (Van der Biezen and Jones, 1998; Dangl and Jones, 2001).

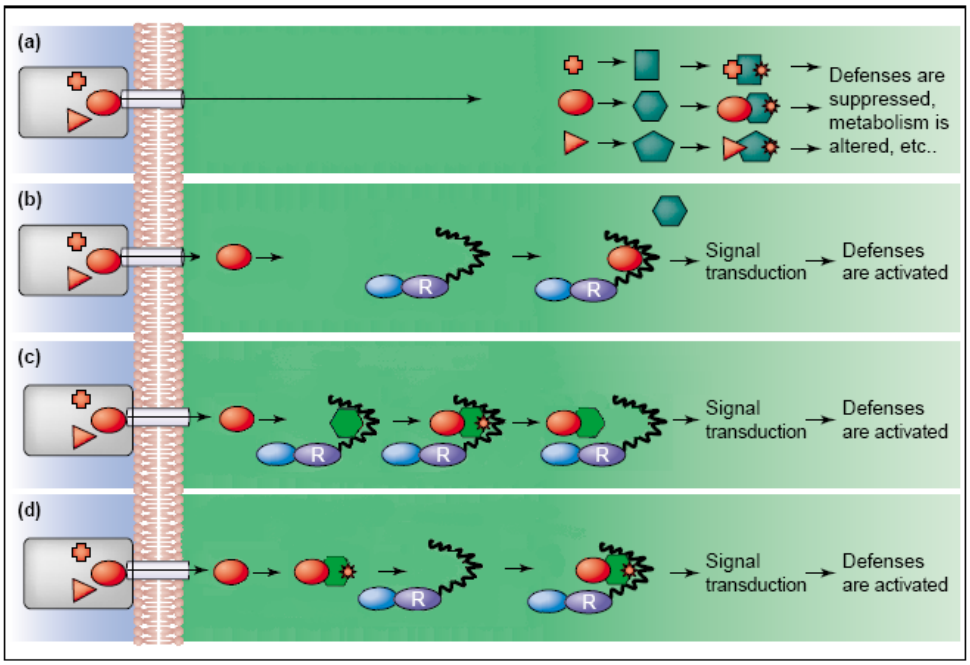

Figure 1.3: Interactions between pathogen Avr proteins and plant R proteins. The hypothetical pathogen (grey) that is attached to a plant cell excretes a suite of virulence proteins (red) (McDowell and Woffenden, 2003). These proteins are translocated into the plant via Type III secretion in the case of bacteria (Nimchuk *et al.*, 2001) and the stylet in the case of aphids (Fouché *et al.*, 1984). They target host proteins (green) that control defence responses, metabolism or other plant processes that affect pathogen virulence. (a) In this case, the plant cell does not express an R protein with a nucleotide-binding site leucine-rich repeat (NBS-LRR protein) that is capable of recognizing any virulence protein. Thus, the plant cannot detect the pathogen efficiently and the defences are suppressed. This generally results in infection and disease caused from the collective action of the virulence proteins. (b) Here the classic receptor–elicitor hypothesis is shown, in which an R protein directly binds a virulence protein. This recognition event activates a complex signal transduction network, which in turn triggers defence responses. (c) The guard hypothesis is illustrated here, in which an R protein (guard) is in complex with the host protein (guardee, red star) and when the attacking' virulence protein change the conformation of the host protein the R protein dissociates and activate the defence response. (d) The guard hypothesis is illustrated here where the 'attacking' virulence protein cause the conformation change in the host protein and the binding (detection) of the R protein (guard). The complex then activates the defence (Dangl and Jones, 2001; McDowell and Woffenden, 2003).

The R proteins will constitutively bind to their host protein and then dissociate after modification to activate a response. Another possible explanation is the formation of a complex including the cellular target, interacting R protein and virulence protein that leads to the activation of a response (Van der Biezen and Jones, 1998; Dangl and Jones, 2001).

A number of single dominant *R* genes have been mapped, and molecular markers linked to these loci have been identified (Venter and Botha, 2000) and reviewed (Yencho *et al.*, 2000; Klinger *et al.*, 2001; Liu *et al.*, 2001; Jena *et al.*, 2002; Liu *et al.*, 2002; Tan *et al.*, 2004). The *Mi*-1 from tomato is the first *R* gene that has been cloned and grants resistance to potato aphid *(Macrosiphum euphoriae)* (Kaloshian *et al.*, 1995), whitefly (*Bemisia tabaci*) (Nombela *et al.*, 2003), and root-knot nematodes (*Meloidogyne* spp.) (Milligan *et al.*, 1998). *Mi-1* belongs to the NBS–LRR class of *R* genes (Rossi *et al.*, 1998) and encodes a cytoplasmic protein of 1, 257 amino acids with putative coiled coil (CC), NBS and LRR domains (Milligan *et al.*, 1998). Resistance mediated by *Mi-1* requires the *Rme1* gene and their gene products may interact in a manner similar to that described for the "guard hypotheses". Rme1 may function as the target of nematode and insect effectors and be guarded by Mi-1 detecting possible conformation change in Rme1. The Mi-1 protein may then activate the defence by "sounding the bell" (Kaloshian, 2004).

The 'Jack-knife' model was proposed in which two different protein complexes are present at the plasma membrane that is involved in elicitor recognition, this is a model of the negative regulation of NBS–LRR proteins by *trans* partners (Moffett *et al.*, 2002; Belkhadir *et al.*, 2004). After infection, the pathogen effectors associate with the target protein and modify an adaptor protein on it. A proportion of the modified complex then associates with the NBS–LRR protein called the trigger complex. It undergoes conformation changes whereby it is activated and relocated to other cellular compartments with other downstream signalling molecules (Belkhadir *et al.*, 2004). The amino-terminus of the NBS-LRR trigger protein is required for protein–protein interactions with an adaptor protein, whereas the NBS domain is responsible for adenosine triphosphate (ATP) hydrolysis and release of the signal. The amino-terminal domain of the LRR appears to modulate activation, whereas specific residues that are

located in the carboxy-terminal domain of the LRR appear to be responsible for elicitor recognition, and hence define this region of the LRR as an interaction platform for upstream activators (Inohara *et al.*, 2002; Chen *et al.*, 2004; Tanabe *et al.*, 2004).

R proteins might rather actively and continually monitor key physiological processes that are targeted by pathogens than act as passive security guards that idly wait for specific signals from an invader (McDowell and Woffenden, 2003).

1.2.4. Hypersensitive response

Hypersensitive response is characterized by the localized cell and tissue death that form local lesions by programmed cell death (PCD). It also leads to the induction of intense metabolic alterations in the cells surrounding the necrotic resions that is the cause of localized acquired resistance (LAR) (Allen *et al.*, 1999). It is triggered with the combined action of nitrous oxide (NO) and ROS with the aim of "die and let live" (Baker *et al.*, 1993). The production and accumulation of antibiotic compounds around these lesions, called phytoalexins has strong antimicrobial activity. Phytoalexins accompany HR for the confinement of biotrophic pathogens preventing further infection (Hahlbrock and Scheel, 1989). Cell walls are also reinforced around the LAR by lignification, callose, and silicon deposition, suberization and hydroxyproline-rich protein production (Barker *et al.*, 1989; Lozovaya *et al.*, 1998).

HR can also cause salicylic acid (SA) and benzoic acid (BA) production that travels from the infection site to nearby tissues as a secondary defence mechanism (Mohase and Van der Westhuizen, 2002). SA and BA are associated with triggering the expression of a set of defence gene families (see 2.1.7) encoding certain pathogenesis related (PR) proteins. PR proteins include lytic enzymes i.e. chitinases, glucanases that degrade fungal and bacterial cell walls and lectin-like, thionin-like and proteinase-inhibitor-like antifungal proteins (Van Wees *et al.*, 2000). SA and BA can also trigger systemic expression of PR proteins in the other parts of the plant. This trigger leads to a long-lasting and broad-spectrum resistance response called the systemic acquired resistance (SAR) that is non-specific (Metraux *et al.*, 1990). This type of defence is

much more energy efficient compared to constitutive defences and allows the essential resources to be available for growth and reproduction (Karban and Baldwin, 1997).

1.2.5. Signalling

After either the resistance gene products detect the pathogen or non-specific recognition occurs (Suzuki *et al.*, 2004) the membrane potential and ion permeability of the plasma membrane changes. The response is quantitatively appropriate, correctly timed and highly coordinated with other plant cell activities (Blumwald *et al.*, 1998)**.** A proposed signal-transduction pathway comprises G proteins to relay the initial elicitor-receptor recognition through a series of pathways in the membrane. In the pathway both the cytosolic Ca^{2+} concentrations and protein kinases/phosphatases change affecting the activity of key enzymes (Figure 1.4) (Blumwald *et al.*, 1998). Plasma membrane H^{+}-ATPase and Ca^{2+} channels are opened for the rapid influxes of H^{+} and Ca^{+} and effluxes of K^{+} and Cl^{-}. The ion fluxes are a prerequisite for MAP kinase activation and reactive oxygen intermediate formation such as O^{2-}, H_2O_2 and OH^{-} via the action of plasma membrane-associated NAD(P)H oxidases and/or apoplastic-localized peroxidases (Orlandi *et al.*, 1992). This triggers a network of signal transduction events. For example, H_2O_2 production in the oxidative burst drives cross-linking in the cell wall (reinforcement of cell wall) and activates protein kinase and phosphatase proteins (Somssich and Hahlbrock, 1998). Metabolic enzymes are activated and deactivated in secondary metabolism pathways that play an important role in the defence. The secondary metabolism is involved in antimicrobial activity by phytoalexin production, the production of endogenous signalling molecules such as salicylic acid, ethylene, lipid-derived metabolites and jasmonates or the modification of the cell wall. An invading pathogen has to bypass many of these signalling components to cause disease successfully (Mysore and Ryu, 2004).

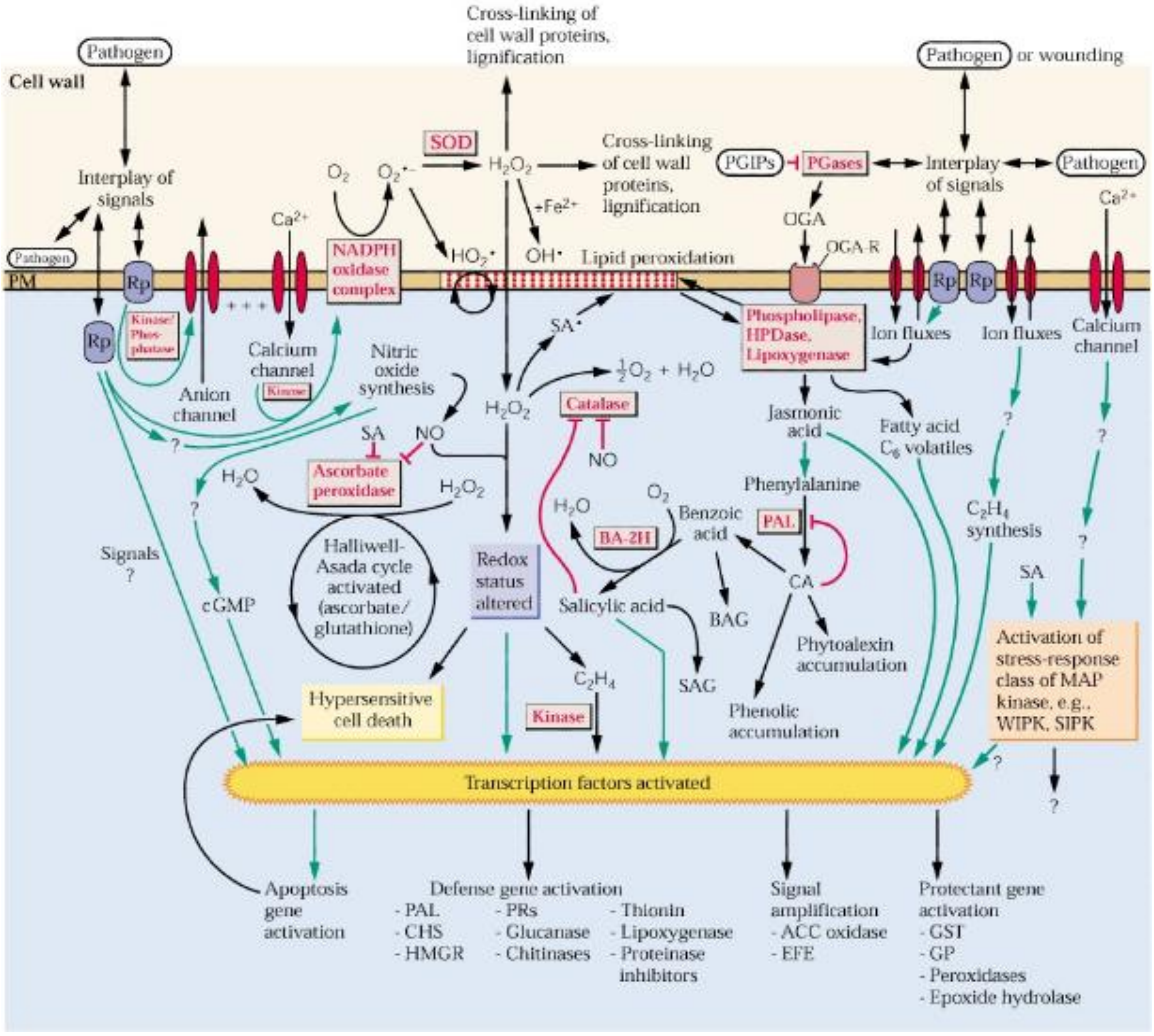

Figure 1.4: A hypothetical model of the signal transduction events. The specific model is for the signal transduction events in plant–pathogen interactions (Blumwald *et al.*, 1998). Following pathogen recognition events by plasma membrane receptor, signal transduction leads to an increase in cytosolic Ca^{2+} concentrations, and effluxes of K^+ and Cl^-. Ca^{2+} activates specific mitogen-activated protein kinases (MAPK). This also induces NADPH oxidase activity with the consequent production of active oxygen species (O_2^-) and H_2O_2. The increase in cytosolic Ca^{2+} concentrations will also activate a protein kinase C (PKC) and a Ca^{2+}/CaM-dependent protein kinase. These kinases can rephosphorylate the H^+-ATPase, returning the enzyme activity to control levels and activate transcription factors (Blumwald *et al.*, 1998). The ROS produced lead to lignification, lipid peroxidation and alters the redox status that in turn causes HR and activates transcription factors. The degraded lipids activate Jasmonic acid that also activates transcription factors (Somssich and Hahlbrock, 1998).

Other general elicitors exist that has the potential to amplify the plants defence response. Some general elicitors originate from the degradation of either the pathogen or the plant's exterior protection (Okada *et al.*, 2002). Pathogens may produce enzymes that degrade the cell wall polymers of the plant (Cervone *et al.*, 1989) such as endopolygalacturonases (EPG). EPG is a cell-wall-degrading enzyme that cleaves the linkages between D-galacturonic acid residues in non-methylated homogalacturonan, a major component of pectin (De Lorenzo *et al.*, 2001). The oligogalacturonides formed in this cleavage acts as endogenous elicitors and stimulate plant defence responses. Plant chitinases and ß-1, 3-glucanases act in the same way on pathogen cell walls to give oligochitins and oligoglucans (Kolattukudy, 1985; Fritig *et al.*, 1998). It is found that lipid based signalling molecules play a role, but not much research has been done on it (Li *et al.*, 2002). Signalling molecules that originate from neighbouring plants include ethylene (Hoffman *et al.*, 1999), methyl jasmonate [MeJA] (Seo *et al.*, 2001) and methyl salicylate [MeSA] (Shulaev *et al.*, 1997).

1.2.6. Resistance genes

Plants respond to pathogen attack with a defence response through a variety of signalling pathways whereby proteins take part in crucial functions (Somssich and Hahlbrock, 1998). Some proteins function to serve as receptors (He *et al.*, 1996; Salmeron *et al.*, 1996) for the perception of the pathogens and others that play a role in the signalling. The outcome is to activate proteins with antimicrobial ability (Fritig *et al.*, 1998). Many other proteins are also produced that serve minor functions, but each protein involved require a gene. For the plant's defence reaction to be functional hundreds of these disease-resistance genes exists. Constitutively expressed and inducible genes that are involved in resistance, have been identified and cloned, but efforts to discover how they work are continuing (Johal *et al.*, 1995).

In early agricultural times plants were cross-bred to transfer these genes to crop plants to obtain resistant traits (McIntosh *et al.*, 1995). Cross-breeding is one of the earliest methods to help plants survive the assaults of insect infestation and diseases. The hope was to pass on the 'resistance' genes from the resistant plant to the plant of interest to acquire nominal genetic permanency thus having obvious advantages over the use of

chemical pesticides (Murray *et al.*, 2005). Cross-breeding works only between closely related plants species and takes decades to get the appropriate resistance (McIntosh *et al.*, 1995). The major disadvantage is the limiting time of effectiveness due to selection pressure that is placed on pest populations to develop means of overcoming the resistance (Toxopeus, 1959; Enjalbert *et al.*, 2005). Nowadays transgenic plants have been produced through genetic manipulation (Mysore and Ryu, 2004). Constitutively expressed genes expressing receptors and signal transduction proteins have not received much attention. Alternatively, genes encoding antimicrobial proteins are being thoroughly studied and when introduced into plants have improved the resistance (Fritig *et al.*, 1998).

Arabidopsis thaliana, one of the best studied plants, serves as a source whereby *R*-genes can be easily identified through genetic analysis. Most major kinds of defence responses present in *Arabidopsis* are present in other plants as well. A relatively large number of defence-related genes were identified from *Arabidopsis* and other plants and stored in a sophisticated web-accessible plant-microbe interaction database (PMIDB; http://0-genetics.mgh.harvard.edu.innopac.up.ac.za/ausubelweb/nsf2010/NSF2010.html). It was created to provide common storage location for experimental data of plant-microbe interactions and is stored in standardized format (Dong, 2001). The development of genomic technologies has started the development of a much more detailed model of how the combination of defence-related genes functions and interacts to combat pathogen attack (Ji *et al.*, 1997). According to the combination of structural motifs, these receptor proteins fall into five major classes (**Error! Reference source not found.** and Figure 1.5) (Martin *et al.*, 2003).

R-proteins are composed of a limited number of common motifs including leucine-rich repeats (LRR's), a nucleotide-binding site (NBS) and a serine/threonine kinase domain. Other proteins involved in plant defence also contain some the mentioned motifs (Fritig *et al.*, 1998). The increasing list of *R*-genes shows that the majority belongs to the NBS-LLR class that is characterized by a nucleotide-binding site near the N-terminus and a leucine-rich repeat region near the C-terminus (Martin *et al.*, 2003). The *Arabidopsis* genome for example contains approximately 200 genes encoding NBS-LRR related motifs and the rice genome are even more prevalent containing 600 NBS-

LRR encoding genes (Meyers *et al.*, 2003). These sequences are diverse and common in plants indicating that they are ancient (Tao *et al.*, 2000).

1.2.7. Pathogenesis related proteins

Plant products related to defence include pathogenesis-related (PR) proteins, which is a major group of proteins with antimicrobial activity (Fritig *et al.*, 1998), accumulate in the apoplast (Bowles, 1990; Van der Westhuizen and Pretorius, 1995; Van der Westhuizen *et al.*, 1998a; Van der Westhuizen *et al.*, 1998b). PR proteins are divided in 17 protein families and are classified mainly according to sequence similarities (Van Loon *et al.*, 1994). The group include lytic enzymes, chitinases, glucanases (degrade fungal and bacterial cell walls) and lectinlike, thioninlike and proteinase-inhibitor-like antifungal proteins (Van Wees *et al.*, 2000). Some PR proteins have a damaging action on the structures of the parasite, either to the plasma membrane (PR-1, PR-5) or to fungal cell walls by β-1.3 glucanases (PR-2) and chitinases (PR-3, PR-4, PR-8 and PR-11). In this process oligochitins and oligogulans can be generated which act as exogenous elicitors in the further perception that amplify the plant defence response (Bergey *et al.*, 1996; Broekaert *et al.*, 1997). Small antimicrobial peptides that are membrane permeabilizers include thionins (PR-13), plant defensins (PR-12) and lipid transfer proteins (PR-14) (Terras *et al.*, 1992; Molina *et al.*, 1993; Epple *et al.*, 1995; García-Olmedo *et al.*, 1995; Broekaert *et al.*, 1997). PR-15, PR-16 and PR-17 are PR-like proteins that are thought to be involved in the signal transduction pathway that regulates HR (Zhou *et al.*, 1998; Okushima *et al.*, 2000; Christensen *et al.*, 2002; Park *et al.*, 2004).

Error! Reference source not found.**:** Five major classes of receptor proteins and the category domain of each (Martin *et al.*, 2003). The domains include Leucine-rich repeats (LRR), Nucleotide binding sites (NBS), Coiled-coil (CC), Trans-membrane domain (TM) and receptor-like protein kinases (RLK's)

Class	Serine/threonine kinase catalytic region	Myristylated motifs in the N-terminus	LRR	Extracellular LRR region with TM domain	NBS	CC	RLK's
1	✓	✓					
2				✓			
3	✓			✓			✓
4			✓		✓	✓	
5		✓	✓		✓		

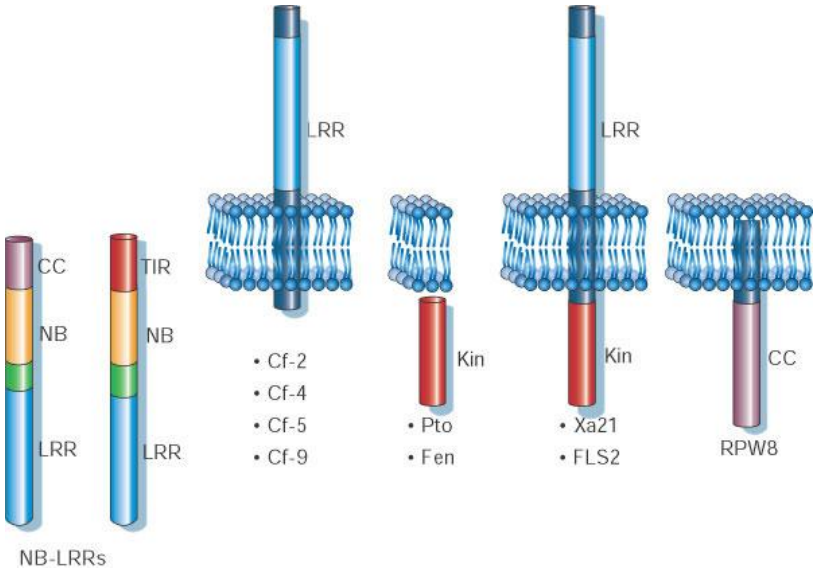

Figure 1.5: Illustration of the structure of the five main classes of plant disease resistance proteins. The largest class of R proteins, the NB-LRR class, are presumably cytoplasmic (although they could be membrane associated) and carry distinct N-terminal domains. The LRR proteins domains of Xa21 and Cf-X proteins are extracellular and carry transmembrane domains. The *Pto* gene however encodes a cytoplasmic Ser/Thr kinase, but may be membrane associated through its N-terminal myristoylation site. The RPW8 proteins are cytoplasmic, but carry a putative signal anchor at the N terminus (Dangl and Jones, 2001).

1.3. The Russian wheat aphid

1.3.1. Morphology

The Russian wheat aphid , (*Diuraphis noxia* Kurdjumov) (Homoptera: Aphididae), is a devastating pest of wheat and barley and causes a major loss in capital in many wheat-producing areas of the world (Smith *et al.*, 1991). The aphid is indigenous to Southern Russia but spread to many other areas in the world and was first recognized as a serious pest of wheat in South Africa in 1978 (Walters *et al.*, 1980). It also spread to the North American continent in the 1980's causing havoc to farmers (Gilchrist *et al.*, 1984; Webster *et al.*, 1987; Morrison, 1988).

Aphids are the largest group of phloem-feeding insects, but he RWA is only one of many aphid genera as can be seen in Figure 1.6 (Miller *et al.*, 1994). The RWA wingless form is about 1.4 to 2.3 mm long and is light green to grey-green. The winged forms of RWA however, are 1.5 – 2.0 mm long and have a pale green abdomen (Walters *et al.*, 1980; Robinson, 1992; Bryce, 1994; Karren, 2003). Several characteristics are important for the identification of the RWA. The shape of the insect is distinctive. The RWA is more elongate (spindle-shaped) than other aphids, which are teardrop-shaped. They have small antennae, a secondary tail projection above regular cauda (tail), covered in a powdery, mealy wax and their cornicles are very short and not obvious (Figure 1.7). These characteristics separate the RWA from the other small grain aphids (Walters *et al.*, 1980; Hein *et al.*, 1998).

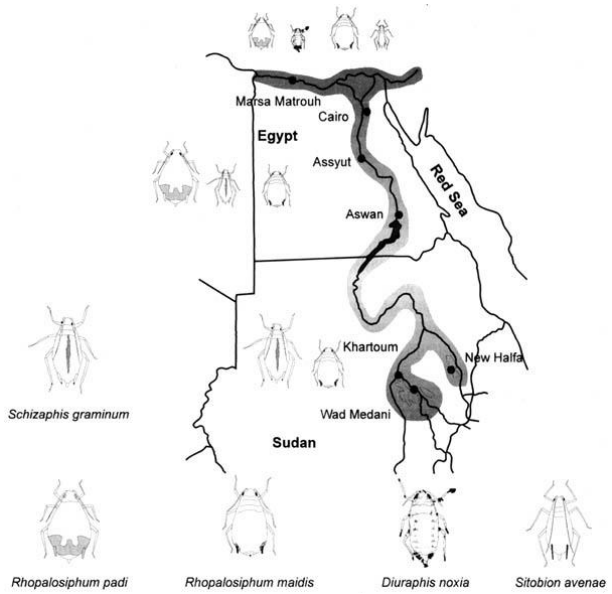

Figure 1.6: The physiological differences of the aphid apecies and the distribution of their populations (Miller *et al.*, 1994).

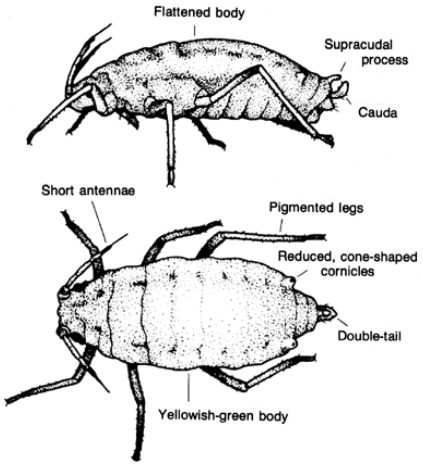

Figure 1.7: Russian wheat aphid identification characteristics (Hein *et al.*, 1998).

1.3.2. Phenotypic symptoms during RWA infestation

The best way to identify the presence of the *D. noxia* on wheat is by its characteristic damage to the wheat. The aphids preferentially colonize the axils or the insides of curled up leaves of the newest growth of the wheat plants, where they are partially protected from aphicides, predators and insecticides. The most visible symptoms are the longitudinal rolling of the colonized leaf and the white streaking on the leaves in warm weather and purple streaking in the colder weather (Walters *et al.*, 1980). Other

symptoms include the reduction of leaf size and wheat biomass, the disruption of the osmoregulatory processes, oxidative stress, and necrotic lesions on the leaves (Van der Westhuizen *et al.*, 1998a; Ni and Quisenberry, 2006).

The young leaves of heavy invested plants may often lay parallel to the ground and the rest of the plant exhibit a flattened appearance. Damage in the later growth stages causes the flag leaf to curl and turn white. This prevents the head from completely emerging and does not allow proper grain maturation (Karren, 2003). Aphids can move from originally infested plants to neighbouring plants allowing the infestation to exhibit a patchy distribution in the field (Walters *et al.*, 1980). Extensive damage is caused to the membranes and chloroplasts by the injection of phytotoxins into the plants by the feeding aphids (Van der Westhuizen and Botha, 1993; Miles, 1999). The toxins prevent the production of chlorophyll, cause the cells to empty and eventually the leaves edges to curl inward (Fouché *et al.*, 1984). The damage is less on resistant wheat cultivars and certain wild grass species (Hewitt *et al.*, 1984; Kindler *et al.*, 1992). Chlorophyll deficiency due to infestation reduces yields by up to 50% in susceptible varieties (Fouché *et al.*, 1984; Burd and Burton, 1992). Infestation also alters the expression of protein patterns (Van der Westhuizen and Botha, 1993) and nutritionally enhances its phloem diet (Telang *et al.*, 1999).

1.3.3. Feeding

RWA uses a variety of chemical and physical stimuli to recognize a suitable host. Initially the host is recognized with sensory neurons on the antennae. Once the aphids land on the wheat plant it searches for an appropriate feeding site by a surface scan with receptors on the proboscis to detect a vein where aphids prefer to feed (Hewitt *et al.*, 1984; Dixon, 1998). Recognition is confirmed with a drop of saliva on the cuticle surface that dissolves the cuticle and dissolved material is sensed by a chemoreceptor on the labium tip. When the plant is recognized as a suitable host penetration commences (Srivastava, 1987). The aphids mainly probe between the cells (intracellular) until it reaches the phloem so that its stylet can penetrate (Pollard, 1973; Fouché *et al.*, 1984). Structural traits of leaves may physically hinder insect herbivores from feeding, including trichome density that are directly involved in RWA resistance

in some cultivars (Fouché *et al.*, 1984; Bahlman *et al.*, 2003). The RWA is mainly found on the newest growth and axils of leaves. Feeding begins at the base of the leaves near the top of the plant. During cooler and rainy conditions the aphids move from the leaves to a more protected area near the crown of the plant and as weather become favourable, the aphids move back up to the leaves (Von Wechmar, 1984; Parker *et al.*, 2001).

Cutin and silica in epidermal cell wall and cuticle, provides additional barriers to penetration (Brett and Waldon, 1990). A needle-like stylet is used to probe into the plant while sheath material is secreted from the salivary gland to form a stylet sheath. This provides rigidity and directional control for the flexible stylets that functions as a "straw" to suck up the phloem (Dixon, 1998). The phloem flow with a pressure of 15-30 atmospheres, sufficient to drive phloem sap up the food canal of the stylets, and is controlled by a piston valve. Aphids are also able to pump phloem sap into their alimentary canal under negative ambient substrate pressures (Dixon, 1998).

The diet of an aphid usually consists solely of the phloem sap of the host's leaves (Telang *et al.*, 1999). The phloem sap contains carbohydrates, amino acids and water but is nutritionally imbalanced. The phloem sap of vascular plants is low in concentrations of nitrogenous compounds, particularly certain essential amino acids (Dadd, 1985). Aphids are highly adapted to survive on phloem sap and accomplish this by manipulating the plants metabolism to suit its needs.

The deficiency in the diet for the aphid is compensated by the biosynthetic contribution of an endosymbiotic bacterium. *Buchnera aphidicola* found in the gut of aphids overproduce the limiting amino acids that benefit the host (Baumann *et al.*, 1997; Baumann *et al.*, 1998; Douglas, 1998). It was first considered that the ingested phloem to have the same composition as the phloem sap of the intact plant (Fisher and Frame, 1984). However Telang *et al.*, (1999) found that the damage inflicted by *D. noxia* on its host causes an increase in the concentration of essential amino acids in ingested sap. Thus, the function of *Buchnera* in *D. noxia* is reduced and might undergo reduced selection for production of these amino acids in the presence of an enriched diet. This may explain why *Buchnera* contain less copies of the plasmid borne genes tryprophan

and leucine when living in *D.noxia* compared to *Shizaphis graminum* (i.e. 1.8 and 0.9 vs. 23.5 and 14.5 of tryptophan and leucine respectively) (Telang *et al.*, 1999).

1.3.4. The Life cycle

The RWA occur mostly in the wingless (apterous) form but the life cycle of the RWA also includes the winged (alates), sexual, and asexual forms (Figure 1.8). When females of most aphid species feed on cereals they reproduce by giving rise to nymphs via parthenogenesis (without being fertilized) rather than giving rise to eggs (Walters *et al.*, 1980; Dürr, 1983; Robinson, 1992). Volunteer grain, including many species of grasses that act as reservoir hosts, serve as a food source during the interval between grain harvest and the emergence of seed crops (Karren, 2003). Newly planted wheat fields are dominantly colonized by apterae from nearby off-season hosts (Hewitt *et al.*, 1984). In the crop season, all Russian wheat aphids are females that do not lay eggs. The females give birth to live nymphs at a rate of four to five per day for up to four weeks and can mature in as little as 7-10 days (Karren, 2003). In South Africa only female RWA's occur, thus there is no genetic diversity in the South African population (Prinsloo *et al.*, 1997).

Because of the high reproductive ability, large infestations can spread rapidly (Karren, 2003). Overcrowding, weather conditions and mature plant growth may stimulate the production of winged forms to enable them to move to new habitats. When another suitable host is found, the aphid begins feeding and reproducing and prefer to live in leaf whorls and tightly rolled leaves (Walters *et al.*, 1980; Karren, 2003).

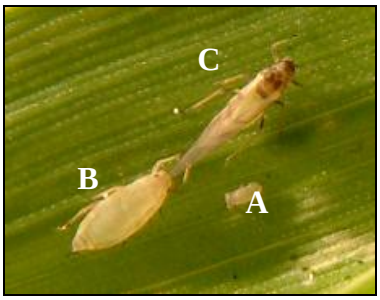

Figure 1.8: Maturity stages of *Diuraphis noxia*. The female stages include the nymph (A), an adult (B) and a winged adult (C) (Photograph taken by Leon van Eck).

1.3.5. Pest management

Cultural practices, biological control and chemical control can be used to restrict the damage done by the Russian wheat aphid. Cultural control includes choosing delayed planting dates, the control of volunteer plants and planting non-host plants that might even yield better economic results. The RWA does not have any important enemies that are adapted to access the aphids in their protected habitats, but ladybirds, wasp parasites and fly species (Hayes, 1998) can be used in restricting the numbers. Chemical control management was at first limited due to the registered insecticides for the control of aphids on wheat that could not reach the aphids in their protected habitat (rolled leaves). Only when the standard dosage rates of a contact insecticide such as parathion were used in combination with a systemic insecticide (Elsidaig and Zwer, 1993) acceptable results were obtained and thereby became a common practice (Walters *et al.*, 1980). Applying insecticides is an effective way of controlling these pests (Peairs, 1990; Hill *et al.*, 1993), but it is a costly and environmentally dangerous practice (Robinson, 1992).

1.3.6. Biotypes

Biotypes are intraspecific classifications based on biological rather than morphological characteristics (Shufran *et al.*, 2007). *D. noxia* collected around the world show biotypic differences (Smith *et al.*, 1992). The biotypic status of *D. noxia* is determined by the phenotypic response of the plant as a result of the aphid's feeding. A *D. noxia* biotype population is independent of geographic location that is able to injure a cultivated plant containing a specific gene(s) which was previously resistant to known aphid populations (Basky, 2003; Smith, 2005).

The original biotype (Biotype A or 1) of the Russian wheat aphid was first found in eastern Colorado and adjacent areas in 1987 (Haley *et al.*, 2004; Burd *et al.*, 2006). The first *D. noxia*-resistant cultivar released in the USA was Halt derived from PI 372129 and contained a single dominant gene *Dn4*, in 1994 (Quick *et al.*, 1996). Biotype development due to selection pressure jeopardizes the durability of plant resistance, which has been the best management of the Russian wheat aphid (Smith *et al.*, 2004;

Burd *et al.*, 2006). *D. noxia* infestations of Prairie Red wheat, a cultivar that also expresses *Dn*4 resistance, were reported in south-eastern Colorado in June 2003 (Haley *et al.*, 2004). This resistance-breaking strain of *D. noxia* has since been designated biotype B or 2 (Burd *et al.*, 2006). Many other discoveries have been made, but although molecular genetic variation within aphid biotypes has been well documented, little are known about phenotypic variation between biotypes (Shufran *et al.*, 2007).

Table 1.2: Russian wheat aphid biotypes. Fourteen natu und worldwide, but not all are yet established and published. Their old name and new name have been provided and the cultivars which are resistant or susceptible to the biotype.

Country		Virulent to this cultivars	Resistant cultivars/ genes	References
USA-Colorado	RWA1		*Dn4*	
USA-Colorado	RWA2	*Dn4*, *Dny*, *Dnx*	02 Altus 162, *Dn7* (94M370)	Haley *et al.*, 2004; Porter *et al.*, 2005; Jyoti *et al.*, 2006
USA-Texas	RWA3	*Dn1*, *Dn2*, *Dn4*, *Dn5*, *Dn6*		Haley *et al.*, 2004
USA-Wyoming	RWA4			
USA-Texas	RWA5	*Dn1*, *Dn2*, *Dn4*, *Dn5*, *Dn6*		Haley *et al.*, 2004
USA-Colorado	RWA6			
USA- Baca county	RWA7	Custer, Yuma, Carson, TAM107	*Dn7*, 'STARS 02RWA2414-11', 'CO03765','CI2401'	Weiland *et al.*, 2008
USA-Montezuma County	RWA8	Custer, Yuma, Carson, TAM107	*Dn7*, 'STARS 02RWA2414-11', 'CO03765','CI2401'	Weiland *et al.*, 2008
USA-Nebraska		*Dn1*, *Dn2*, *Dn4*, *Dn5*, *Dn6*		Haley *et al.*, 2004
SA		CVS MV Magdalena, MV Magvas and MV 17		Du Toit, 1989a; Basky, 2003
Hungary		Halt, Dn (from PI 137739), *Dn2*, (from PI 262660), and *Dn4*.		Basky, 2003
Syria		*Dn4*	02 Altus 162 containing *Dn4*	Puterka *et al.*, 1992; Smith *et al.*, 2004; Porter *et al.*, 2005
Russia		PI 372129 *Dn4*		Puterka *et al.*, 1992; Smith *et al.*, 2004
Chile		*Dn4*	*Dn2*, *Dn5* (from PI 294994), *Dn6* (from PI 243781 or CI 6501), *Dnx* (from PI 220127), and *Dny* (from PI 220350)	Puterka *et al.*, 1992; Smith *et al.*, 2004
Czech Republic		*Dn4*	*Dnx*	Smith *et al.*, 2004
Ethiopia		*Dn4*	*Dny*	Smith *et al.*, 2004

1.4. Wheat

1.4.1. Background

Wheat (*Triticum aestivum* L.) is a grass that has been domesticated in southwest Asia around 10,000 years ago (Kellogg, 2001). Wheat is now second to maize in total worldwide production and is still seen as the most important food grain (Belderok *et al.*, 2000). It has major economical value not only as food but is also used in fermentation yielding beer, bio fuel etc and is planted as forage crop (Belderok *et al.*, 2000; Payne *et al.*, 2001).

Cereals belong to the grass family (Gramineae), which is subdivided into several genera. Wheat, barley, rye and oats are representatives found in temperate zones where wheat and barley are grown in relatively fertile soil compared to rye and oats that are more adapted to poor (acid and dry) soils (Belderok *et al.*, 2000).

The grass family includes approximately 10,000 species classified into 600 to 700 genera (Clayton and Renvoize, 1986; Watson and Dallwitz, 1999). The Russian cytogeneticist Avdulov (1931) found that a large group of temperate grasses had much larger and fewer chromosomes, with a base number of $x = 7$, compared to other grasses (Avdulov 1931 as cited by Kellogg, 2001). A French anatomist Prat (1932) then confirmed this group by looking at the shape and structure of epidermal cells to find that the subsidiary cells of the stomata have outer walls that are parallel rather than curved (Prat, 1932 as cited by Kellogg, 2001). The Grass Phylogeny Working Group (GPWG) decided to represent this group by only three genera, *Avena* (oats), *Bromus*, and *Triticum* (wheat), but it also includes species such as barley (*Hordeum*), rye (*Secale*) as well as all the cool season grasses commonly placed in subfamily Pooideae (GPWG, 2000). Genes in the nuclear genome of all grasses are syntenious, thus whole chromosomes of rice can be lined up with chromosomes of wheat or maize (Gale and Devos, 1998). However, major rearrangements have occurred among blocks of linked genes (Bennetzen and Kellogg, 1997; Kellogg, 2001).

The genus *Triticum* consists of several species and can be divided into three basic natural groups. The number of chromosomes in the vegetative cells can distinguish

each group. Diploid, tetraploid and hexaploid wheat species carry 14, 28 and 42 chromosomes in their vegetative cells, respectively (Belderok *et al.*, 2000). *T. monocuccum* also known as einkorn, is the best known ancestor of our modern cultivated wheat containing two sets of seven chromosomes. Rice, wheat and maize coevolved from this common ancestor ~55-75 million years ago (Kellogg, 2001). This species can be genetically described as AA plants (diploid). Tetraploid wheats, emmer (*T. dicoccum)* and durum (*T. durum)* are crosses between wild einkorn wheat and an unknown wild grass containing a diploid set of chromosomes that differ from the AA genome (Figure 1.9). This wild grass genome is indicated with a letter B and the hybrid has a tetraploid genome described as AABB. Hexaploid wheat in turn evolved through the hybriduzation of tetraploid wheat (AABB) and a wild diploid grass for example *Aegilops tauschii* (DD) giving spelt and bread wheat (*Triticum aestivum).* This species can be genetically described as AABBDD (Devos and Gale, 1997; Belderok *et al.*, 2000; Hancock, 2004). Common wheat is thus an allohexaploid consisting of seven groups of chromosomes (Figure 1.9), each group containing a set of three homologous chromosomes belonging to the A, B and D genomes (Gill *et al.*, 2004).

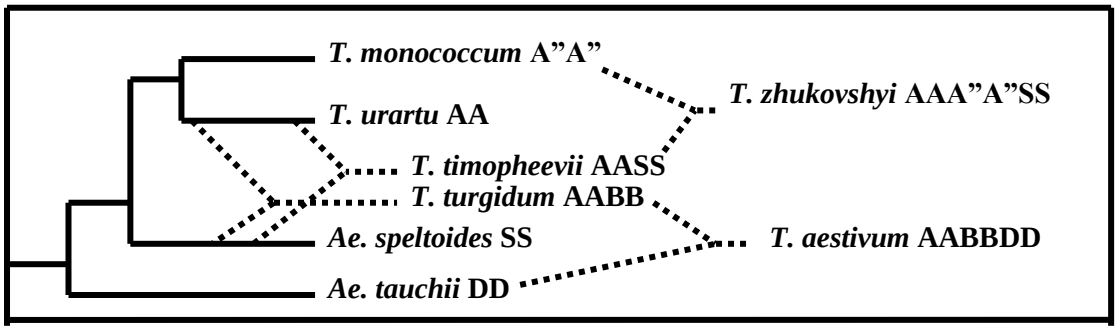

Figure 1.9: Phylogeny of polyploid species of *Triticum*. Genomes of each species are indicated by capital letters (Dvorák *et al.*, 1988; Dvorák and Zhang, 1990; Dvorák and Zhang, 1992).

Plant genomes have a much higher complexity than the human genome (3,000 Mb) as they can have multiple distant genomes in their nucleus. Their genomes tend to be repetitive resulting in a larger genome. Bread wheat is an allohexaploid with a genome size of 16,000 Mb (Soderlund *et al.*, 2002). About 90 % of the wheat genome consists of repeated sequences and 70 % of known transposable elements (TEs) (Li *et*

al., 2004). Table 1.3 shows five different plant genomes, their sizes, the percentage repetitive DNA and polyploidy to get a perspective of the size of the bread wheat genome. It is beneficiary to have a gene map for the plant community to organize markers and genes identified. The majority of the wheat genome has been mapped (Röder *et al.*, 1998) and wheat chromosomes 1D and 7D can be seen in Figure 1.10 showing the RWA resistance genes (*Dn*, *Dn2*, *Dn4*, *Dn5*, *Dn6*, *Dn8*, *Dn9* and *Dnx*) and the linked molecular markers (Liu *et al.*, 2002). The sequencing of the wheat genome will better the understanding of the relationship among grass lineages (Freeling, 2001) and the resistance mechanisms not yet fully understood.

Table 1.3: Attributes of a few plant genomes (Soderlund *et al.*, 2002).

Genome	*Size(Mb)*	*% Repetitive*	*Description*	References
Arabidopsis	125	14	Diploid	*Arabidopsis* Genome Initiative, 2000
Rice	380	76	Diploid	Chen *et al.*, 2001
Maize	2,500	83	ancient tetraploid	Miguel *et al.*, 1996
Barley	5,000	88	Diploid	Vicient *et al.*, 1999
Bread wheat	16,000	88	Hexaploid	Devos and Gale, 1997; Gill *et al.*, 2004

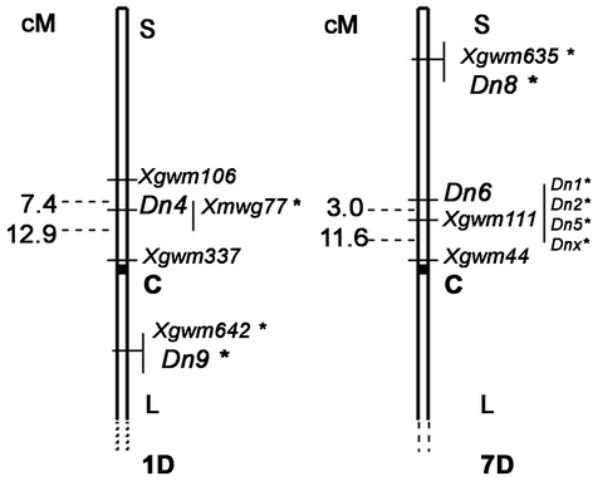

Figure 1.10: Genetic linkage maps of wheat chromosomes 1D and 7D showing RWA resistance genes (*Dn*) and the linked molecular markers. *S, L* = short or long chromosome arm, *C* = centromere position. *The orientation between markers and genes is unresolved (Liu *et al.*, 2002).

1.4.2. Inheritance

Cross breeding have been implemented through the years in order to generate resistant cultivars, but cross breeding gave short-term success due to the lack of understanding plant resistance mechanisms (McIntosh *et al.*, 1995). The search for understanding the mechanism and finding genes involved are of utmost importance.

Painter first reports three categories of host responses that occur after RWA infestation in 1958 (Painter, 1958). The responses include tolerance, antibioses and antixinosis. Tolerance is when the plant survives under levels of infestation that will kill or severely injure susceptible plants in other words resistant plants will show less damage (Kindler *et al.*, 1995), while antibiosis is the ability of resistant plants to adversely affect the biology of the insect decreasing its body size, longevity and reproduction (Unger and Quisenberry, 1997). Antixinosis is defined as the non-preference of plants for insect oviposition, shelter or food, thus a RWA would prefer another plant to an antixenotic plant (Painter, 1958; Rafi *et al.*,1996).

The first gene found in wheat to be involved in resistance against RWA was *Dn* (Du Toit, 1989b). This single dominant gene occurs in the resistant wheat cultivar TugelaDN (Nkongolo *et al.*, 1991a). Ten more genes have been found to date that provide resistance to wheat and are indicated in Table 1.4. Several independent dominant genes control the inheritance of these resistant genes to RWA (Du Toit, 1989b; Nkongolo *et al.*, 1991a; Saidi and Quick, 1996). It is suspected that *Dn*, *Dn2*, *Dn5* and *Dnx* form a linkage group on wheat chromosome 7DS, while *Dn7* is located in a defence-gene-rich region of wheat chromosome 1DL (Liu *et al.*, 2001; Liu *et al.*, 2002; Anderson *et al.*, 2003; Liu *et al.*, 2005). *Dn2* is a single dominant gene found in PI 262660 (South African wheat accession SA2199) (Du Toit, 1987), and confers mostly tolerance with only a low level of antibiosis (Smith *et al.*, 1992). The antixenotic single dominant *Dn7* gene was transferred from rye into a wheat background via a RS/1BL translocation and confers a higher level of resistance than *Dn4*, although linkage studies have shown that *Dn7* and *Dn4* may be orthologous (Anderson *et al.*, 2003).

The *Dnb1* and *Dnb2* genes are resistance genes from barley that were transformed into wheat and can be found in the STARS-9301B line (Mornhinweg *et al.*, 1995). Since the possible occurrence of new RWA biotypes limits the use of the known genes, additional sources of RWA resistance genes are necessary (Assad *et al.*, 1999).

The high yielding wheat cultivar Tugela ('Kavkaz'/'Jaral') was released in South Africa in 1985 (Tolmay *et al.*, 2006). This hard red intermediate wheat also has a medium growth period but is susceptible to RWA feeding. Upon realizing this, the Small Grain Institute, Bethlehem, South Africa made use of backcrossing to create Tugela near isogenic lines containing the *Dn*, *Dn2* or *Dn5* resistance genes. TugelaDN (Tugela*4/PI 137739), Tugela *Dn2* (Tugela*4/PI 262660) and Tugela *Dn5* (Tugela*4/PI 294994) were the result of consecutive backcrosses using single selection in each generation. The final lines were then selfed twice to produce homozygous resistant lines (Lacock *et al.*, 2003; Tolmay *et al.*, 2006).

Table 1.4: Resistance genes found in wheat cultivars. The[boilerplate: UNIVERSITEIT VAN PRETORIA / UNIVERSITY OF PRETORIA / YUNIBESITHI YA PRETORIA]e provided (Botha *et al.*, 2006b).

Resistance gene	Line/ Number	Gene response	Reference	Host response	Reference
Dn	PI 137739	Single dominant gene	Du Toit, 1989b; Nkongolo *et al.*, 1991a		
Dn2	PI 262660	Single dominant gene	Du Toit, 1989a; Dong and Quick, 1995	Tolerance by Tugela *Dn2*	Wang *et al.*, 2004
Dn3	*Triticum taushii*	Single resistant gene	Nkongolo *et al.*, 1991a		
Dn4	PI 372129	Single dominant gene	Du Toit, 1989a; Nkongolo *et al.*, 1991b	Tolerance by Halt and PI 372129	Meyer and Peairs, 1989; Hawley *et al.*, 2003
Dn5	PI 294994	Single dominant gene/ One dominant & one recessive gene/ Two dominant genes	Marais and Du Toit, 1993; Elsidaig and Zwer, 1993; Saidi and Quick, 1996		
Dn6	PI 1243781	Single dominant gene	Saidi and Quick, 1996	Antibiosis by Halt, Ankor, Stanton and CO 940626 carrying *Dn4* and *Dn6*	Hawley *et al.*, 2003; Randolph *et al.*, 2006
Dn7	Rye	Unknown	Marais *et al.*,1994	Antixenosis and antibiosis	Peng and Lapitan, 2005
Dn8	PI 294994	Unknown	Liu *et al.*, 2001		
Dn9	PI 294994	Unknown	Liu *et al.*, 2001		
Dnx	PI 220127	Single dominant gene	Liu *et al.*, 2001		
Dny	PI220350		Liu *et al.*, 2001		
Dnb1& Dnb2	STARS-9301B		Mornhinweg *et al.*, 1995		

1.5. Wheat-RWA interaction

1.5.1. General

After the Russian wheat aphid overcame environmental conditions, predators and insecticides it still has to face the problem of feeding. Aphids are phloem-feeding insects and thus developed a mechanism to retrieve nutrients from the phloem (Miles, 1999). This would not have hindered the wheat plant much, but the aphid injects phytotoxins that causes the wheat to produce less seed or even die (Telang *et al.*, 1999; Karren, 2003). The wheat plant thus developed mechanisms to detect and deter the RWA. This fight for survival for both RWA and the wheat plant is a daily interaction and pressures evolutionary changes to be 'one step ahead' in the evolutionary arms race (Chapin, 1991; Bray, 1993). The main survival pressure is to prevent recognition by the wheat plant.

The wheat plant defends itself with physical and chemical mechanisms which is present in naturally resistant wheat, but can be induced with breeding and with genetic manipulation. Ni and Quisenberry (1997) reported that RWAs prefer wheat lines with shorter trichomes. Lines with longer trichomes positioned along the leaf veins were less preferred although the trichome density was less (Ni and Quisenberry, 1997). The leaf veins are the preferred feeding site of RWA and the trichomes hinder their probing. The discovery was supported by the finding of Bahlmann *et al.*, 2003, that the resistant wheat line TugelaDN has more trichomes on the leaf veins than other non-resistant lines (Bahlman *et al.*, 2003).

The RWA continually evolve new biotypes to overcome the pressure from the wheat resistant lines (Haley *et al.*, 2004). It was found that many of the resistant wheat cultivars produced became susceptible after a while (Smith, 2005). This could be due to new biotypes and still many new biotypes are being identified and reported.

1.5.1.2. R-protein elicitors

When an aphid is feeding on a plant very complex signaling occurs. These signals may be caused by physical damage to the plant, by mechanical stress or by biochemical recognition. When an aphid is probing a plant with its stylet, cells are damaged along the feeding path, which may be recognized as a signal to activate defence related genes. Stylet movement between cells may disrupt cell-to-cell contact, which may be seen as mechanical stress to activate genes (Walling, 2000). Phytotoxins are also injected by the RWA through the stylet (Fouché *et al.*, 1984) that in most cases leads to the activation of the resistance response (Musser *et al.*, 2002).

Aphids secrete two types of saliva at the feeding site along the stylet path. The first is a rapid gelling, sheath saliva, that consists of protein, phospholipids and conjugated carbohydrates. These compounds form a protective barrier along the stylet path in order that the stylet does not come in contact with the plant's apoplast. The second type of saliva is watery, digestive saliva that contains pectinase, cellulases, amylases, proteases, lipases, alkaline and acidic phosphatases and peroxidases. These salivary compounds may act as elicitors in inducing resistance (Miles, 1999).

Invading organisms induce plant responses through two biochemical signaling pathways, which are jasmonic acid (JA) and salicylic acid (Walling, 2000). The mode of feeding determines which signaling pathways will be induced (Karban and Baldwin, 1997; Walling, 2000). Chewing insects primarily activate the wound-inducible JA-mediated cascade(s) (Thaler *et al.*, 1996; Karban and Baldwin, 1997; Thaler, 1999; Thaler *et al.*, 1999; Bostock *et al.*, 2001). Phloem-feeding whiteflies and aphids, for instance, produce little injury to plant foliage, but induce the SA-dependent pathway by other means (Walling, 2000).

De Ilarduya's results published in 2003 indicate and support earlier findings that both JA and SA signal pathways are activated by aphid feeding (Botha *et al.*, 1998; de Ilarduya *et al.*, 2003). The JA pathway is activated by the physical and mechanical damage of feeding (wounding) and the SA pathway by the biochemical recognition of saliva (elicitor perception) (Walling, 2000; Moran and Thompson, 2001). There are indications that JA- and ethylene induced resistance happens before SA- and ethylene

induced pathways (de Ilarduya *et al.*, 2003). Interactions between JA and SA pathways have been reported co-inducing or co-repressing large number of genes (Schenk *et al.*, 2000). It is thus possible that phloem-feeding insects might elicit novel defence mechanisms and signal pathways in plants (Van de Ven *et al.*, 2000; Moran and Thompson, 2001).

1.5.1.3. Resistance genes

Monogenic resistance (major gene resistance) is a single gene that control resistance and can usually be easily identified even in seedlings since it is usually very specific towards a certain pest or pathogen (Jones and Clifford, 1983). The monogenic RWA resistance genes already discovered are *Dn-Dn4*, *Dn6-Dn9*, *Dnx* and *Dny* (Table 1.4). Polygenic resistance involves a number of genes at different loci, each having a small individual effect in a combined outcome (Jones and Clifford, 1983). This type of resistance is usually a general resistance against all races of a given pathogen. *Dn5* has not jet been determined as monogenic or polygenic and its origin is not known either (Heyns *et al.*, 2006).

The resistance mechanisms against the Greenburg (*Schitzaphpis graminum*) and the RWA are independent from each other and are probably regulated by different genes. These different genes then independently regulate antibiosis, antixenosis and tolerance (Castro *et al.*, 1999; Castro *et al.*, 2001).

The protein profile of cereal plants begin to change within 48 hours, when they are infested with the RWA (Van der Westhuizen and Botha, 1993). When Tugela (susceptible) and TugelaDN (resistant) were compared, it was observed that RWA infestation resulted in an increase in the number of proteins in the resistant cultivar and a decrease in the susceptible cultivars. Although the aphids prefer to feed at the bases of the leaves, the profiles of all the different parts of the resistant leaf are similar, indicating a global expression of “protective components” (Van der Westhuizen and Botha, 1993).

The levels of salicylic acid is up regulated to a greater extent in resistant plants about 48 hours post infestation (pi) having the role of a signalling molecule and the activation of the SAR. Similarly, peroxidise gene expression is also increased within

48 hours pi and accumulate in the apoplast that cause the thickening of cell walls and the release of ROS (Van der Westhuizen *et al.*, 1998b: Mohase and Van der Westhuizen, 2002). Catalase activity gets inhibited as SA content increases, ROS accumulate and indicate that the HR has been activated (Mohase and Van der Westhuizen, 2002). The presence of RWA phytotoxin increases the expression of esterase and superoxide dismutase genes to have a "detoxification" effect in susceptible wheat plants after RWA infestation. Detoxification is necessary to prevent extended damage to the plant (Ni and Quissenberry, 2003).

It was found that the expression of genes related to tobacco and barley PR proteins (PR-2, PR-Q and PR-5) was induced in the apoplasm of resistant lines of wheat (Van der Westhuizen and Pretorius, 1996). Resistant plants respond to RWA infestation by producing high concentrations of β-1,3-glucanase that accumulate mostly in the cells in the vascular bundles and especially in the chloroplasts (Van der Westhuizen *et al.*, 2002). Seven isoforms of β-1,3-glucanases have also been observed in the apoplast (Van der Westhuizen *et al.*, 1998a). The intra- and extracellular forms might manage to attack the pathogen before it has entered the cell. Three forms of chitinase isoforms are present in wheat and are expressed in response to either RWA feeding, ethylene induction or mechanical wounding (Botha *et al.*, 1998). Thus, one can assume that the resistance response associated with RWA infestation is unique to the pest and not a result of the probing or general SAR.

1.5.2. Photosynthesis

The conversion of light energy into chemical energy is fundamental for life. Photosynthetic reaction centers, composed of special protein-chlorophyll complexes in the core of light-harvesting photosystems (Figure 1.11), play a major role in the energy conversion process (Buttner *et al.*, 1992). Oxygenic photosynthesis of chloroplasts involves two photosystems: the oxygen-evolving photosystem II (PSII) that originated from purple bacteria and the ferredoxin reducing photosystem II (PSI) that originated from the green sulphur bacteria (Buttner *et al.*, 1992).

In the chloroplast of eukaryotic cells, photosynthesis takes place as a major energy harvesting reaction for biological systems. The primary pigments, Chlorophyll *a* (Chl

a) and Chl *b* located in the thylakoid membrane, absorb different light and accumulate the energy in excited electrons in the thylakoid membrane. Secondary pigments, carotenoids (Carotenes and Xanthophyll) are located in the chloroplast membrane and outer membrane to absorb the light waves not efficiently absorbed by Chl (Nelson and Yocum, 2006).

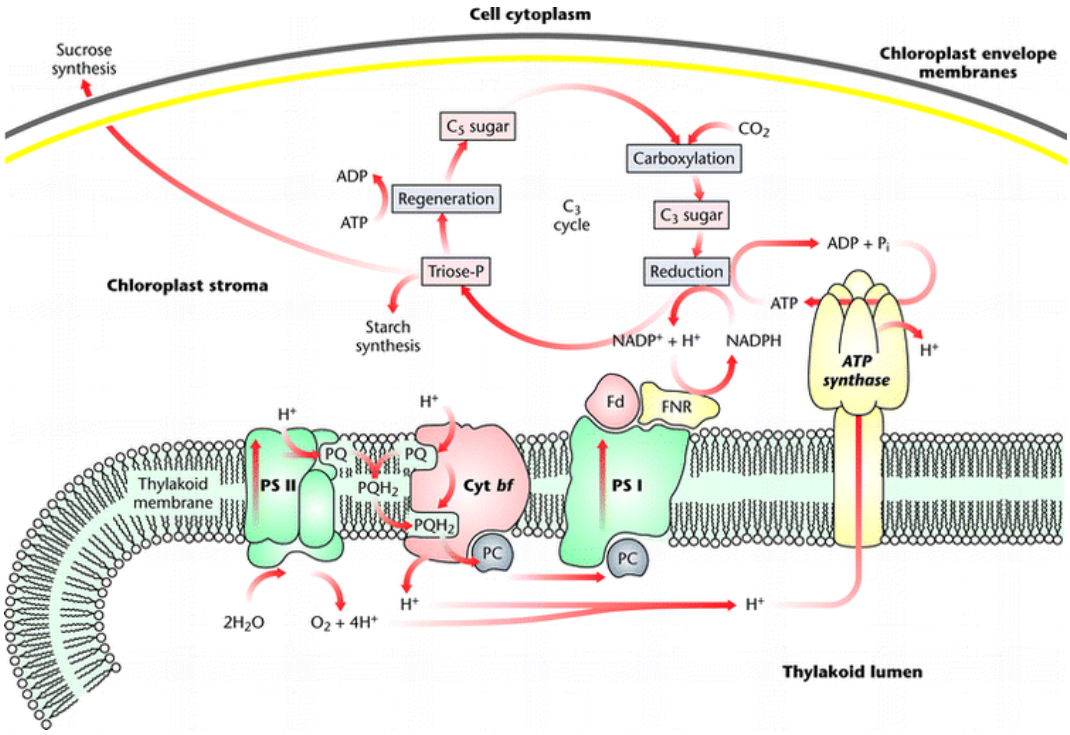

Figure 1.11: Photosystem I and II location and function. The thylakoid membrane with PSI and PSII are indicated with the movement of reactions to produce sugars through the Calvin cycle (www.ualr.edu/botany/botimages.html, assessed on 10.9.2009).

A PSII reaction center complex consists of D-1 and D-2 polypeptides, five chlorophyll *a*, two pheophytin *a*, one *B*-carotene, and one or two cytochrome *b*-559 heme(s) (Nanba and Satoh, 1987). In the Photosystem II the P680 reaction centre captures photons and the light energy is used to carry out oxidation (splitting) of water molecules. When the electrons are released from the water, the water molecule is broken into oxygen, which is released into the atmosphere, and hydrogen ions, which are used to power ATP synthesis. The electrons, excited at the antenna molecule P680, are passed down a chain of electron-transport proteins and receive extra electrons from PSI. More hydrogen ions are pumped across the membrane as these electrons flow down the chain providing more protons for ATP synthesis. The

electrons are then transported on a NADPH molecule to enzymes that build sugar from water and carbon dioxide (Nelson and Yocum, 2006).

A photosystem I reaction center complex consists of 6 polypeptides containing two of subunit I that associate with P700, subunit PSI-D, subunit PSI-E, quinones and fluorenones. Twenty chlorophyll *a* molecules and a cytochrome 522 heme form the complex P700 molecule (Bengis and Nelson, 1977). Photosystem I also uses photons, but at 700 nm wavelength, to excite electrons from its antenna molecule P700. The electron produced by PSIi is transferred to PSI, excited, captured by ferredoxin and used to reduce $NADP^+$ to NADPH. ATP is produced via chemiosmosis as three hydrogen ions, which supply the energy, pass three at a time from the thylakoid to the stroma. ATP and NADPH are then used in the light-independent reactions together with hydrogen atoms extracted from water by PSII to convert carbon dioxide to glucose and in the process release oxygen as a by-product (Fromme, 1996; Nelson and Yocum, 2006).

1.5.2.1. Fluorescence induction

Chlorophyll *a* fluorescence has been found to have close correlation with light reactions in the photosynthetic tissues. Measuring Chl *a* fluorescence is thus a non-invasive technique to study the light induced electron transfer in the multi-protein pigment complexes of photosynthesis (Schreiber, 2002; Laza´ r, 2003). Fluorescence induction (FI) is the measurement of the Chl fluorescence yield of photosynthesis tissue as a function of time when kept under continuous light (Kautsky and Hirch, 1931 as cited by Joly *et al.*, 2005). When photosynthetic samples are excited from a dark adapted state, that correspond to the basal level of fluorescence Fo (O) to a maximum level Fm (P), the PSII complex change from a closed state to an open state (Neubauer and Schreiber, 1987; Strasser and Govindjee, 1992) The fluorescence rise from O-level to an intermediate level J (I_1) within the first 2 ms after the onset on continuous light and to a second intermediate level I (I_2) within 32 ms (Laza´ r, 2003). The P-level is only reached after several hundred ms (Strasser *et al.*, 1995; Laza´ r, 2003). The OJIP technique have been widely used to measure changes in the electron transport of photosystem II due to external conditions (Barthélemy *et al.*, 1997; Posp´ıšil and Dau, 2000; Joly *et al.*, 2005).

1.5.2.2. Regulation of plant systems

Phloem-feeding insects (PFI) not only significantly reduce photosynthesis in their host plants (Macedo *et al.*, 2003) by down-regulating the expression of photosynthesis-related genes (Heidel and Baldwin, 2004; Voelckel *et al.*, 2004; Zhu-Salzman *et al.*, 2004; Qubbaj *et al.*, 2005; Yuan *et al.*, 2005), but also cause a changed carbohydrate metabolism by resource allocations from a growth to a defence metabolism (Heidel and Baldwin, 2004). The changes in metabolism are due to the pathogen that manipulates the carbohydrate metabolism of the plant for its own advantage. The plant also upon recognition of the aphid increase carbon supply to the areas of need such as defence reactions (Berger *et al.*, 2004). In resistant cultivars such as TugelaDN, genes required for Rubisco synthesis are upregulated, which might be necessary for sustaining energy production for resistance (Van der Westhuizen and Botha, 1993). Source–sink relationships and water relations are also modified within the plant, because PFI must extract large volumes of phloem sap to attain adequate nitrogen (Douglas, 2006). By inducing genes involved in carbon assimilation and mobilization to increase sugar uptake, the sugars are depleted and thereby localized metabolic sinks are created (Moran and Thompson, 2001; Moran *et al.*, 2002; Zhu-Salzman *et al.*, 2004). PFI also modifies nitrogen allocation in their hosts by up-regulating genes involved in nitrogen assimilation in particular genes encoding enzymes required for synthesis of tryptophan and other essential amino acids (Sandstrom *et al.*, 2000; Heidel and Baldwin, 2004; Zhu-Salzman *et al.*, 2004; Thompson and Goggin, 2006).

1.5.2.3. The effect of RWA feeding on photosynthesis

Photosynthesis is significantly reduced by RWA feeding as the expression of genes involved in chloroplast synthesis and function is suppressed (Botha *et al.*, 2006a). Photosynthesis-related genes are strongly suppressed by MeJA signaling, and to a lesser extent by SA (Zhu-Salzman *et al.*, 2004).

RWA feeding destroys the cell membranes, damages the chloroplasts, and thus, effective photosynthetic capacity declines (Fouché *et al.*, 1984). The decrease of total chlorophyll (*a* and *b*) and carotenoids (luteins), which play an important role in the

PSI, were observed by Heng-Moss *et al.*, (2003) in susceptible cultivars after RWA feeding. This suggests that RWA feeding causes the reduction of the photosynthetic rate in susceptible lines and the PSII complex is suggested to be a target for RWA damage (Heng-Moss *et al.*, 2003).

The indicative damage to the PSII reaction centre is the reduction of chlorophyll *a* and the damage to the reaction centre protein D, which is important for the assembly and stability of PSI. PSI has 20 copies of chlorophyll *a* compared to the 5 copies of PSII, making PSI much more vulnerable to chlorophyll *a* loss. When PSI-D was damaged or levels reduced the plants showed leaf chlorosis, lower chlorophyll content and P700 levels, and a high non-photochemical quenching, suggesting inefficient electron transport (Haldrup *et al.*, 2003). Low PSI-D levels affects the redox state of chloroplast thioredoxin *f* and *m*, which is one of the main participants in regulating cellular redox balance by reducing disulfide bridges. Chloroplast thioredoxin *f* and *m* regulate the enzymatic activity of certain enzymes including Fru-1,6-bisphosphatase and NADP malate dehydrogenase that are involved in photosynthetic carbon metabolism (Ruelland and Miginiac-Maslow, 1999). The catalytic extrinsic CF_1 segments of cpATPase is activated in the light when carbon fixation is possible, but when PSI-D is low thioredoxin *m* oxidation might turn cpATPase off (Haldrup *et al.*, 2003).

Oxidized thioredoxin changed to the thiol disulfide redox state because of a defective PSI system may be the indirect cause of the death of plants due to RWA feeding. This is because thioredoxin is one of the main switches for the initiation of CO_2 assimilation and photoprotection. The direct cause of damage and decreased growth is the inability of defective PSI to down-regulate the PSII levels accordingly (Haldrup *et al.*, 2003). The electron transport on the acceptor site of photosystem II reaction centre is therefore blocked and causes an over-reduction in the system (Burd and Elliott, 1996). This suggests that RWA feeding could reduce protein synthesis making photoinhibition irreversible (Haldrup *et al.*, 2003).

1.5.2.4. Photosynthetic regulation in resistant plants

When herbivores eat plants, changes in the energy conversion might occur (Buttner *et al.*, 1992). The maintenance of the chloroplast machinery is one of the determining factors in enabling resistant varieties to overcome the stress during RWA feeding (Botha *et al.*, 2006a).

Botha *et al.* (2006a) obtained 200 non-redundant expressed sequence tags (ESTs) from the RWA resistant wheat transcriptome elucidated in response to RWA. Eighteen point nine percent of the genes were involved in photosynthesis including chloroplast genes for the Rubisco *rbcL*, red chlorophyll catabolic reductase, the Photosystem I P700 apoprotein, thioredoxin *m* and chloroplast ATP synthase (Botha *et al.*, 2006a).

TMP14 Thylakoid membrane phosphoprotein (14 kDa) is a novel subunit of plant PSI and is designated by Khrouchtchovaa *et al.*, as PSI-P (Khrouchtchovaa *et al.*, 2005). Its EST was induced in resistant cultivars in respect to RWA feeding (Van Eck, 2007). The TMP14 subunit is found with PSI-D as phosphorylation subunits of PSI (Hansson and Vener, 2003). It is probably involved in the interaction with LHCII and together with PSI-D ensures PSI function by accepting electrons from PSII (Khrouchtchovaa *et al.*, 2005). An induction of TMP14 would be a strategy to overcome pest attack to keep PSI stable and energy production going and a reduction might be to force energy flow in a different direction.

Photosystem I P700 is bound by PsaA and PsaB in PSI and function as the primary electron donor. PSI converts photonic excitation into a charge separation, which transfers an electron from the donor P700 chlorophyll pair to the spectroscopically characterized acceptors A0, A1, FX, FA and FB in turn. Photosystem I P700 induction ensures electron excitation and reduction might force the synthesis of ROS for HR (Grotjohann and Fromme, 2005).

Chloroplast ATPase (cpATPase) is found to be essential for photosynthesis (Maiwald *et al.*, 2003) by playing a direct role in the translocation of protons across the membrane as a key component of the proton channel. The F-type ATPases has a CF_1 segment functioning as the catalytic core and a CF_0 segment functioning as the

membrane proton channel. cpATPase gene expression is up regulated upon RWA feeding and was found to be significantly higher expressed in resistant TugelaDN when compared to the susceptible Tugela cultivar. It seems that cpATPase is important for maintenance of photosynthetic activity in resistant wheat during RWA feeding (Botha *et al.*, 2006a).

Fructose-1,6-bisphosphatase catalyzes the reaction converting D-fructose 1,6-bisphosphate to D-fructose 6-phosphate in carbohydrate biosynthesis http://au.expasy.org/cgi-bin/get-similar?type=pathway&name=. The induction of FBP would overcome the step inhibited by thioredoxin (Berg *et al.*, 2002).

Glutathione S-transferase covalently links glutathione to a hydrophobic substrate to form less reactive and more polar glutathione S-conjugates. Endobiotic and xenobiotic compounds such as peroxidised lipids are detoxified by GST (Neuefeind *et al.*, 1997). Induced GST would detoxify RWA virulent factors and enzymes affected by the redox state.

The mentioned genes play important roles in the defence response of wheat plants against RWA infection (Botha *et al.*, 2006a). They are thus promising candidates to consider for expression manipulation to ultimately find a means of generating resistant wheat lines.

1.6. Virus-induced gene silencing (VIGS)

1.6.1. General background

Virus-induced gene silencing is an important tool for the analysis of gene function in plants (Burch-Smith *et al.*, 2004). The objective of VIGS is to eliminate the encoded protein and thereby observe the revealed symptoms (Lu *et al.*, 2003). This can be achieved at the level of RNA silencing of endogenous plant genes by virus-mediated, transient expression of homologous gene fragments (Holzberg *et al.*, 2002). The selected plants are infected with a virus carrying target sequences with homology to a host nuclear gene. The viruses activate the host's sequence-specific RNA degradation system that targets the RNAs of the viral genome for degradation. This dsRNA is cleaved to produce small guide molecules called short interfering RNA (siRNA) that associate with its antisense strand to the RNAi silencing complex (RISC) to guide the degradation of homolougous sequences (Bartel, 2004). As the virus contains transcribed plant sequences, homologous host mRNA is also targeted for destruction resulting in the absence of accumulated transcript (Scofield *et al.*, 2005). This happens as target sequences align with the transcribed mRNA and the double stranded mRNA will then trigger its own degradation preventing translation to protein (Vance and Vaucheret, 2001). Post-transcriptional gene silencing by suppressing the accumulation of an RNA species in the cytoplasm was first discovered in plants by Lindbo and Dougherty, 1992. This powerful, reverse genetic approach has since then became an important tool for functional genomics for dicotyledones plant species. It was then also demonstrated in 2002 to be affective in monocotyledones species with the use of barley stripe mosaic virus (BSMV) (Holzberg *et al.*, 2002).

1.6.2. Advantages

VIGS eliminates most of the limitations of traditional approaches and is most commonly used as an *Agrobacterium*- or *in vitro* transcription-based transient assay to rapidly generate phenotypes (Ratcliff *et al.*, 2001; Dinesh-Kumar *et al.*, 2003). VIGS can be used on *Nicotiana benthamiana*, tomato, tobacco, barley, wheat, *Arabidopsis* and the number of plant species amenable is increasing (Burch-Smith *et al.*, 2004). When compared to other functional genomics approaches, VIGS is low cost and rapid, ranging 3-4 weeks from infection to silencing and one can identify a loss-of-

function in phenotype for a specific gene within a single generation (Burch-Smith *et al.*, 2004) (Table 1.5). VIGS avoids plant transformation by being a transient method as it does not rely on the generation of transgenic plants. Thereby, phenotypes showing loss-of-function that might result in death at early stages of development can often be avoided. Another advantage of VIGS is that it overcomes functional redundancy. Either specific members of a gene family or all the members can be silenced using a targeting sequence by respectively selecting unique sequences in family members or of the most highly conserved region of the gene family (Scofield *et al.*, 2005).

Table 1.5: Comparison of VIGS with other functional gen -Smith *et al.*, 2004).

Method	Description	Transformation required	Space requirements	Cause of loss of function	Used to study entire families	Cost
VIGS	Plants infected with virus carrying fragment of endogenous gene	No	Small for single gene studies, Large for large-scale screen	PTGS of gene homologous to targeting sequence	Yes	Low; limited sequencing to ascertain specificity; PCR to confirm silencing
Chemical/ physical mutagenesis	Seeds or plants treated with chemical mutagen (e.g. EMS) or radiation (e.g. fast-neutron)	No	Large	Point mutations (EMS) Deletions (fast-neutron)	No	High; mapping and sequencing
TILLING	Chemical mutagenesis with mutations identified by SNP analysis	No	Large	Point mutations (EMS)	No	High; extensive PCR and sequencing
T-DNA insertion	Plants transformed with *Agrobacterium* have T-DNA inserted into their genomes	Yes	Large	Ectopic activation of neighbouring genes or disruption of coding sequence or UTR	No	Moderate; sequencing and PCR
Transposon activation	Plants transformed with transposon that is mobilized to produce insertions or excision footprints	Yes	Large	Disruption of coding sequence	No	Moderate; sequencing and PCR

1.6.3. The Barley stripe mosaic virus

Viruses are among the most damaging of plant pathogens but an early observation was that virus-infected plants were subsequently resistant to infection by the same or a closely related strains of the same virus (McKinney, 1929). The phenomenon was termed 'cross-protection', the technique used to engineer resistance described as pathogen-derived resistance (PDR) (Beachy, 1997) and it was later found that the molecular basis was PTGS (Ratcliff *et al.*, 1997).

BSMV is a member of the hordeivirus family that infects many agriculturally important monocotyledon species (Figure 1.12) (McKinney and Greeley, 1965) including barley, oats, wheat and maize. When the BSMV is injected into wheat leaves it first flows along major veins then escapes to spread cell-to-cell through the mesophyll tissue and infect the epidermal cells. The invasion of the epidermis is an important characteristic of BSMV (Haupt *et al.*, 2001). Further characteristics of the BSMV vector that makes it suitable for the development of monocot-overexpression and RNA-silencing include: the availability of infectious BSMV cDNA clones (Petty *et al.*, 1989), mechanical transmissibility (Palomar *et al.*, 1977), and wide host range (McKinney and Greeley, 1965).

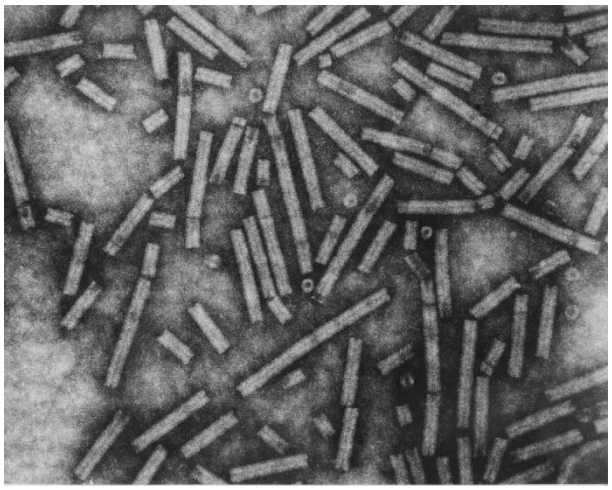

Figure 1.12: Electro micrograph of BSMV (Photographed by IACR Rothamsted) http://www.rothamsted.ac.uk/ppi/links/pplinks/virusems/d10.gif, assessed on 19.2.2010

A VIGS vector has been developed for silencing in barley, a monocotyledonous plant (Holzberg *et al.*, 2002). The BSMV is a positive-sense RNA virus that has a tripartite genome consisting of RNAs α, β and γ (Palomar *et al.*, 1977). BSMV can express GFP in systemically infected barley tissue as a fusion to the C-terminus of the BSMV γb protein (Haupt *et al.*, 2001; Lawrence and Jackson, 2001). In the same way endogenous host genes can be silenced by this γb fusion vector, expressing plant cDNAs such as phytoene desaturase (PDS) or any other protein downstream of the γb open reading frame (ORF) (Figure 1.13). Foreign inserts downstream of the γb gene can be arranged in either sense or antisense orientations. A γb stop codon was included in the vectors to prevent the cDNA inserts from being translated in plants and thereby interfering with γb activity (Holzberg *et al.*, 2002).

Coat protein deletion on the β-RNA enhances the ability of BSMV to silence PDS. It has been proven that BSMV can be widely applied for endogene silencing (Holzberg *et al.*, 2002). A new vector was developed with greater efficiency of silencing that contains 40-60 base pair direct inverted repeats that generate dsRNA upon transcription (Lacomme *et al.*, 2003).

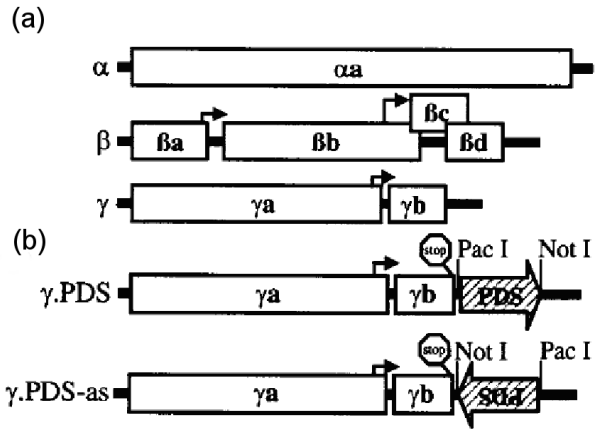

Figure 1.13: Genomic organization of BSMV. (a) Genomic RNAs (α, β and γ) of BSMV, strain ND-18. (b) Genomic organization of BSMV RNA γ modified to express untranslatable sense and antisense PDS fragments. Open boxes indicate ORFs; hatched boxes indicate untranslated ORFs. Arrows indicate subgenomic promoters. Arrow-shaped boxes indicate orientation of PDS inserts. The positions of selected restriction enzyme sites are indicated (Holzberg *et al.*, 2002).

Because it is necessary to evaluate whether the VIGS viral vectors were effective in transforming the plant a gene must be included to discern phenotypic changes. The phytoene desaturase endogene is frequently used to evaluate VIGS viral vectors (Kumagai *et al.*, 1995; Ruiz *et al.*, 1998; Angell and Baulcombe, 1999; Ratcliff *et al.*, 2001). PDS is an enzyme necessary for the biosynthesis of carotenoids, which is colored compounds that protect the green pigment, chlorophyll from photolysis also known as photo-bleaching. Thus when PDS synthesis is interfered by VIGS, photo-bleaching can be observed and used as a discernable phenotypic change (Sandmann and Boger, 1989; Bartley and Scolnik, 1995). BSMV vectors were designed to express untranslatable fragments of PDS mRNA in either the sense or antisense orientation. When this BSMV vector is inoculated into plants the plants exhibited photo-bleaching in systemically infected leaves and phytoene (substrate for PDS) accumulate (Holzberg *et al.*, 2002).

The isolation and analysis of genes involved in disease resistance in wheat is extremely difficult. This is due to the fact that the common bread wheat *(Triticum aestivum)* is hexaploid and thus the wheat genome is extremely large with high ratios of physical to genetic distance and difficulties stem from genetic redundancy due to polyploidy (Arumuganathan and Earle, 1991). That is why wheat is not suitable to be transformed by T-DNA using current methods and why the over-expression of genes in wheat is so difficult. Although genes have been over-expressed in wheat in the past, it was done with difficulty. In the future we may find more optimal methods to transform wheat or methods to manipulate gene expression. Increasing our understanding of wheat's defence response is thus of great importance and will allow us to create wheat lines with increased resistance to RWA and the environment.

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Chapter 2:

The effect of silencing selected photosynthetic related genes on aphid fecundity, plant health and photosynthetic efficiency

2.1. Introduction

Chloroplasts, harboring the photosynthesis machinery, are not only the sole supplier of energy for the plant (Buttner *et al.*, 1992) but are also involved in plant pathogen interactions (Botha *et al.*, 2006a). Electrons exited by photon energy are transported from the acceptor site of the photosystem II to various acceptors (Blankenship, 2002). Oxygen ($O_{2)}$, one of the electron acceptors receive electrons directly from reduced ferredoxin in the Mehler reaction to form superoxide radicals (Mehler, 1951). The superoxide radicals in turn are converted to H_2O_2 and highly reactive hydroxyl radicals ($HO^{\cdot}$) (Orlandi *et al.*, 1992). Reactive oxygen intermediates such as O^{2-}, H_2O_2 and OH^- formed as a product of photosynthesis and from other sources must be regulated to prevent oxidative damage to the chloroplast (Foyer *et al.*, 1994). The regulation is not only controlled by the chloroplast, but also by the nucleus that regulates antioxidant gene expression. Antioxidants buffer redox imbalances which forms part of the defence mechanism. The rate of ROS formation and the strength of the cytosolic antioxidant system determines if the ROS signals created could reach the nucleus for gene regulation (Baier and Dietz, 2005). Russian wheat aphid resistant wheat such as TugelaDN depends on ROS signals for an effective defence response, but for the susceptible Tugela cultivar ROS imbalance is lethal (Botha *et al.*, 2006a).

Toxins injected into wheat by the RWA targets the chloroplast for degradation. This cause chlorotic streaking as chlorophyll *a* and PSI-D gets damaged (Fouché *et al.*, 1984). Lower chlorophyll content and P700 levels and a high nonphotochemical quenching suggests inefficient electron transport (Haldrup *et al.*, 2003). Low PSI-D levels affect the redox state of chloroplast thioredoxin which in turn regulate the enzymatic activity of various enzymes. Enzymes affected include Fru-1,6-bisphosphatase and NADP malate dehydrogenase that are involved in photosynthetic carbon metabolism and cpATPase which are involved in carbon fixation (Haldrup *et al.*, 2003). The expression of various photosynthesis related genes is inhibited in susceptible wheat lines and induced in resistant lines (Botha *et al.*, 2006a; Van Eck, 2007). Included are the mentioned P700, cpATPase and FBPase, but also TMP14 that together with PSI-D form subunits of PSI (Hansson and Vener, 2003) and Glutathione S-transferase (GST) that detoxifies endobiotic and xenobiotic compounds. The direct

targeting of these genes by the RWA and the strategic regulation thereof by resistant wheat plants will affect the ROS balance and the survival of the wheat plant (Botha *et al.*, 2006a).

To elucidate the roles of strategically regulated photosynthesis genes in the existing RWA resistance mechanisms TMP14, FBPase and P700 were selected to be silenced. The VIGS system was optimized for the purpose and molecular, biological and physiological methods were used to analyse the effect on plant resistance. TugelaDN, Tugela *Dn2* and Gamtoos *Dn7* were selected as representatives respectively harbouring the defence mechanisms antibiosis, tolerance and antixenosis. The same methods were also used to compare how the three representatives respond to aphid feeding.

2.2. Materials and Methods

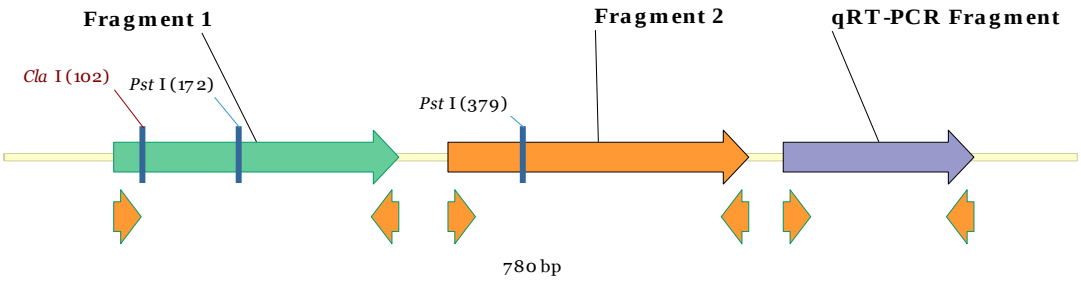
2.2.1. Cultivation of wheat plants

Near-isogenic lines (NILs) of the hexaploid wheat (*Triticum aestivum* L.) were used which includes RWA susceptible Tugela and resistant TugelaDN (PI137739 with the South African wheat accession SA1684) and Tugela *Dn2* (PI 262660 with the South African wheat accession SA2199) that were obtained from the Small Grain Institute, Bethlehem, South Africa (Liu *et al.*, 2001; Tolmay *et al.*, 2006). The resistant Gamtoos-*Dn7* (98M370) was obtained from Prof. N.L.V. Lapitan, Colorado State University, CO, USA. With successive rounds of backcrossing NILs are produced that are closely related, but still contain the gene that has its origin in a different donor. Plants were grown to a 3-leaf stage under greenhouse conditions in a 1:1 soil mixture of sterilized potting soil (Chemserve, Olifantsfontein, South Africa) and soil (Experimental farm, University of Pretoria, South Africa). Growth conditions included a temperature of ± 21°C, a pathogen free environment and a watering interval of 2 days.

2.2.2. Sequences of genes to be silenced

Three gene sequences were selected to establish their putative involvement in enabling plants to cope with the stress associated with RWA feeding. They were Thylakoid membrane phosphoprotein 14 (Tmp14) (Van Eck, 2007), Fructose-1,6-biphosphatase

(FBPase) (Botha *et al.*, 2006a) and p700 chloroplast gene (Botha *et al.*, 2006a). Since the available cDNA clones were inadequate in length, the sequences were used to BLAST the wheat genome database at http://www.compbio.dfci.harvard.edu (assessed on 15.2.2010) for adequate clone lengths. For each gene three fragments were designed, two fragments with sizes ranging between 200 and 500 bp for the use in silencing and the third fragment of size 50-250 bp for qRT-PCR quantification. Each gene was silenced in two separate plant sets, each with a different fragment, the one to confirm the other. **Error! Reference source not found.** illustrate an example of how primers were constructed.



Error! Reference source not found.**:** Schematic representation of the three fragments used in silencing studies. Fragment one and two are for the use of silencing and the qRT-PCR fragment are for transcript quantification.

2.2.3. Virus constructs

Barley stripe mosaic virus constructs were obtained from Dr. Steve Scofield (USDA, Purdue University, West Lafayette, Indiana 47907 (S.R.S., A.S.B.), USA). The alpha and beta plasmids were used unmodified while the gamma plasmids were used to clone the genes of interest into (Figures 2.2, 2.3, 2.4). Appropriate restriction enzymes (see 2.1.6) were used to linearize the plasmids downstream of the insert. Reverse transcriptase reactions were then performed on these plasmids using the mMessage mMachine® kit (Ambion, USA) containing a capped GTP to produce three RNA constructs needed to form a virus body. The RNA mixture of 1 µl of each viral construct were mixed in 22 µl FES buffer (0.754 % (w/v) Glycine, 1.045 % (w/v)

K_2HPO_4, 1 % (w/v) Sodium Pyrophosphate, 1 % (w/v) Bentonite and 1 % (w/v) Celite 545 AW Course.

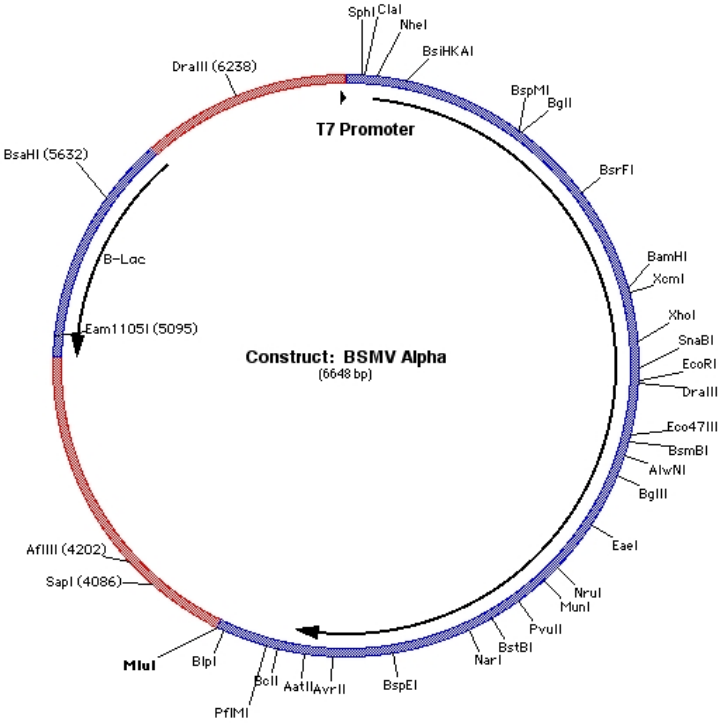


Figure 15: BSMV Alpha (α) construct.**:** BSMV Alpha (α) construct (6,648 bp) (Scofield *et al.*, 2005).

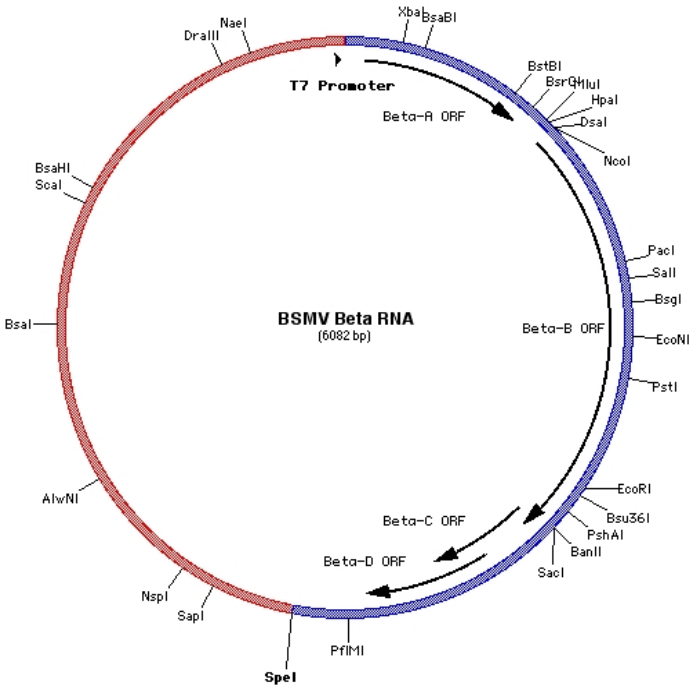


Figure 16: BSMV Beta (β) construct (6,082 bp) (Scofield *et al.*, 2005).

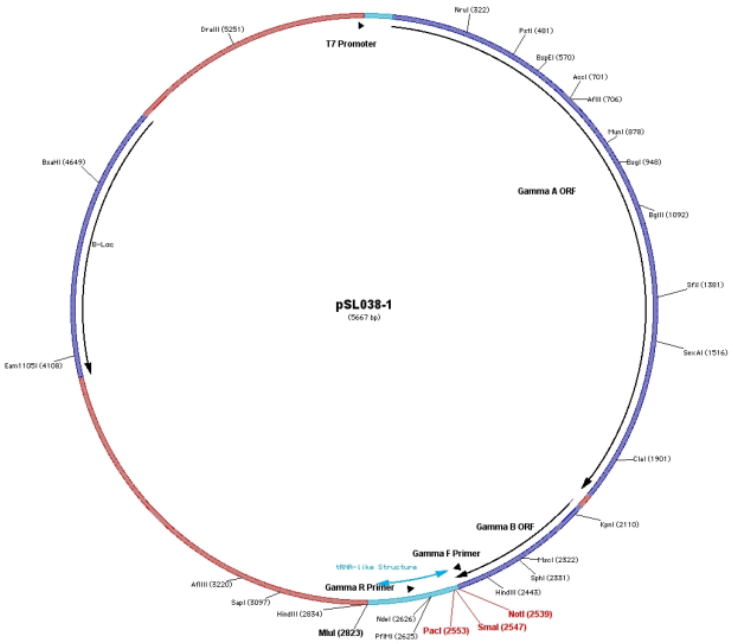


Figure 17.4: BSMV Gamma (γ) construct (5,667 bp) (Scofield *et al.*, 2005).

2.2.4. Plant infection

Barley stripe mosaic virus infection was done by rubbing 25 µl of the RNA, FES buffer mixture prepared in 3.2.2 onto the first and second leaf of the wheat plant. After infection, the plants were watered immediately and placed in sealed polycarbonate containers which have a length of 720 mm, a height of 320 mm and width of 320 mm that prevent any spreading of the viruses by insects and other means. The front and the back of the containers had fine sieve material covering the openings in stead of the poly carbonate panels to allow air movement. With all feeding trials four *D. noxia* adults of the biotype SA1 were applied with a paint brush to the second leaf of each plant and incubated for 35 days except for the control plants that remained uninfested.

2.2.5. RNA extraction

Diethyl pyrocarbonate (DEPC) water was used for RNA extraction and all other RNA related work as well as the treatment of glassware where after the glassware were baked at 200 ˚C. DEPC water was prepared by treating distilled water with 0.1 % (v/v) DEPC for 1 h and was autoclaved thereafter (Sambrook *et al.*, 1989). RNAse away (Molecular

BioProducts, Inc.) was used to prevent RNAse degradation of RNA samples by treating surfaces, gloves and equipment.

Plant material, composed only of the youngest leaf of the plant, was harvested into liquid N_2 and stored at -80 °C prior to RNA isolation. The plant material was ground to a fine powder in liquid nitrogen for the extraction of total RNA using a modified method of Chomczynski and Sacchi (1987). Approximately 0.1 g frozen ground leaf material per sample was homogenized in 1 ml guanidinium isothiocyanate (GITC) extraction buffer (4 M guanidine isothiocyanate, 25 mM sodium acetate, pH 6.0, and 0.8 % (v/v) β-mercaptoethanol) (Chomczynski and Sacchi, 1987). The mixture was incubated for 10 min at room temperature and centrifuged at 10,000 rpm (9200 g) for 20 min. The supernatant was transferred to a new tube where after sodium acetate (0.2 M, pH 4) and phenol: chloroform (1:1) were added before it was mixed and incubated at room temperature for 10 min. After centrifugation at 10,000 rpm (9200 g) the supernatant was transferred to a new tube, 1 volume isoproponol was added to 1 volume supernatant and mixed and incubated at -20 °C for 2 hours. The samples were centrifuged at 13,000 rpm (12400 g) for 30 min, the supernatant was discarded and washed with 75 % (v/v) ethanol. It was then centrifuged for a further 10 min at 10,000 rpm (9200 g) at 4 ^{o}C and the pellet washed with 75 % (v/v) ethanol. The RNA pellet was then dissolved in DEPC treated water.

The DNA was removed by treatment of samples with 1 U RNase free DNase and 4 µl RT buffer (0.25 M Tris-HCl, 0.5 M KCl, 30 mM $MgCl_2$, 25 mM DTT) per 15 µl RNA sample. The total RNA was then cleaned using the RNeasy® mini kit as prescribed by the manufacturers (Qiagen, USA).

The RNA concentration was determined with the Nanodrop® ND-1000 spectrophotometer (Nanodrop technologies) and expressed as $\mu g.ml^{-1}$ (Sambrook *et al*., 1989). To confirm the quality and quantity of the extracted RNA, 200 ng RNA of each time point was separated on a 1 % (w/v) agarose gel using TAE buffer (0.4 M Tris pH 8.0, 18 mM acetic acid, 10 mM EDTA pH 8.0) containing Goldview™ Nucleic Acid Stain (Beijing sBs Genetech Co. Ltd.) for visualization.

2.2.6. Cloning of fragments

The A1, A2, B1, B2, D1 and D2 fragments of the specific genes were amplified using the PCR technique with the indicated primers (Table 2.1) and cloned into the pSLO39b (pBSMV Gamma) plasmid by digesting the plasmid and the fragments using Not1, Pac1 or Sma1 restriction enzymes (New England Biolabs$_{inc.}$). Reaction conditions were: 1X NEBuffer 3, 100 ng.ml^{-1} BSA and incubation at 37 ºC; 1X NEBuffer 1, 100 ng.ml^{-1} BSA and incubation at 37 ºC; 1X NEBuffer 4 and incubation at 25 ºC respectively. Ligations of the specific fragment and plasmid were performed over-night at 4 °C using T4 DNA ligase (Fermentas) according to manufacturers' instructions. Five µl of the ligation reaction was mixed with 40 µl heat shock competent DH5 *E. coli* cells (NEB) in a chilled eppendorf and incubated on ice for 30 min. The mixture was heat shocked for 30 sec at 42 ºC. One ml of SOC medium (2 % (w/v) Tryptone, 0.5 % (w/v) Yeast extract, 8.6 mM NaCl, 2.5 mM KCl, 20 mM $MgSO_4$, 20 mM glucose) was added and transferred to an eppendorf and incubated at 37 °C for one hour. The transformed cells were plated at 1:100 and 1:1000 dilutions on LB plates (1 % (w/v) Bacto-Tryptone, 0.5 % (w/v) Yeast-extract, 1 % (w/v) NaCl, 1 % (w/v) agar, and 50 µg.ml^{-1} ampicillin). The plates were then incubated overnight at 37 °C and positive ampicillin resistant screening was used to select for transformed colonies containing recombinant plasmids. Colonies were picked up and added to a PCR mixture and amplification was done using primers selecting for the correct fragment on size.

2.2.7. Screening for recombinant transformants

The specific colonies were first inoculated and grown overnight in LB medium (1 % (w/v) Bacto-Tryptone, 0.5 % (w/v) Yeast-extract, 1 % (w/v) NaCl, and 50 µg.ml^{-1} ampicillin). The cells were harvested and the transformed plasmids were then extracted from the selected colonies with the plasmid miniprep extraction kit (GeneJet^{TM} Plasmid miniprep kit, Fermentas) according to the manufacturers' instructions.

PCR were performed on plasmids with specific primers (3.2.2; Table 2.1) and amplicons were separated on a 1 % (w/v) agarose gel to confirm the presence of an insert.

2.2.8. Agarose gel electrophoresis

A 1 % (w/v) agarose gel was prepared and loading buffer (0.25 % (w/v) bromophenol blue, 0.25 % (w/v) orange G, 0.375 M ficoll) was added to all samples at a 1:3 ratio. The gel was run at 12 $V.cm^{-1}$ for 1 h using 1X TAE as running buffer. To visualize the separated DNA or RNA, the gel was exposed to ultraviolet light with a 305 nm wavelength and photographed.

2.2.9. Polymerase chain reaction

The PCR reaction (10 µl total volume) was done on the GeneAmp® PCR System 9700 and contained 10 pmol of both forward and reverse primers, 1.25 U Amplitaq, 1 µl 10X buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl, and 0.01 % (w/v) gelatin), 2.5 mM $MgCl_2$ and 0.2 mM deoxy nucleotide triphosphate mix (dNTP's). The amplification procedure was as follows; one cycle at 94 °C for 5 min, 30 cycles including 94 °C for 30 s, for 30 s at specific temperature of primer T_m, 72 °C for 1 min and one step at 72 °C for 7 min.

2.2.10. Quantitative real time PCR (qRT-PCR)

qRT-PCR was performed using the iScript™ One-step RT-PCR Kit SYBR® Green (BioRad, USA). The reaction mixture was prepared on ice. The 20 µl mixture included 17 $ng.\mu l^{-1}$ total RNA template, 10 µl iScript Reverse Transcriptase Reaction Mix, 10 pmol of each of the forward and reverse primers (Table 2.1) and 0.4 µl iScript Reverse Transcriptase. The amplification procedure was as follows; one cycle at 50 ^oC for 10 min for cDNA synthesis, one cycle at 95 ^oC for 5 min for iScript inactivation, ten cycles at 95^oC for 10 s, (primer T_m ^oC specific) for 30 s for PCR cycling and data collection and a melt curve analysis step that included 1 min at 95 ºC, 1 min at 55 ºC and 10 sec at 55 ºC (80 cycles, increasing each by 0.5 ºC each cycle). A 10-fold total RNA dilution series ranging from 10^{-1} to 10^{-5} was prepared in triplicate to generate standard curves. The expression of 16S rRNA and HSP90 genes were used as references as they do not show any regulation under RWA feeding (Botha *et al.*, unpublished results). The data generated were analyzed using the iCycler (BioRad) to

generate PCR Amp/Cycle - and Melt Curve graphs. Transcript expression was calculated by reading the concentration RNA from the standard curve using the Ct values obtained from each sample (Pfaffl, 2007).

Transcript quantification was performed on the silenced plants compared with control plants to confirm silencing. The regulation of the transcripts in non silenced plants with aphid feeding was also analyzed.

2.2.11. Primer designing

Primers were designed using Primer designer 3, version 0.4.0 (http://frodo.wi.mit.edu/primer3/). The primers used to amplify specific fragments for cloning contained Pac1 and Not1 restriction enzyme palindromes on the 5' end of the forward and reverse primers, respectively. This allowed the fragments to be digested with Pac1 and Not1 restriction enzymes and cloned into the virus vector in an antisense orientation.

Table 6 **:** Primer sequences.

Primer name	Primers	Sequence
TMP14 qRT-PCR	A1-F	5’-TTCGCTAAGGTCAGGAACAT-3’
TMP14 qRT-PCR	A1-R	5’-ATCGTCTAGCCGCTGATTAT-3’
TMP14 Fragment 1	A2-F	5’-ATATTAATTAACTGCGTACAAGAACCTGCTATT-3’
TMP14 Fragment 1	A3-R	5’-TATGCGGCCGCCTATTGCGTCAATATGTCCAACT-3’
TMP14 Fragment 2	A_F3-F	5'-ATATTAATTAACTTCCGAAGGCATCGATCTCCAT-3'
TMP14 Fragment 2	A_F3-R	5'-TATGCGGCCGCCTACAAGCCGTTGAAGAACTCTG-3'
FBFase qRT-PCR	B1-F	5’-CGTCCGTGTCATTCAGTGTA-3’
FBFase qRT-PCR	B1-R	5’-CGTGCCATCATCAGTGGATT-3’
FBFase Fragment 1	B2-F	5’-ATATTAATTAACTGTACGTGGAGAAGTGCAAGT-3’
FBFase Fragment 1	B3-R	5’-TATGCGGCCGCCTACCTCCTCCATCAGGAATGAC-3’
FBFase Fragment 2	B_F3-F	5'-ATATTAATTAACTCAACGAGGTGTTCGTCAATG-3'
FBFase Fragment 2	B_F3-R	5'-TATGCGGCCGCCTACGGCGGTATCTTGATATCTG-3'
P700 qRT-PCR	D1-F	5’-GAGAATACGCCTCCTCAACG-3’
P700 qRT-PCR	D1-R	5’-GAAAACCGCTCCACAGAAC-3’
P700 Fragment 1	D2-F	5’-ATATTAATTAAAGCAAGACGTGCTTCTATTC-3’
P700 Fragment 1	D2-R	5’-TATGCGGCCGCAATCGTCCGCAGGTACATAA-3’
P700 Fragment 2	D3-F	5’-ATATTAATTAATGGATGCTACTACCGTACTT-3’
P700 Fragment 2	D3-R	5’-TATGCGGCCGCAACGATCCTCCTCCGATAAT-3’

2.2.12. Measuring Chl *a* fluorescence

Chlorophyll fluorescence of the resistant and susceptible wheat lines was measured using the OS-30p Chlorophyll Fluorometer from Opti-sciences (USA). Readings were taken at time 0 h, being the time just before infection, and 10 days post infection (d.p.i.). For each parameter readings were taken from three plants to serve as biological repeats and three readings were taken per plant on the newest leaf ± 50 mm from the stem of each plant which serves as technical repeats. Selected leaves were allowed to adapt to the dark for 3 min using the provided clips before the maximum quantum yield (variable Chl fluorescence (Fv)/maximal fluorescence yield (Fm)) and the OJIP kinetics were measured.

The equation applied for the measurement of quantum yield is as follows: Fv/Fm = (Fm-Fo)/Fm where Fo (O) is the basal level of fluorescence, Fm (P) is the maximal level of fluorescence and Fv is the excitation difference. The fluorescence rise from O-to P-level passes through two intermediate steps J (I1) and I (I2) (Barthélemy *et al.*, 1997; Posp´ıšil and Dau, 2000; Joly *et al.*, 2005). The OJIP readings provide information on the physical condition of the plant's photosynthesis and kinetic profile obtained illustrate possible restrictions in the excitation energy.

First the wheat lines were analyzed in triplicate without any infection to determine if the basal level of photosynthesis differed. This could indicate a pre recognition advantage in the resistant lines compared to the susceptible line. Aphids were then allowed to feed on the plants and after ten days the change in photosynthesis were analyzed between the lines to measure any differences in photosynthesis. Photosynthesis was also measured in the silenced plants for a use as a biological assay in the conformation of silencing. The same was done for silenced plants with aphids to determine if photosynthesis further decrease.

2.2.13. Extraction and analysis of Chl *a* and Chl *b*

Approximately 50 mm of new leaf material was sampled of each of the three biological repeats, weighed and corrected to the mass of 0.054 g ± 0.007 g. Each sample was then placed in 1.5 ml 80 % (v/v) acetone and left overnight in the dark. Chlorophyll *a* (Chl *a*) and Chlorophyll *b* (Chl *b*) content were measured spectrophotometrically at 664 and 647 nm respectively (Porra *et al.*, 1989). Chlorophyll concentrations were expressed as $mg.l^{-1}$.

2.2.14. Statistical analysis of data

All the data obtained from chlorophyll content, chlorophyll fluorescence, aphid fecundity and real time PCR were analyzed with two way ANOVA statistics. The ANOVA statistics was done to determine if the data have significant statistical value. At least three biological repeats and two technical repeats were obtained for each data point. The *F* values obtained were referenced to the *F* distribution table and if the

values were higher than the value calculated by using degree of freedom, the data set was accepted. The robustness values for the data sets are calculated and presented with the data.

2.2.15. Assessing plant health and aphid fecundity

Changes in the plant's health were assessed and a score was assigned ranging from 0 % to 100 % (Tables 2.2 and 2.3) and phenotypic symptoms were photographed using a Canon SLR 300. The plant's health was documented to illustrate the effect of the aphid on the performance of the different wheat lines and the effect silencing of the genes has on resistance. It is also used to illustrate what effect the BSMV virus has on the plant.

Aphid fecundity served as a bioassay for the silencing of the genes of interest and indicates whether the gene play a significant role in the defence response in response to aphid feeding. Aphids were counted by eye at a five day-interval after infection of the plant up to 35 days after infection. Three biological repeats were assayed.

Table 7: Plant health documented. Plant health is scored according to the percentage of green leaf area vs. white areas. From 100 % to 60 % are explained.

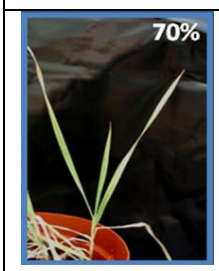
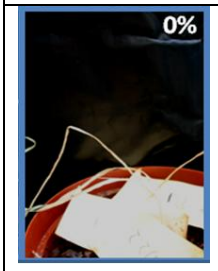
		The plant has no phenotypic effects and leaf areas are green.
		Slight color changes can be observed and leafs are slightly curled.
		Plant seem healthy but leafs start to show phenotypic signs of photobleaching.
		Plant still looks healthy with increased photobleaching and increased leaf curling.
		Considerable photo bleached areas, a visible increase in leaf curling and no new plant growth can be observed.

Table 8: Plant health documented in percentages. Plant health is scored according to the percentage of green leaf area vs. white areas. From 50 % to 0 % are explained.

		Plant has equal photobleaching and green areas.
		Plant start dying with 40% green areas left.
		Plant starts drying out with 30% green area left.
		Plant still show green patches.
		Plant still standing with minor green leaf area.
		Plants collapse and are dry.

2.3. Results

2.3.1. Cloning of gene fragments into viral vectors for silencing

Plasmids received from Dr. Scofield were transformed into DH5 α (*E.coli*) competent cells that were allowed to grown for the purpose of increasing the amount of plasmid for further work. The plasmids were then extracted with the GeneJet ™ Plasmid Miniprep kit (Fermentas) and run on a 1 % agarose gel (Figure 18).

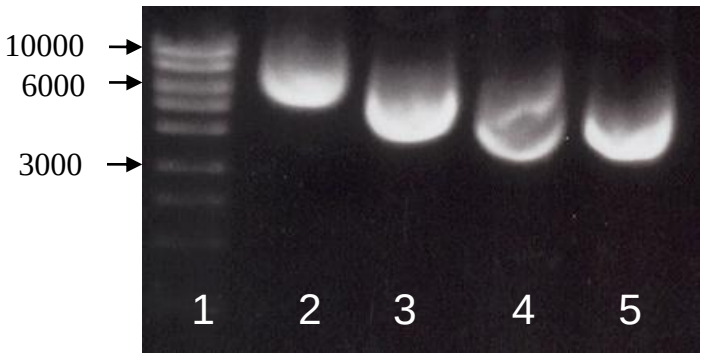


Figure 18: Vector plasmids obtained after a miniprep using GeneJet ™ Plasmid Miniprep kit (Fermentas). Where 1 = molecular size marker (MassRuler™, high range DNA ladder, Fermentas); 2 = BSMV Alpha (α) construct (6,648 bp); 3 = BSMV Beta (β) construct (6,082 bp); 4 = BSMV Gamma (γ) construct (5,667 bp); 5 = BSMV Gamma (γ) construct containing the PDS insert (5,734 bp).

The agarose gel revealed that the plasmids received from Dr. Scofield were of good quality and could be used for cloning and preparing RNA transcripts.

The fragments to be used during gene silencing studies were amplified using primers indicated in Table 6: Primer sequences.. The fragments of the TMP14 gene (A1 and A2) are shown in Figure 19, fragments of the FBPase gene (B1 and B2) in Figure 19, and fragments of the P700 gene (D1 and D2) are shown in Figure 20.

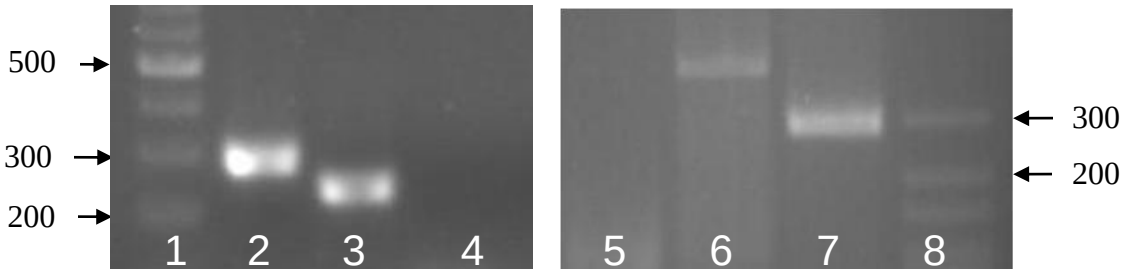


Figure 19: PCR fragments after amplification and separation on a 1 % (w/v) agarose gel. Where 1 = molecular size marker (MassRuler^{TM}, high range DNA ladder, Fermentas); 2 = PCR amplified fragment 1 of the TMP14 gene (276); 3 = PCR amplified fragment 1 of the FBPase gene (224); 4 and 5 = Control PCR; 6 = FBPase fragment 2 (409 bp); 7 = TMP14 fragment 2 (300 bp); 8 = molecular size marker (O'GeneRuler^{TM}, Ultra low DNA ladder, Fermentas).

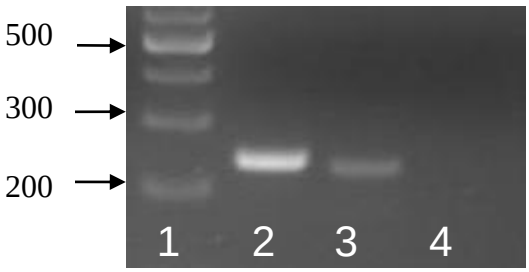


Figure 207: PCR fragments after amplification and separation of a 1 % (w/v) agarose gel. Where 1 = molecular size marker (MassRuler^{TM}, high range DNA ladder, Fermentas); 2 = PCR amplified fragment 1 of the p700 gene (246); 3 = PCR amplified fragment 2 of the p700 gene (234); 4 = Control PCR.

Primers were designed to amplify fragments with the specific sizes, i.e. 276 and 300 bp for the TMP14 gene, 224 and 409 bp for the FBPase gene and 246 and 234 bp for the P700 gene (Table 6: Primer sequences.). The bands observed in lane 2, 3, 6 and 7 from Figure 19 and lanes 2 and 3 from Figure 20 indicate that the PCR products obtained are the correct sized gene fragments.

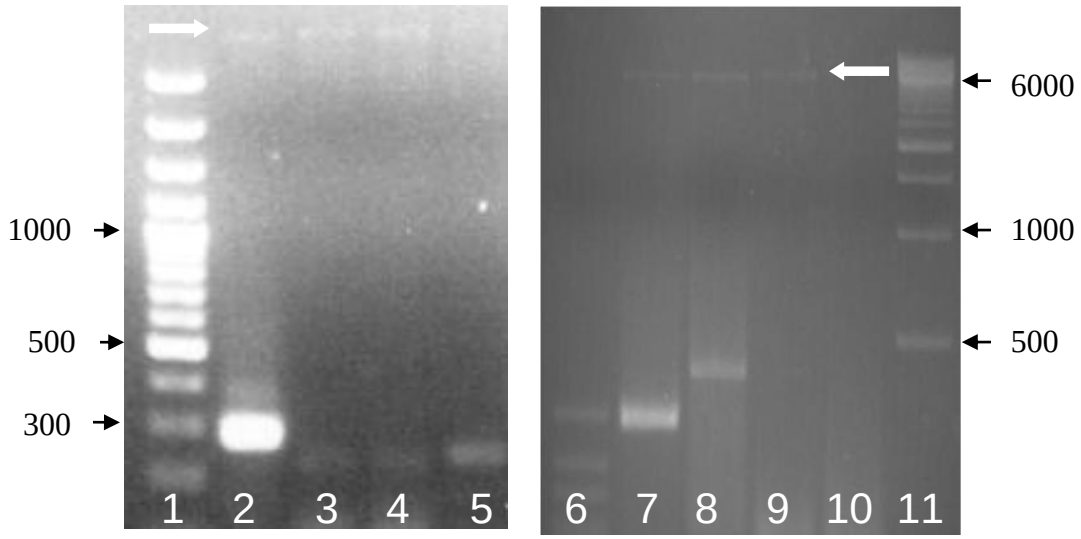


Figure 21: PCR confirmation of specific gene fragments cloned into plasmids. Where 1 = molecular size marker (MassRuler^{TM}, high range DNA ladder, Fermentas); 2 = TMP14 fragment 1 (276 bp); 3 = FBPase fragment 2 (224 bp); 4 = p700 fragment 1 (246 bp); 5 = p700 fragment 2 (234); 6 = molecular size marker (O'GeneRuler^{TM}, Ultra low DNA ladder, Fermentas); 7 = TMP14 fragment 1 (300 bp); 8 = FBPase fragment 2 (409 bp); 9 = plasmid without insert; 10 = negative control PCR; 11 = molecular size marker (O'GeneRuler^{TM}, 500 bp DNA ladder, Fermentas). The white arrows indicate the plasmids used as templates for the amplification of the fragments and has a size of ± 6,000 bp.

PCR confirmation was done on each plasmid to determine if the correct fragments were inserted (Figure 21). Fragments of the TMP14, FBPase and P700 genes were of sizes 276 and 300 bp, 224 and 409 bp, and 246 and 234 bp, respectively.

2.3.2. Preparation of BSMV constructs

Bands in lanes 2, 3 and 4 observed in Figure 22 with sizes 6,648, 5,082 and 5,743 bp respectively indicated that the plasmids were of the correct sizes and of good quality meaning no plasmid breakage could be observed.

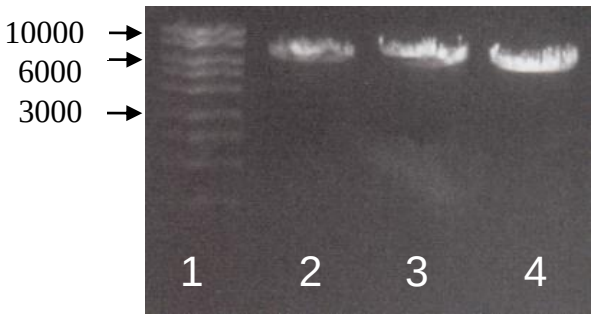


Figure 22: Plasmids linearized using Mlu1 restriction enzyme and separated on a 1 % (w/v) agarose gel. Where 1 = molecular size marker (MassRuler^{TM}, high range DNA ladder, Fermentas); 2 = BSMV Alpha (α) construct (6648 bp); 3 = BSMV Betha (β) construct (6082 bp); 4 = BSMV Gamma (γ) construct containing PDS insert (5743 bp);

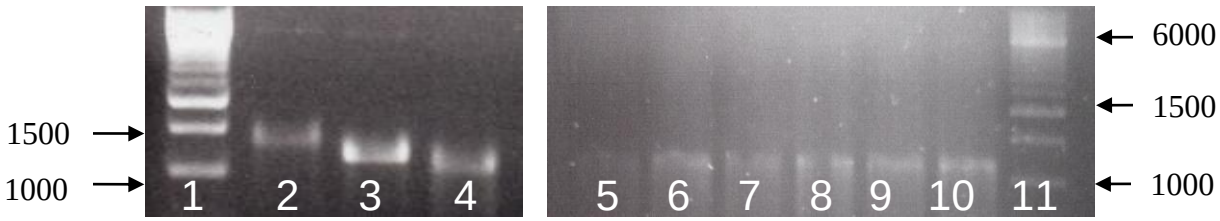


Figure 23: Viral RNA produced from the liniarized plasmids using the mMachine mMessage kit and separated on a 1 % (w/v) agarose gel. Where 1 = molecular size marker (MassRuler^{TM}, high range DNA ladder, Fermentas); 2 = BSMV Alpha (α) RNA molecule; 3 = BSMV Betha (β) RNA molecule; 4 = BSMV Gamma (γ) RNA molecule containing PDS insert; 5 and 6 = BSMV Gamma (γ) RNA molecule each containing one of the TMP14 fragments; 7 and 8 = BSMV Gamma (γ) RNA molecule each containing one of the FBPase fragments; 9 and 10 = BSMV Gamma (γ) RNA molecule each containing one of the P700 fragments 11 = (MassRuler^{TM}, high range DNA ladder, Fermentas).

The three different RNA constructs of the tripartheid BSMV virus and the RNA constructs of the cloned fragments can be seen on Figure 23, ranging between 1,200 and 1,400 bp in size.

2.3.3. Confirmation of gene silencing using quantitative real-time PCR

The virus RNA containing the genes to be silenced was applied to the resistant plants. Four aphids were applied to each plant except for the negative control and thereafter aphid fecundity was analyzed. After 10 days the leaves were sampled and total RNA was extracted from uninfested plants, the infected plants and plants that were infested as well as infected (Figure 24) (Refer to 2.2.8).

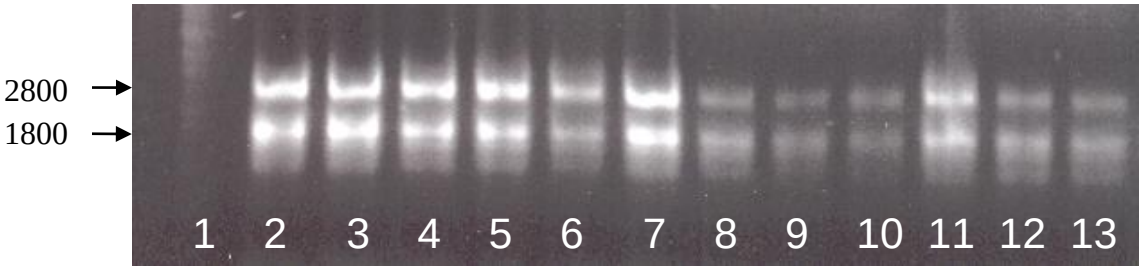


Figure 24: RNA extracted of wheat plants using GITC extraction method and separated on a 1 % (w/v) agarose gel. Where 1 = molecular size marker (MassRuler^{TM}, high range DNA ladder, Fermentas); 2 = Tugela 0 dpi; 3 = Tugela 10 dpi, 4 = Tugela + aphid 10 dpi; 5 = TugelaDN 0 dpi; 6 = TugelaDN 10 dpi; 7 = TugelaDN + aphid 10 dpi; 8 = TugelaDN + BSMV:TMP14_a + aphid 10 dpi; 9 = TugelaDN + BSMV:TMP14_b + aphid 10 dpi; 10 = TuglelaDN + BSMV:FBPase_a + aphid 10 dpi; 11 = TugelaDN + BSMV:FBPase_b + aphid 10 dpi; 12 = TugelaDN + BSMV:p700_a + aphid 10 dpi; 13 = TugelaDN + BSMV:p700_b + aphid 10 dpi.

Figure 24 illustrate RNA extracted from Tugula and TugelaDN. The RNA can be clearly seen on the gel and are of good quality as the 28S and 5S bands are clearly visible. A260/A280 measurements indicated the RNA of the samples is of good quality since the 260/280 value were 2 in most instances, except for one of the TugelaDN:FBPase silenced plants (Table 9). Thereafter the RNA concentrations of the samples were diluted to a concentration of ± 20 ng/ µl for RT-PCR analysis.

Table 9: RNA concentrations of all extracted and corrected RNA UI = u

Sample	A260	A280	260/280	Sample	A260	A280	260/280	Sample	A260	A280	260/280
Tugela_UI_3.2	3.479	1.573	2.21	Tugela_UI_3.1	0.370	0.167	2.22	Tugela_UI_2.1	2.706	1.237	2.19
Tugela _I_4.1	9.365	4.314	2.17	Tugela_I_5.1	5.783	2.617	2.21	Tugela_I_1.1	3.024	1.429	2.12
TugelaDN_UI_3.2	0.339	0.169	2.01	TugelaDN_UI_4.1	1.662	0.759	2.19	TugelaDN_UI_4.2	1.071	0.492	2.18
TugelaDN_I	5.736	2.619	2.19	TugelaDN_I_2.1	3.012	1.398	2.15	TugelaDN_Aphids_1.1	0.756	0.352	2.15
TugelaDN:TMP14_1	5.249	2.370	2.21	TugelaDN:TMP14_1	4.150	1.861	2.23	TugelaDN:TMP14_1	2.050	0.895	2.29
TugelaDN:TMP14_2	2.916	1.363	2.14	TugelaDN:TMP14_2	2.080	1.014	2.05	TugelaDN:TMP14_2	1.580	0.72	2.19
TugelaDN:FBPase_1	0.892	0.432	2.07	TugelaDN:FBPase_1	0.274	0.107	2.56	TugelaDN:FBPase_1	0.801	0.353	2.21
TugelaDN:FBPase_2	1.374	0.657	2.09	TugelaDN:FBPase_2	2.787	1.27	2.19	TugelaDN:FBPase_2	0.1412	0.066	1.43
TugelaDN:P700_1	4.993	2.453	2.03	TugelaDN:P700_1	5.326	2.432	2.19	TugelaDN:P700_1	2.810	1.332	2.11
TugelaDN:P700_2	2.91	1.36	2.14	TugelaDN:P700_2	3.202	1.157	2.04	TugelaDN:P700_2	4.878	2.344	2.09
Tugela *Dn2*_UI_1.1	3.132	1.475	2.12	Tugela *Dn2*_UI_4.2	4.949	2.352	2.10	Tugela *Dn2*_UI_5.1	5.149	2.441	2.11
Tugela *Dn2*_I_5.1	8.55	3.966	2.16	Tugela *Dn2*_I_1.1	0.831	0.387	2.15	Tugela *Dn2*_I_2.1	2.697	1.280	2.11
Tugela *Dn2*:TMP14_1	2.923	1.323	2.21	Tugela *Dn2*:TMP14_1	6.413	2.902	2.21	Tugela *Dn2*:TMP14_1	2.505	1.21	2.07
Tugela *Dn2*:TMP14_2	2.616	1.234	2.12	Tugela *Dn2*:TMP14_2	6.315	2.965	2.13	Tugela *Dn2*:TMP14_2	3.120	1.443	2.15
Tugela *Dn2*:FBPase_1	3.523	1.753	2.01	Tugela *Dn2*:FBPase_1	2.524	1.132	2.23	Tugela *Dn2*:FBPase_1	4.800	2.212	2.17
Tugela *Dn2*:FBPase_2	1.856	0.897	2.07	Tugela *Dn2*:FBPase_2	3.256	1.653	1.97	Tugela *Dn2*:FBPase_2	3.360	1.563	2.15
Tugela *Dn2*:P700_1	2.817	1.275	2.21	Tugela *Dn2*:P700_1	3.754	1.676	2.24	Tugela *Dn2*:P700_1	1.066	0.520	2.05
Tugela *Dn2*:P700_2	3.205	1.512	2.12	Tugela *Dn2*:P700_2	3.300	1.564	2.11	Tugela *Dn2*:P700_2	2.133	0.974	2.19
Gamtoos *Dn7*_UI_1.1	2.159	1.001	2.16	Gamtoos *Dn7*_UI_2.1	2.057	0.951	2.16	Gamtoos *Dn7*_UI_3.1	4.582	2.138	2.14
Gamtoos *Dn7*_I_5.1	3.776	1.790	2.11	Gamtoos *Dn7*_I_3.1	2.074	0.954	2.17	Gamtoos *Dn7*_I_4.1	2.839	1.357	2.09
Gamtoos *Dn7*:TMP14_1	1.536	0.692	2.22	Gamtoos *Dn7*:TMP14_1	0.269	0.127	2.12	Gamtoos *Dn7*:TMP14_1	4.519	2.045	2.21
Gamtoos *Dn7*:TMP14_2	4.779	2.673	1.79	Gamtoos *Dn7*:TMP14_2	3.123	1.435	2.18	Gamtoos *Dn7*:TMP14_2	2.259	1.124	2.01
Gamtoos *Dn7*:FBPase_1	0.322	0.145	2.22	Gamtoos *Dn7*:FBPase_1	3.349	1.565	2.14	Gamtoos *Dn7*:FBPase_1	1.925	0.875	2.20
Gamtoos *Dn7*:FBPase_2	4.279	1.981	2.16	Gamtoos *Dn7*:FBPase_2	4.990	2.354	2.12	Gamtoos *Dn7*:FBPase_2	2.049	0.956	2.14
Gamtoos *Dn7*:P700_1	4.878	2.312	2.11	Gamtoos *Dn7*:P700_1	2,417	1.221	1.98	Gamtoos *Dn7*:P700_1	2.476	1.232	2.01
Gamtoos *Dn7*:P700_2	0.516	0.24	2.15	Gamtoos *Dn7*:P700_2	1.190	0.567	2.10	Gamtoos *Dn7*:P700_2	3.557	1.686	2.11

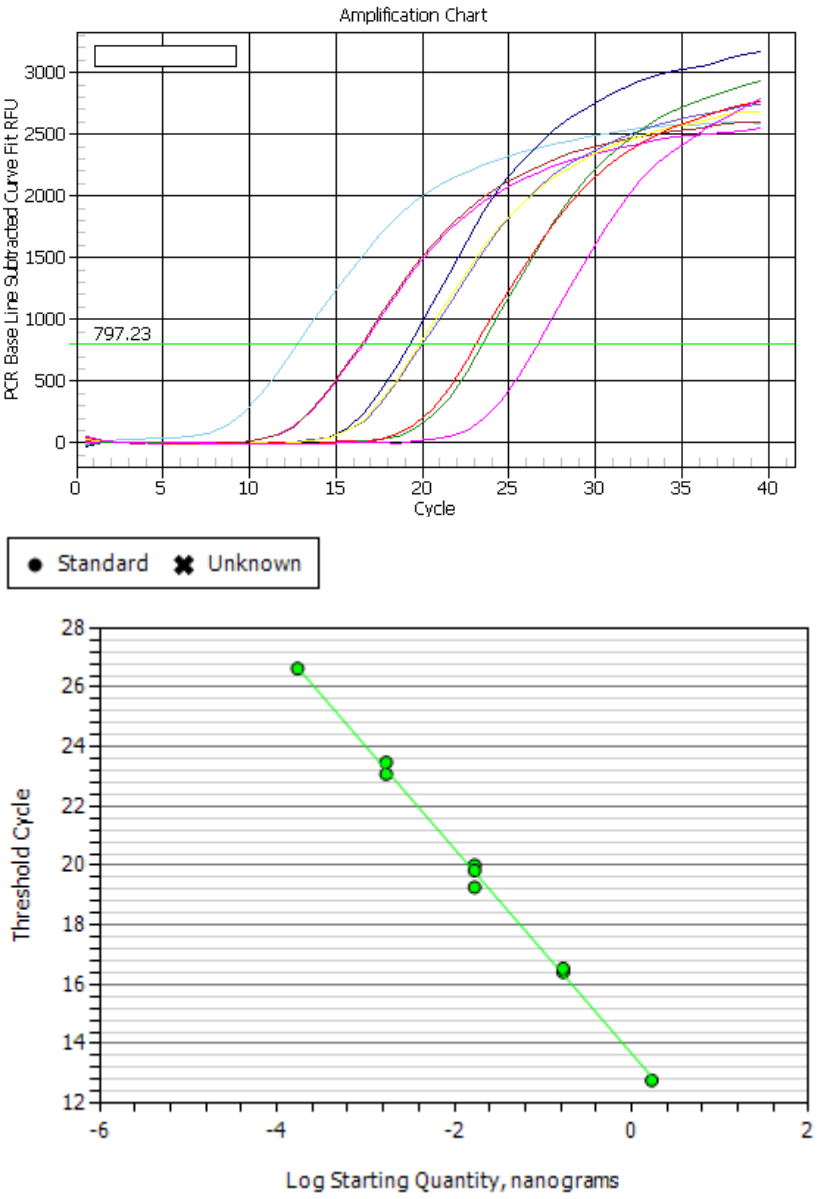


Figure 25: Standard curve graph for the 16S rRNA primer set.

The TugelaDN sample was used for the serial dilution during construction of the primer standard curves. Both 16S rRNA and HSP90 were used as reference genes. Standard curves must have a correlation coefficient above 0.98 and PCR efficiency between 95 % and 105 %. The standard curve graph of 16S rRNA has an acceptable correlation coefficient of 0.998, a PCR efficiency of 98.2 % and a slope of -3.366 (Figure 25).

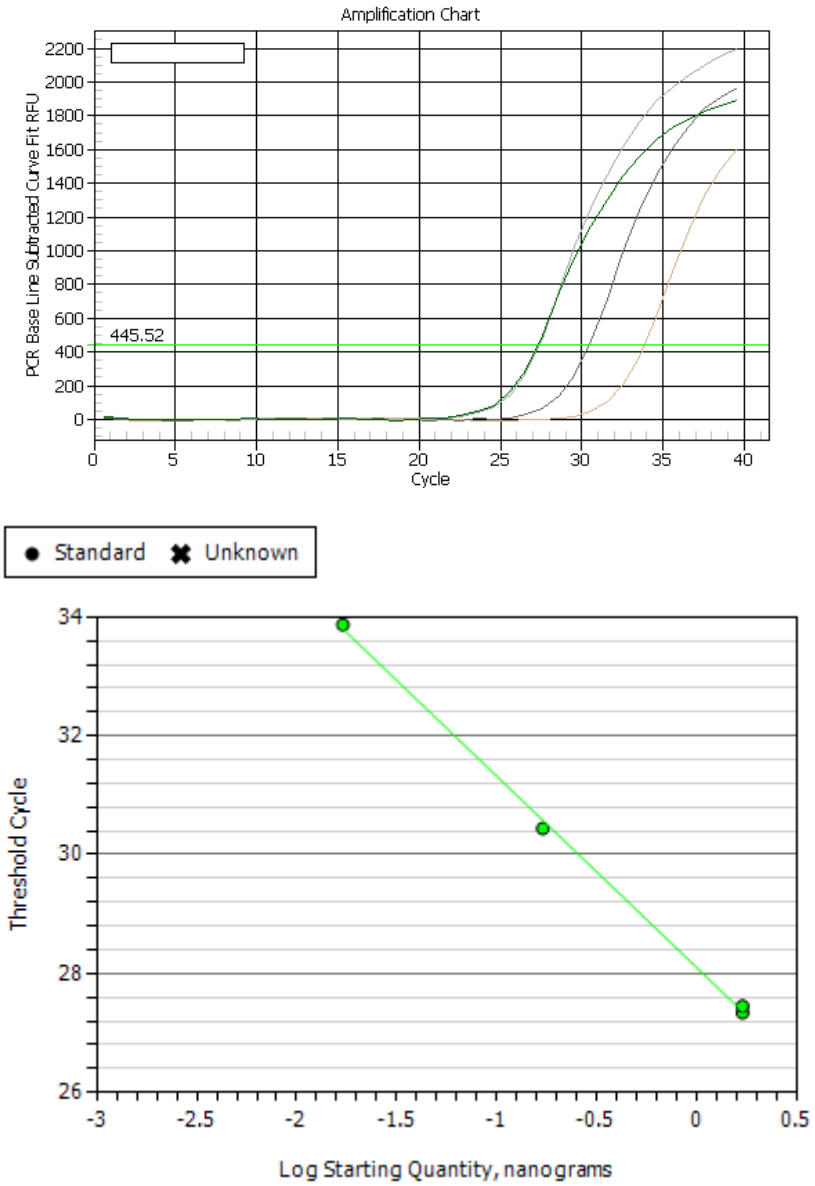


Figure 26: Standard Curve Graph for primer set HSP90.

The PCR efficiency of the HSP90 standard curve was 104.8% and correlation coefficient of 0.993 that are both in the accepted margins. The graph illustrates good quality with a slope of -3.211 (Figure 26).

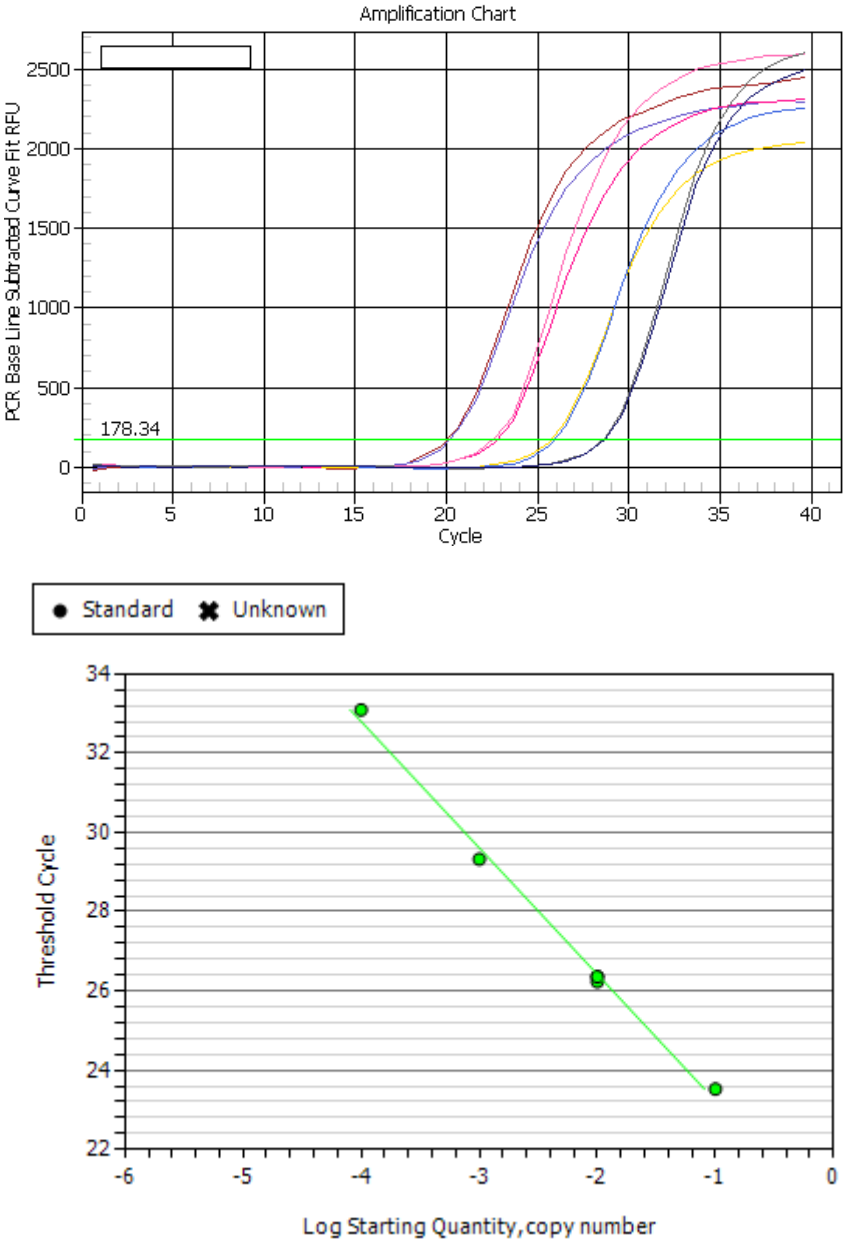


Figure 27: Standard curve graph for primer set FBPase.

The standard curve graph of the FBPase gene indicated a PCR efficiency of 105.7%, correlation coefficient of 0.995 and a graph slope of -3.193 (Figure 27).

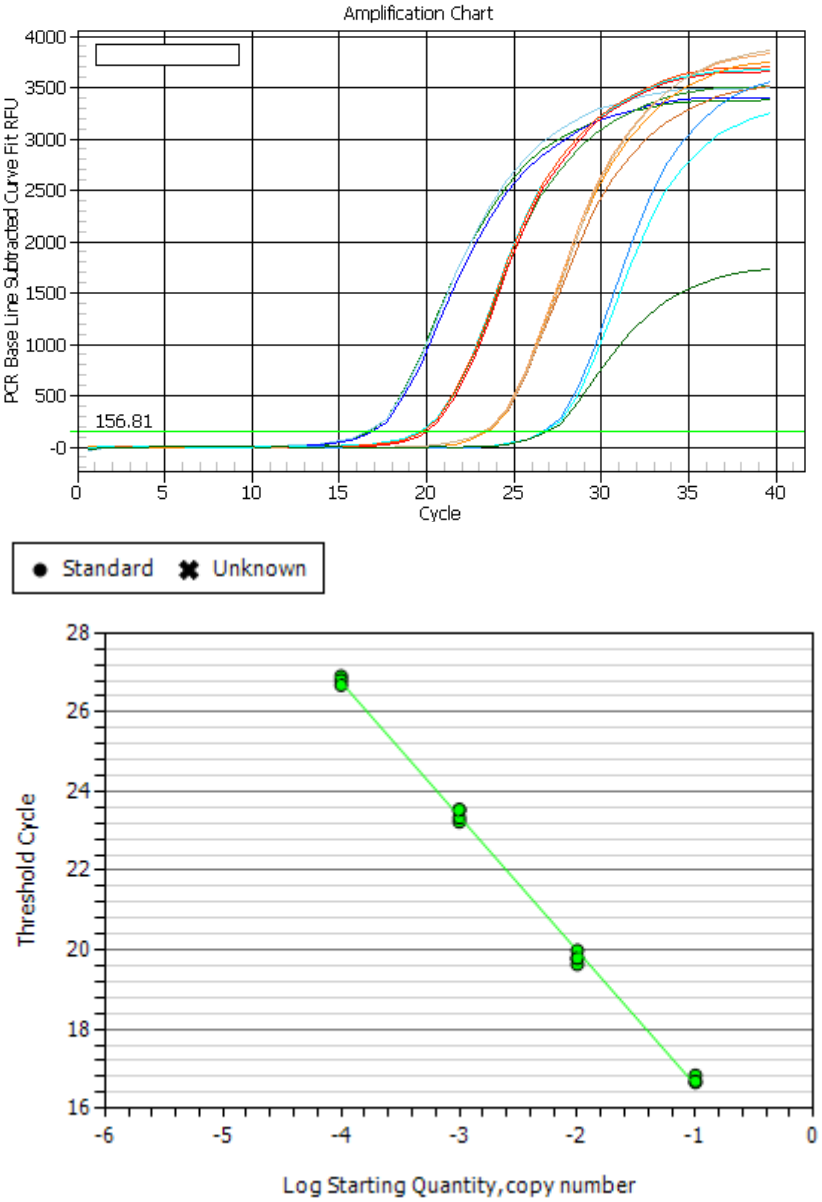


Figure 28: Standard curve graph for primer set TMP14.

The standard curve graph of the TMP14 gene indicated a PCR efficiency of 97.1%, a correlation coefficient of 0.998 and a graph slope of -3.392 (Figure 28).

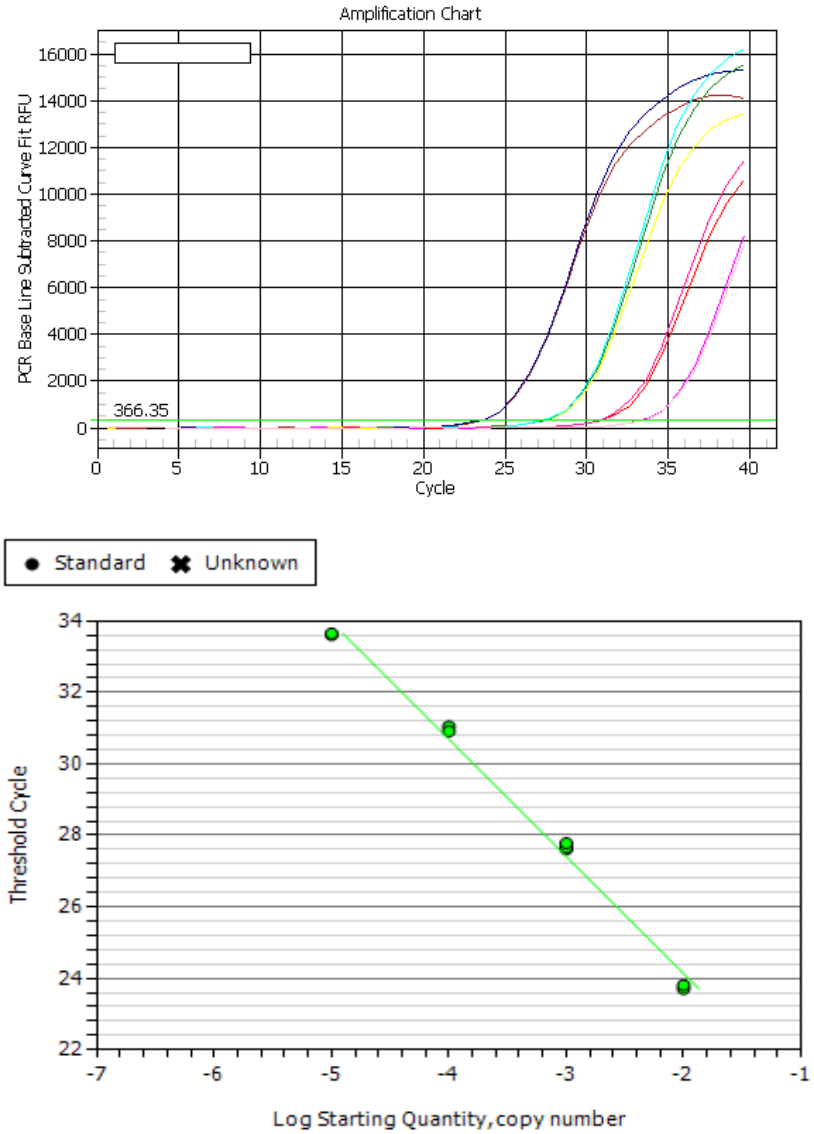


Figure 29: Standard Curve Graph for primer set P700.

The P700 standard curve has a slope of -3.281 with a correlation coefficient 0.992 and a PCR efficiency of 101.7% (Figure 29).

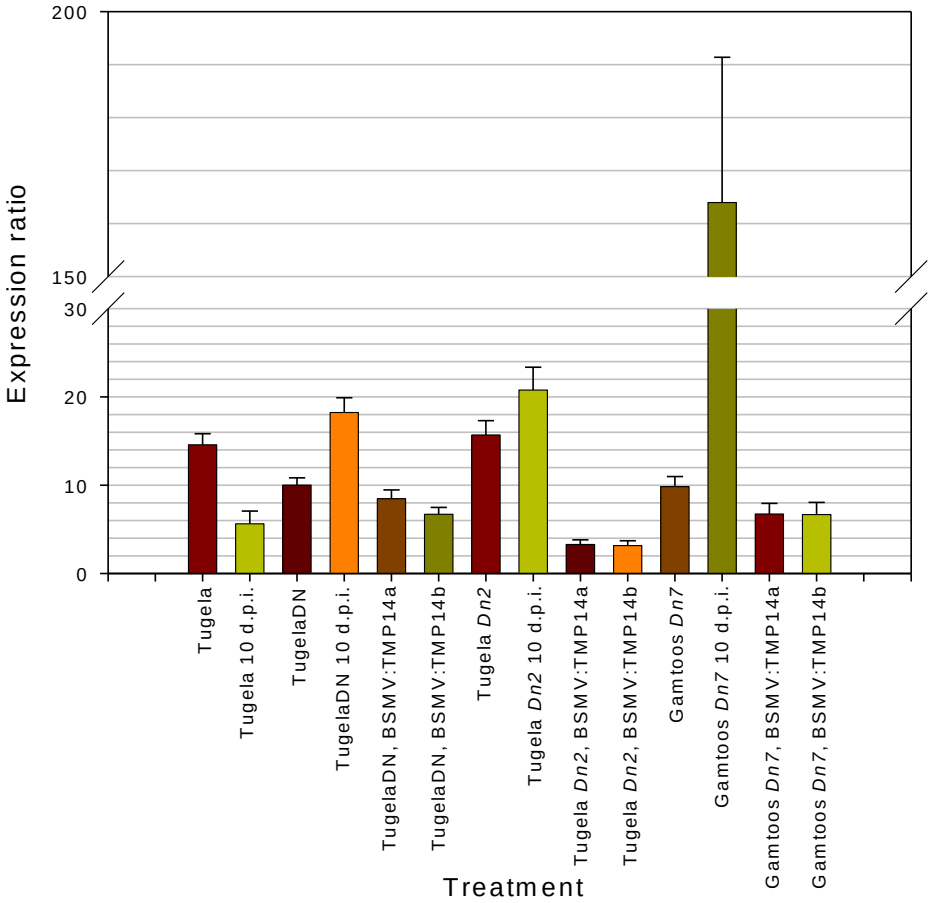


Figure 30: RT-PCR results for the confirmation of TMP14 silencing. Values were expressed against the HSP90 control gene.

The TMP14 mRNA levels in Tugela plants infested with RWA has decreased significantly from an expression ratio of 14.6 to 5.6, but in the resistant TugelaDN the levels increased from 10 to 18.2. Gamtoos *Dn7* responded to the aphid feeding with a TMP14 expression level increase of 9.8 to 164. The expression in Tugela *Dn2* increased from 15.7 to 20.7. Resistant plants infested with RWA (10 d.p.i.) and infected with BSMV:TMP14 showed a drastic decrease in TMP14 expression levels averaging a 2.5 fold change for TugelaDN, 6.9 for Tugela *Dn2* and 24.8 for Gamtoos *Dn7* (Figure 30).

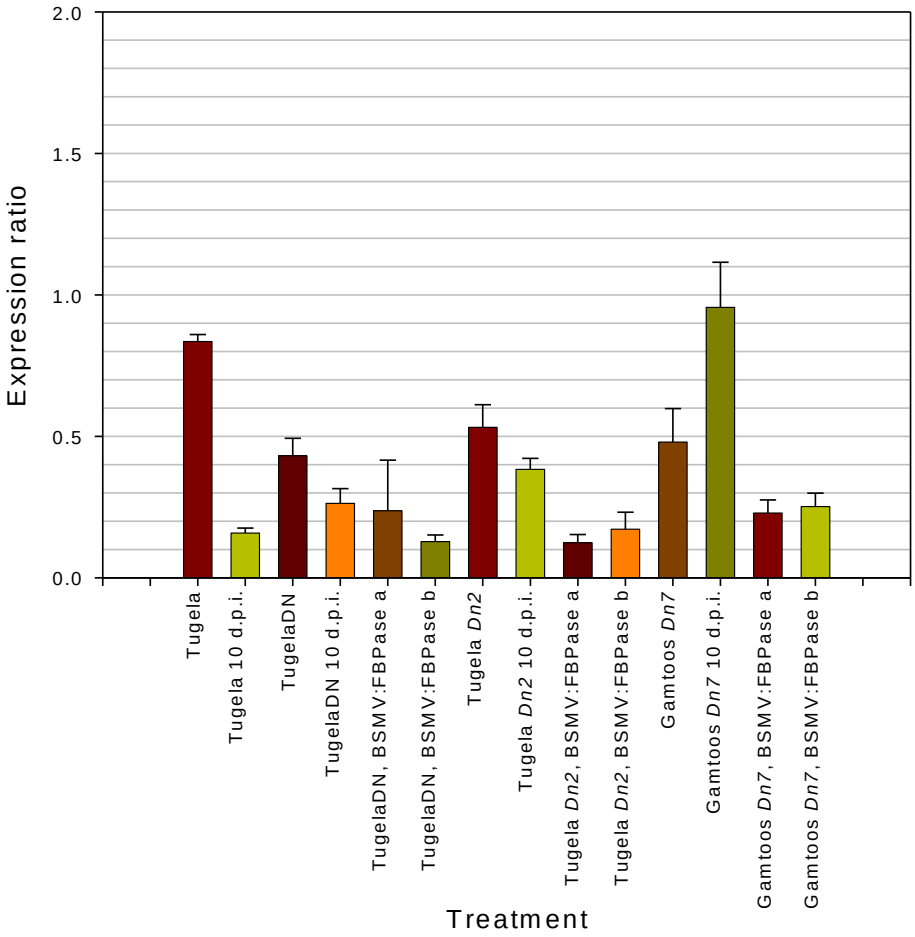


Figure 31: RT-PCR results for the confirmation of FBPase silencing. Values were expressed against the HSP90 control gene.

A decrease in FBPase mRNA levels was observed in resistant TugelaDN (fold change of 1.6) and Tugela *Dn2* (fold change of 1.39) plants infested with RWA compared to non infested plants. However when the mRNA levels of the susceptible Tugela plants were analysed it indicated a fold change decrease of 5.28 in FBPase mRNA levels. The FBPase mRNA levels in Gamtoos *Dn7* infested plants increased significantly compared to uninfested plants (fold change of 1.99). When the expression of the FBPase was silenced in infested TugelaDN plants (10 d.p.i.) using BSMV:FBPase the levels effectively decreased with a fold change of 1.44. For Tugela *Dn2* and Gamtoos *Dn7* plants (10 d.p.i.) silenced with BSMV:FBPase it decreased with a fold change of 2.45 and 4.2 respectively (Figure 31).

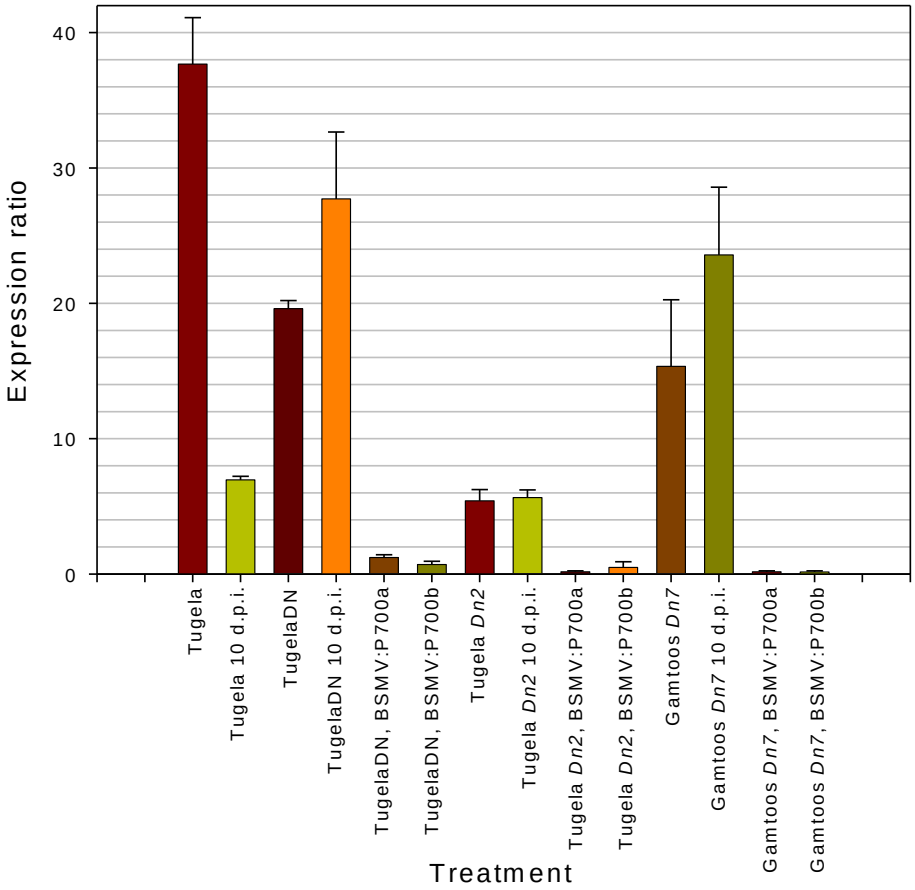


Figure 32: RT-PCR results for the confirmation of P700 silencing. Values were expressed against the HSP90 control gene.

The effect of feeding by the SA1 RWA biotype on the expression of P700 is indicated in figure 2.19. With the resistant TugelaDN and Gamtoos *Dn7* the P700 mRNA levels increases 10 d.p.i. with a fold change of 1.4 (significant) and 1.54 respectively but with Tugela *Dn2* it stays more or less the same (Figure 32). The P700 level of Tugela with infection on the other hand reduces drastically compared to the control and the other lines with a fold change of 5.3. The silencing of P700 in the three resistant cultivars causes a drastic decrease of P700 mRNA levels.

2.3.4. Phenotypic symptoms of RWA infestation

Plants were assessed phenotypically to observe any symptoms associated with RWA infestation, i.e. chlorotic streaking or necrotic lesions. The Scheepers plants, that served as susceptible host food, showed chlorotic streaking on the surface of the leaves and a high population of RWA's which indicate that the plant is susceptible to the SA1 RWA biotype (Figure 33A). The TugelaDN plants on the other hand had only a few live RWA and showed only necrotic lesions on the leaves which indicate that the TugelaDN is resistant to the SA1 RWA biotype (Figure 33B). The SAM RWA biotype however did multiply to large populations on TugelaDN plants and did show chlorotic streaking (Figure 33C). This means that TugelaDN is not resistant to the SAM RWA biotype.

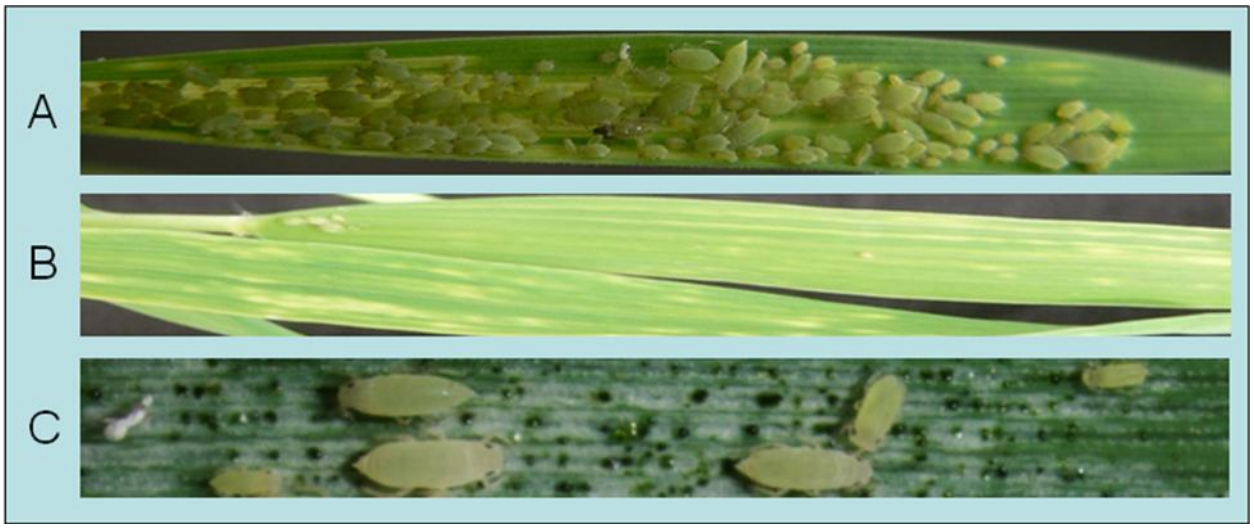


Figure 33: Photographs of different chlorotic streaking. A) Scheepers with SA1 RWA biotype expressing chlorotic streaking. B) TugelaDN with SA1 RWA biotype causing necrotic lesions. C) TugelaDN with SAM RWA biotype showing chlorotic streaking in the phenotype.

2.3.5. Phenotypic symptoms of control gene silenced plants

The virus constructs containing the fragments of the PDS gene (BSMV:PDS) to be silenced were applied to the plants serving as a control test and the virus without any insert (BSMV:00) served as a negative control. Plants treated with only the FES buffer served as mock controls. After 10 days the leaves were screened for PDS silencing (Figure 34).

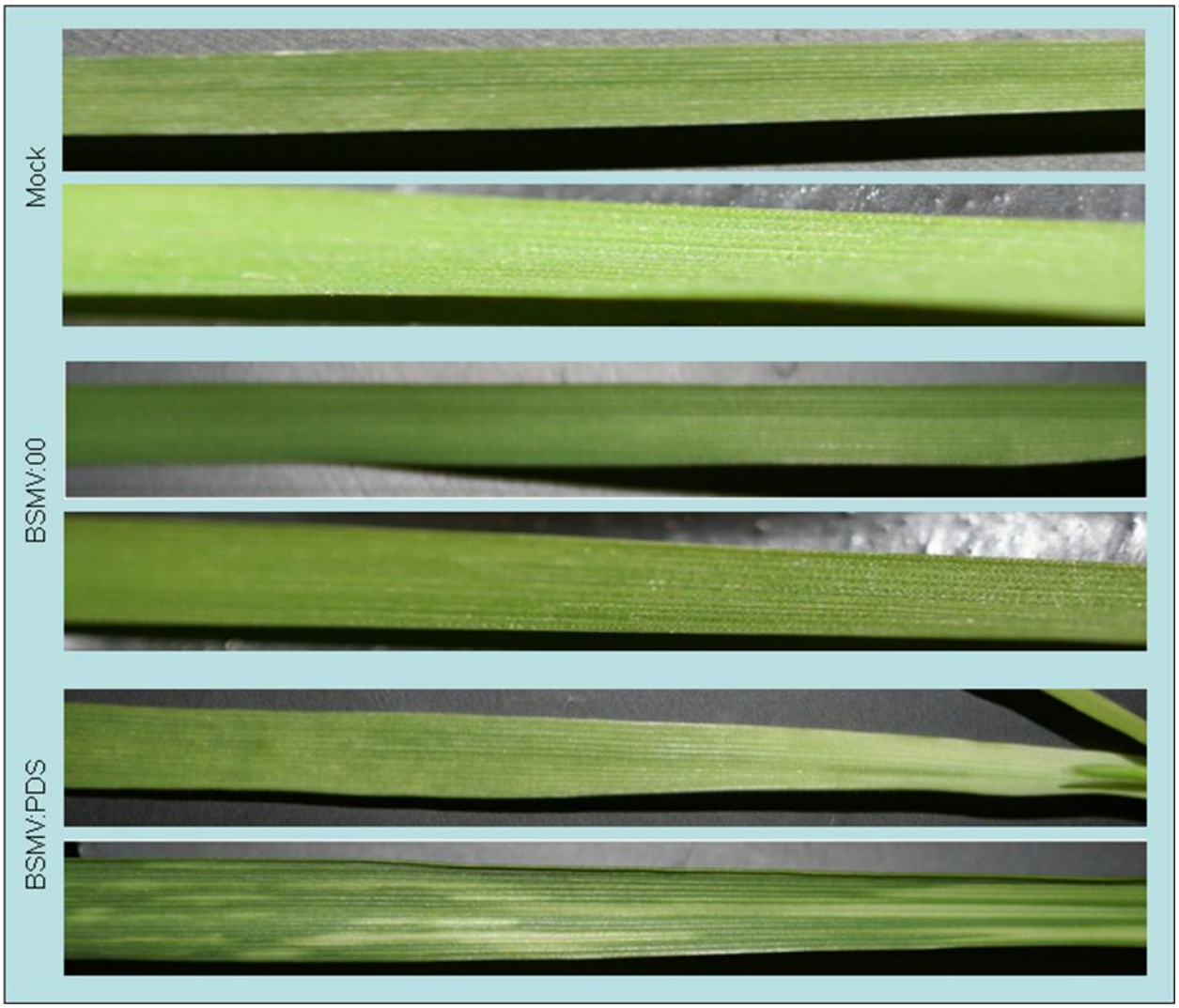


Figure 34: Photographs of leaves from wheat treated with BSMV virus vectors at 14 days after infection. Plants with mock infection, plants with the BSMV virus vectors containing no insert (BSMV:00) and plants with BSMV virus vectors containing a PDS insert (BSMV:PDS) are shown.

Phenotypic symptoms include photo bleaching of the leaves of the PDS silenced plant compared to the control plants. The PDS silencing served as a visual test to indicate whether BSMV successfully silenced candidate genes and serve as a positive control for the silencing series. The PDS silenced plants were scored at 90 % plant health. BSMV:00 plants showed no symptoms and were scored at 100 % plant health.

2.3.6. Photosynthetic changes

To determine the effect of RWA feeding on photosynthetic capacity, fluorescence was measured (Figure 35). Measurements were made before viral infection and RWA infestation to indicate whether pre recognition advantage exist in the resistant lines compared to the susceptible line. Measurements were also made with aphid infestation to indicate differences in photosynthetic responses.

2.3.6.1 Uninfested and infested plants

OJIP readings indicated no significant difference in the photosynthetic rates of the four uninfested wheat lines Tugela, TugelaDN, Tugela *Dn2* and Gamtoos *Dn7*. Although the measurements for Tugela were lower, the difference in measurements was not significant (Figure 35A). This observation was also confirmed with the Fv/Fm readings where no significant changes were observed (Figure 35B). All the plants tested were scored on the phenotypic scale indicated in section 2.2.15. and only 100 % healthy plants were used.

Readings of the same four cultivars, 10 days after infestation with RWA, indicated major differences in photosynthetic rates in response to the RWA feeding. In the susceptible line Tugela, the photosynthesis decreased notably and with TugelaDN it decreased even more. With Gamtoos *Dn7* however, photosynthesis decreased more than Tugela but not as much as TugelaDN (Figure 2.22). With the Tugela *Dn2* line one observes a totally different phenomenon. Instead of decreasing photosynthesis, it was upheld to function at normal levels. The Fv/Fm readings show similar changes and thus confirmed the OJIP readings (Figure 35).

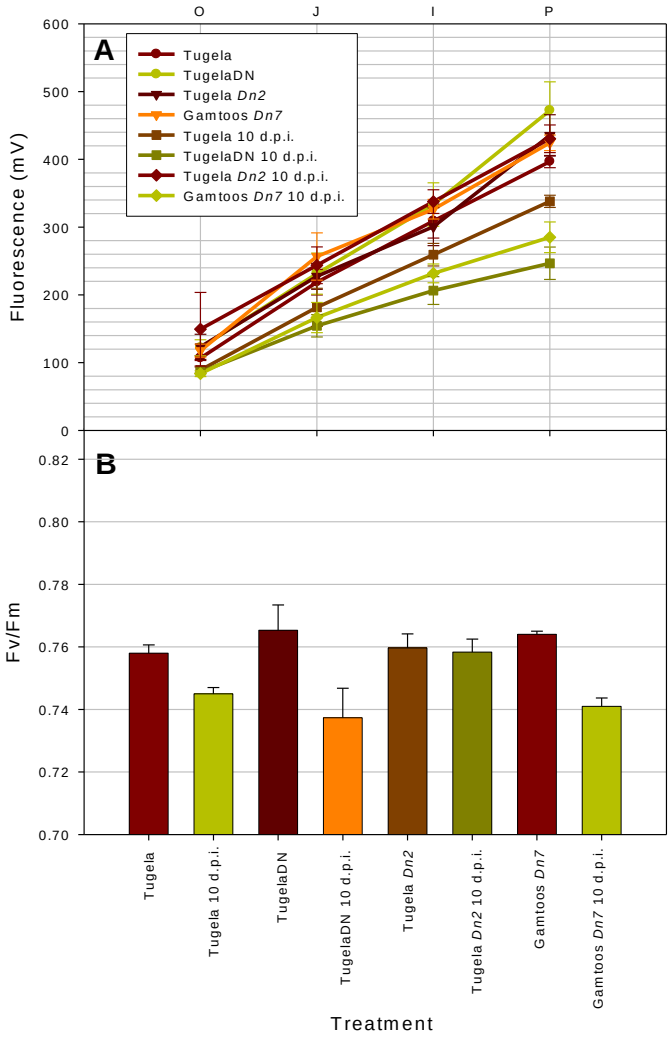


Figure 35: Relative variable fluorescence (V) indicates photosynthesis of wheat lines during RWA feeding. Three biological- and three technical repeats were used per data point. **(A)** OJIP kinetics; **(B)** Maximum quantum yield (Fv/Fm).

2.3.6.2 Silenced plants

To confirm if changes in photosynthesis could be observed on fluorescent level due to the silencing of photosynthesis related genes, uninfested plants were independently treated with virus constructs containing the respected genes. Fluorescence readings were obtained from the plants 10 days after treatment (10 d.p.i.). The results of the susceptible Tugela is documented in Figure 2.23, the resistant plants TugelaDN, Tugela *Dn2* and Gamtoos *Dn7* are documented in Figure 2.24, 2.25 and 2.26 respectively. Due to aphid feeding that has an affect on photosynthesis, infected plants were also independently treated with virus constructs containing the respective genes. Ten days after treatment fluorescence readings were obtained to confirm if further changes in photosynthesis could be observed in the fluorescence level.

Tugela

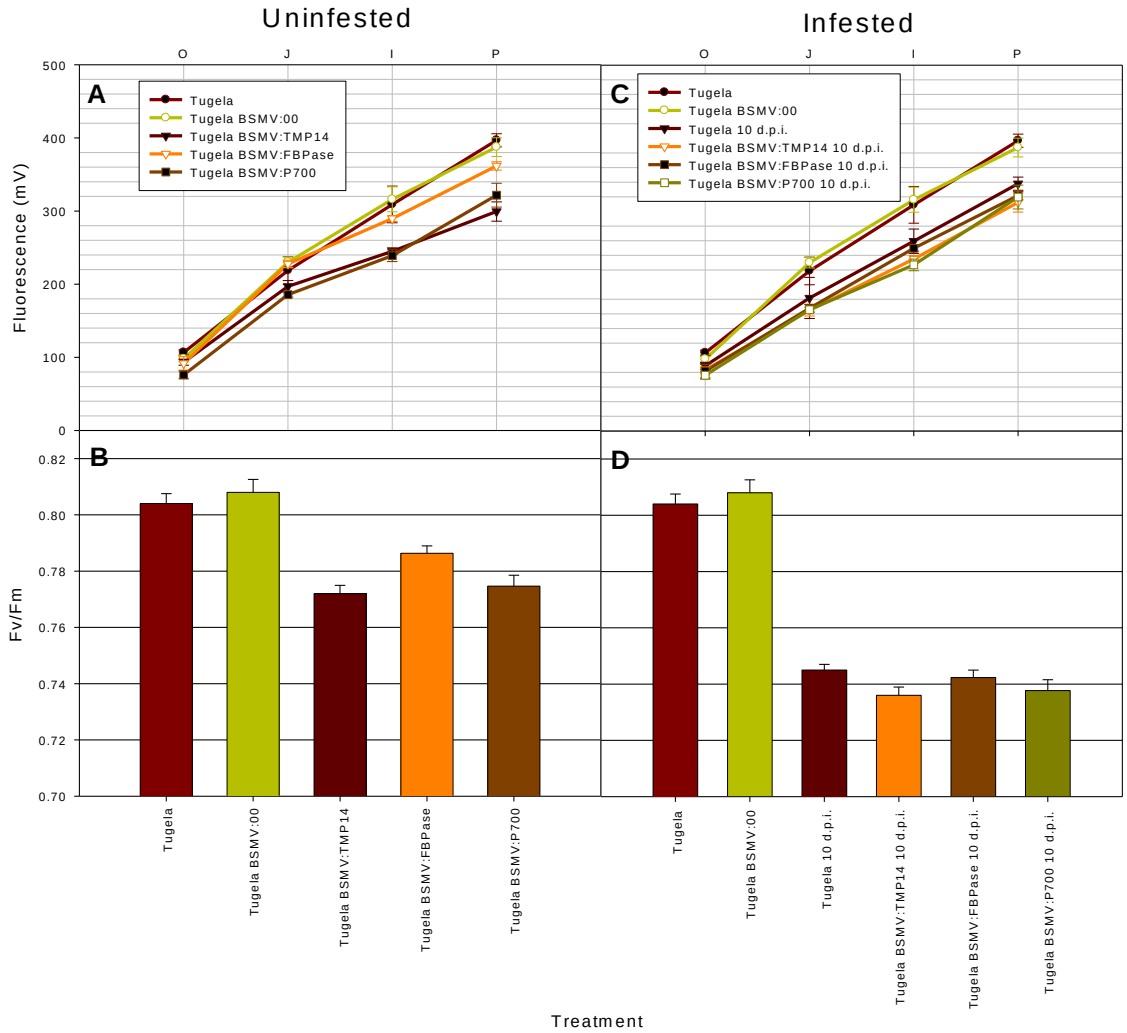


Figure 36: Relative variable fluorescence (V) indicates photosynthetic changes of silenced Tugela plants. **(A)** OJIP kinetics of uninfested plants; **(B)** Maximum quantum yield (Fv/Fm) of uninfested plants; **(C)** OJIP kinetics of infested plants; **(D)** Maximum quantum yield (Fv/Fm) of infested plants.

Plants treated with the empty virus construct BSMV:00 had a similar OJIP profile as the uninfected plants. When FBPase was silenced in the Tugela plants the fluorescence profile observed as OJIP kinetics did not differ from Tugela plants treated with BSMV:00 (Figure 2.23A). When TMP14 and P700 were silenced and the OJIP readings indicated the first two states stayed the same, but the 'I' and 'P' states decreased slightly. These findings were confirmed with the Fv/Fm readings (Figure 2.23B). A significant decline in fluorescence was observed in aphid infested Tugela plants relative to the uninfested Tugela plants. However, the decline observed in

infested plants treated with BSMV:TMP14, BSMV:FBPase and BSMV:P700 compared to non-treated plants was small and not statistically significant.

TugelaDN

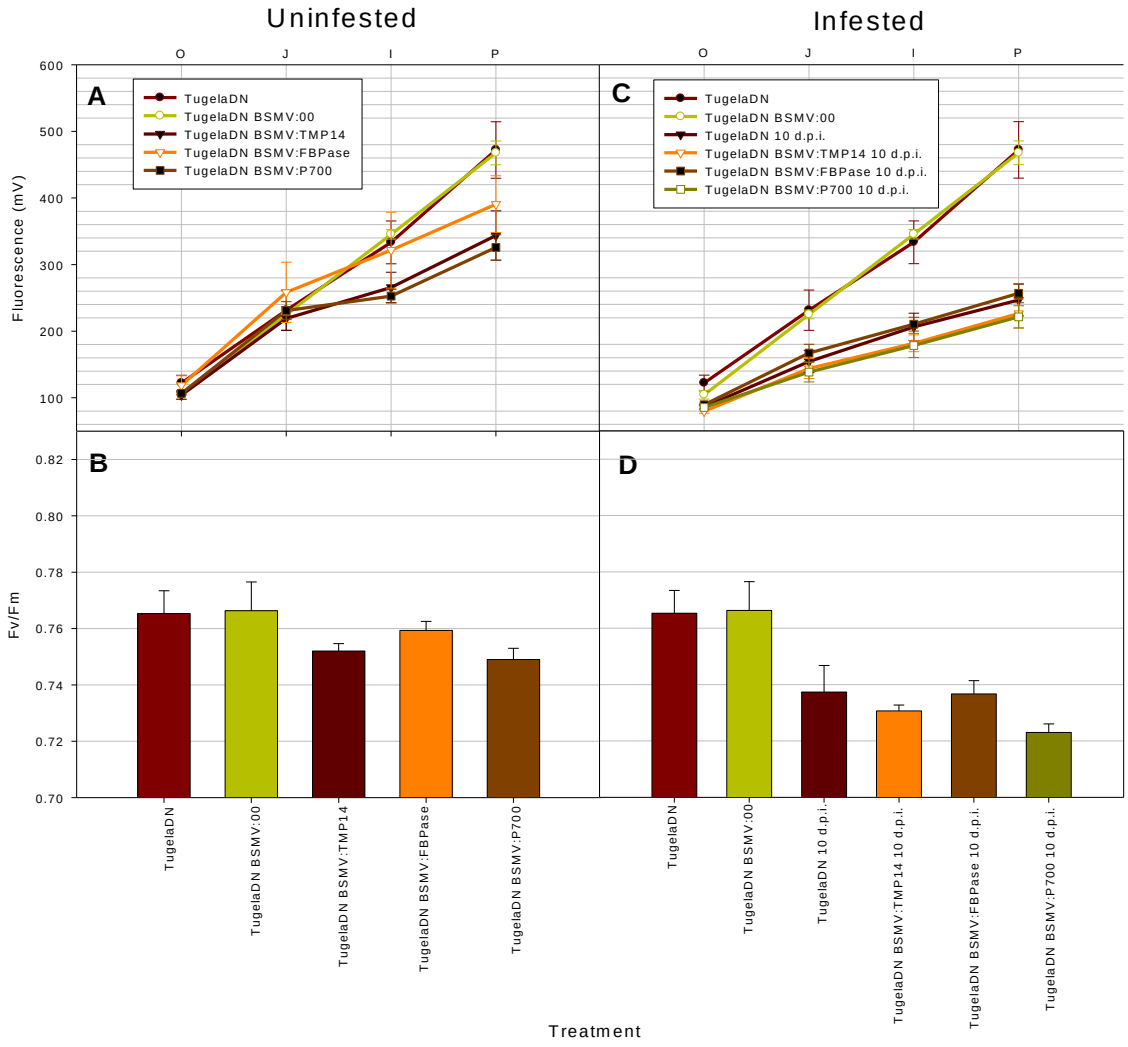


Figure 37: Relative variable fluorescence (V) of silenced TugelaDN plants. **(A)** OJIP kinetics of uninfested plants; **(B)** Maximum quantum yield (Fv/Fm) of uninfested plants; **(C)** OJIP kinetics of infested plants; **(D)** Maximum quantum yield (Fv/Fm) of infested plants.

Plants treated with the empty virus construct BSMV:00 had a similar OJIP profile as the uninfected plants. In the TugelaDN plants where FBPase was silenced the fluorescence profile observed as OJIP kinetics changed slightly but did not decline significantly (Figure 2.24A). In the plants where TMP14 and P700 were silenced on the other hand, the first two states stayed the same, but the 'I' and 'P' states decreased considerably. The Fv/Fm readings also indicate a decline in photosynthesis in the treated plants compared to the control plants (Figure 2.24B). All aphid infested TugelaDN plants show a dramatic decrease in photosynthesis compared to uninfested

plants. Readings of BSMV:TMP14, BSMV:FBPase and BSMV:P700 plants infested with aphids indicated an even further decrease in photosynthesis but not enough to be statistically significant. The Fv/Fm readings supported the OJIP readings.

Tugela *Dn2*

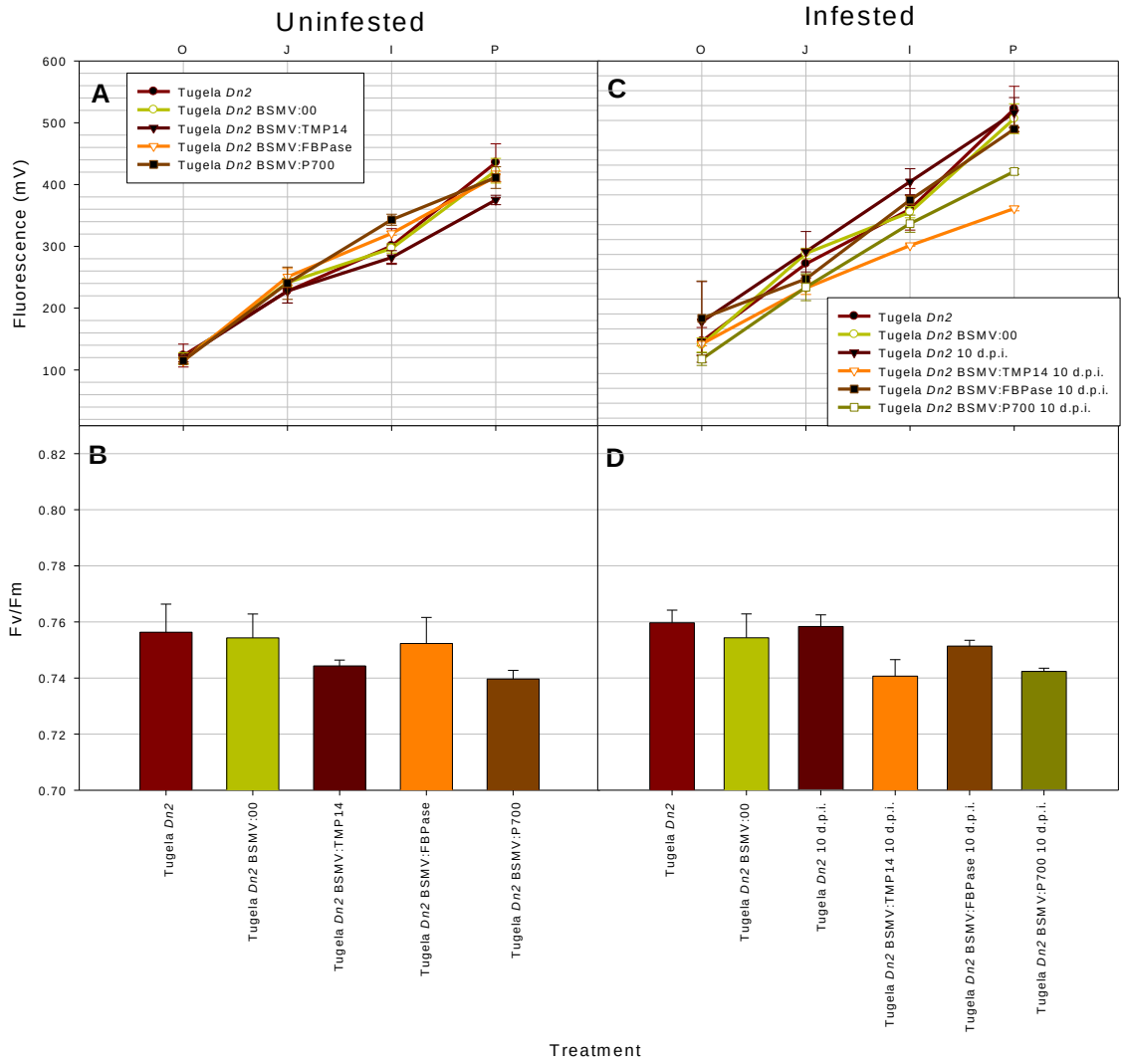


Figure 38: Relative variable fluorescence (V) indicates photosynthetic changes of silenced Tugela *Dn2* plants. **(A)** OJIP kinetics of uninfested plants; **(B)** Maximum quantum yield (Fv/Fm) of uninfested plants; **(C)** OJIP kinetics of infested plants; **(D)** Maximum quantum yield (Fv/Fm) of infested plants.

Plants treated with the empty virus construct BSMV:00 had a similar OJIP profile as the uninfected plants. Silencing of FBPase in Tugela *Dn2* did not affect photosynthesis significantly only minor profile changes occur. TMP14 silencing affects the OJIP profile only slightly where ‘O’ and ‘J’ stays the same and ‘I’ and ‘P’ decline, but with P700 silencing it did not differ much from the BSMV:00 control. The Fv/Fm readings indicated a significant decline for BSMV:TMP14 and

BSMV:P700, but not for BSMV:FBPase (Figure 2.25B). The photosynthesis of aphid infested Tugela *Dn2* plants was maintained, but the photosynthetic rate of BSMV:TMP14 plants decreased significantly which was confirmed with the Fv/Fm readings. Photosynthesis decreased slightly less with BSMV:P700 and minimally with BSMV:FBPase as revealed by the Fv/Fm readings.

Gamtoos *Dn7*

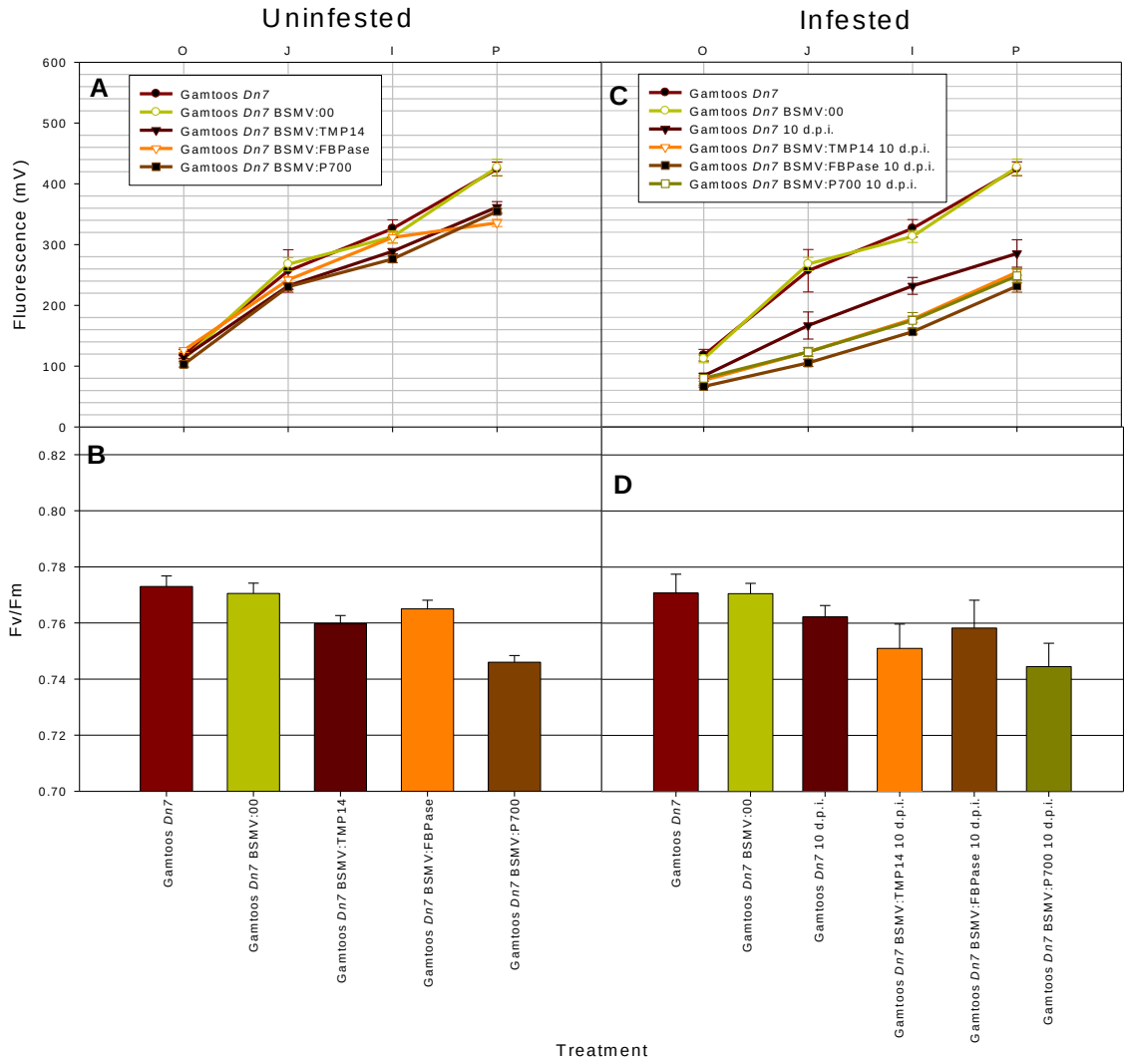


Figure 39: Relative variable fluorescence (V) indicates photosynthetic changes of silenced Gamtoos *Dn7* plants. **(A)** OJIP kinetics of uninfested plants; **(B)** Maximum quantum yield (Fv/Fm) of uninfested plants; **(C)** OJIP kinetics of infested plants; **(D)** Maximum quantum yield (Fv/Fm) of infested plants.

Figure 2.26A and 2.26B indicate that plants treated with the empty virus construct BSMV:00 had a similar OJIP profile as the uninfected plants. After silencing of the Gamtoos *Dn7* plants, the photosynthetic rates didn't change significantly. In the Gamtoos *Dn7*:BSMV:FBPase plants a decrease was only found in the 'P' stage. However in Gamtoos *Dn7* plants where TMP14 and P700 were silenced a slight decrease in the 'I' and 'P' stages was observed. The Fv/Fm readings revealed a significant decrease in the photosynthetic state in BSMV:P700 infected plants and a

less significant decline for BSMV:TMP14 and BSMV:FBPase infected plants. Figure 2.26C and 2.26D indicated photosynthesis of infested Gamtoos *Dn7* plants silenced with the selected genes relative to the control (Gamtoos *Dn7*). The OJIP profiles clearly showed a decrease in the photosynthetic capacity due to aphid infestation and a futher decrease due to the genes that are silenced. The Fv/Fm readings on the other hand indicated only a significant decline for BSMV:P700 and BSMV:TMP14 infected plants compared to infested plants.

2.3.7. Chlorophyll measurements

Chlorophyll measurements were made to support the fluorescence measurements and to indicate what effect aphid infestation had on the chlorophyll content of different wheat lines.

2.3.7.1 Uninfested versus infested plants

The chlorophyll content of uninfested and infested plants of each selected wheat line was compared to indicate what changes RWA infestation initiate. The differences between resistant and susceptible wheat lines were analysed to determine if the resistant lines have mechanisms to prevent chloroplast degeneration. The different resistant wheat lines were also compared to determine if their defence mechanisms have different effects on chloroplast protection. Figure 2.27 indicate that all the wheat lines had more or less the same chlorophyll content before aphid infestation but after infestation the Tugela plant's chlorophyll decreased significantly. TugelaDN and Gamtoos *Dn7*'s chlorophyll decreased only slightly (not statistically significant) and Tugela *Dn2*'s chlorophyll did not change.

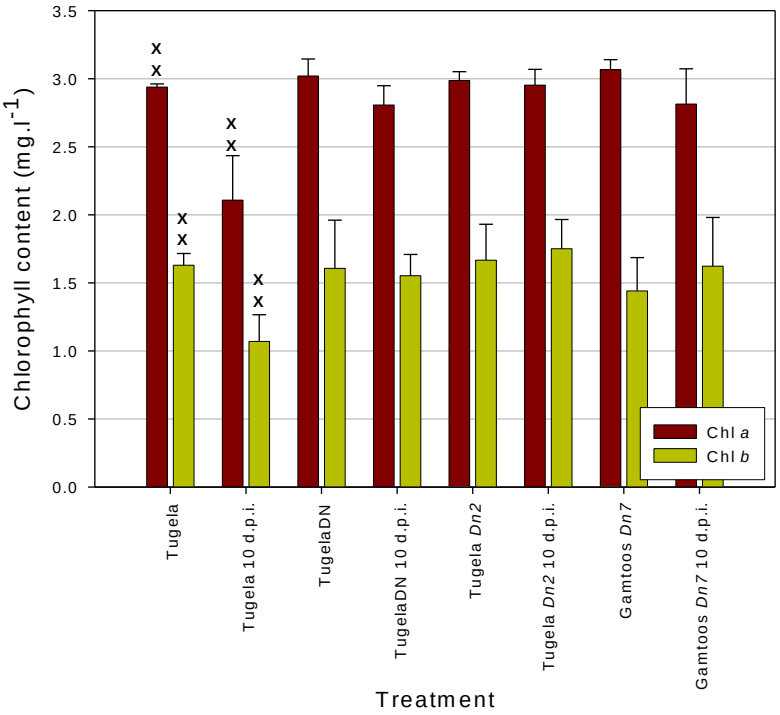


Figure 40: Chlorophyll measurements of uninfested and infested lines. The chlorophyll of Tugela, TugelaDN, Tugela *Dn2* and Gamtoos *Dn7* were measured and indicated as $mg.l^{-1}$. The Tugela and Tugela 10 d.p.i. indicated a significant decrease and are indicated with "x"($p \geq 0.05$).

2.3.7.2 Silenced plants

The genes selected for silencing were photosynthesis related genes and would thus had a significant influence on the chlorophyll content of the leaves. Uninfested plants were therefore treated with virus constructs containing the respected genes. Chlorophyll was then extracted after 10 days (10 d.p.i.) and measured to reveal the effect the silenced genes have on the chlorophyll content of individual wheat lines. Chlorophyll was also measured of RWA infested plants treated with virus constructs containing the respective genes to indicate the chlorophyll levels of the wheat lines during the trials. Too low levels would mean that the health of the plants is not well enough to analyse the resistance of the plants.

Tugela

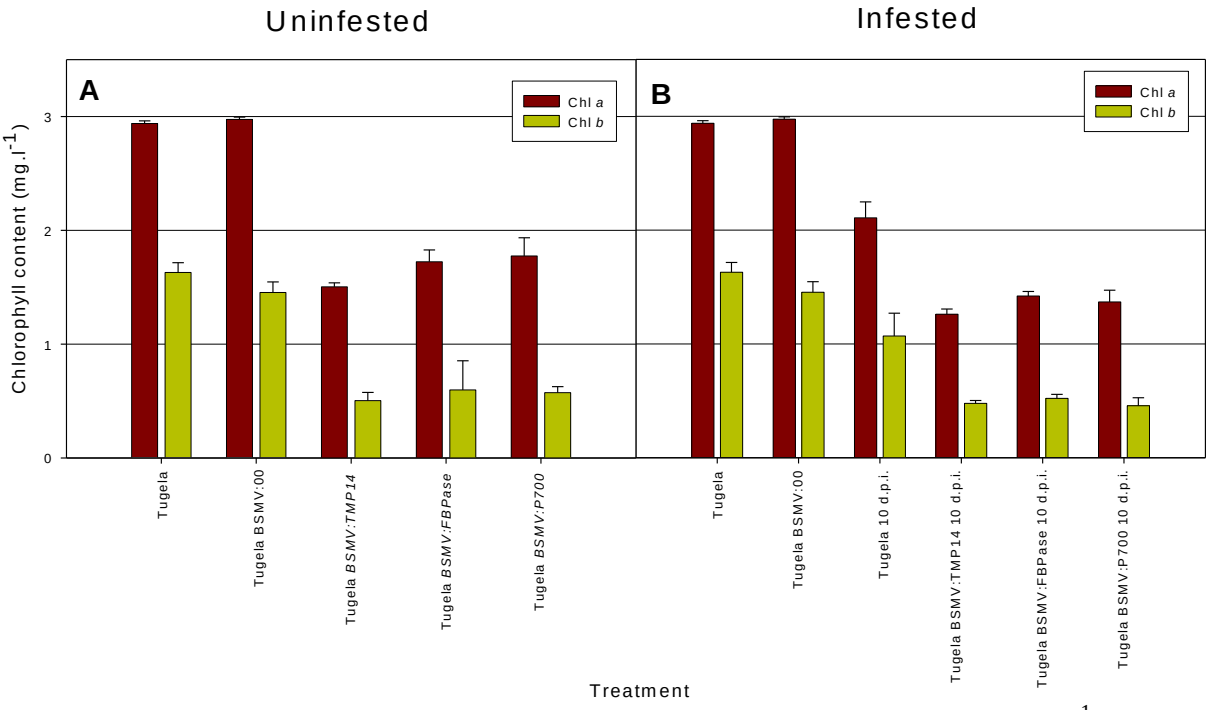


Figure 41: Chlorophyll measurements of silenced Tugela plants indicated as $mg.l^{-1}$. **(A)** Uninfested Tugela plants treated with silencing constructs; **(B)** Infested Tugela plants treated with silencing constructs.

Chlorophyll content measurements (Figure 2.28) indicate a significant statistical decrease in chlorophyll *a* and *b* with TMP14, FBPase and P700 silenced Tugela plants. Tugela infected with BSMV:00 had a chloropyll content similar to Tugela plants (Figure 41A). A major decline in chlorophyll was observed for Tugela plants under RWA feeding. Even further decline in chlorophyll occurred on plants that were also infected with BSMV:TMP14, BSMV:FBPase and BSMV:P700.

TugelaDN

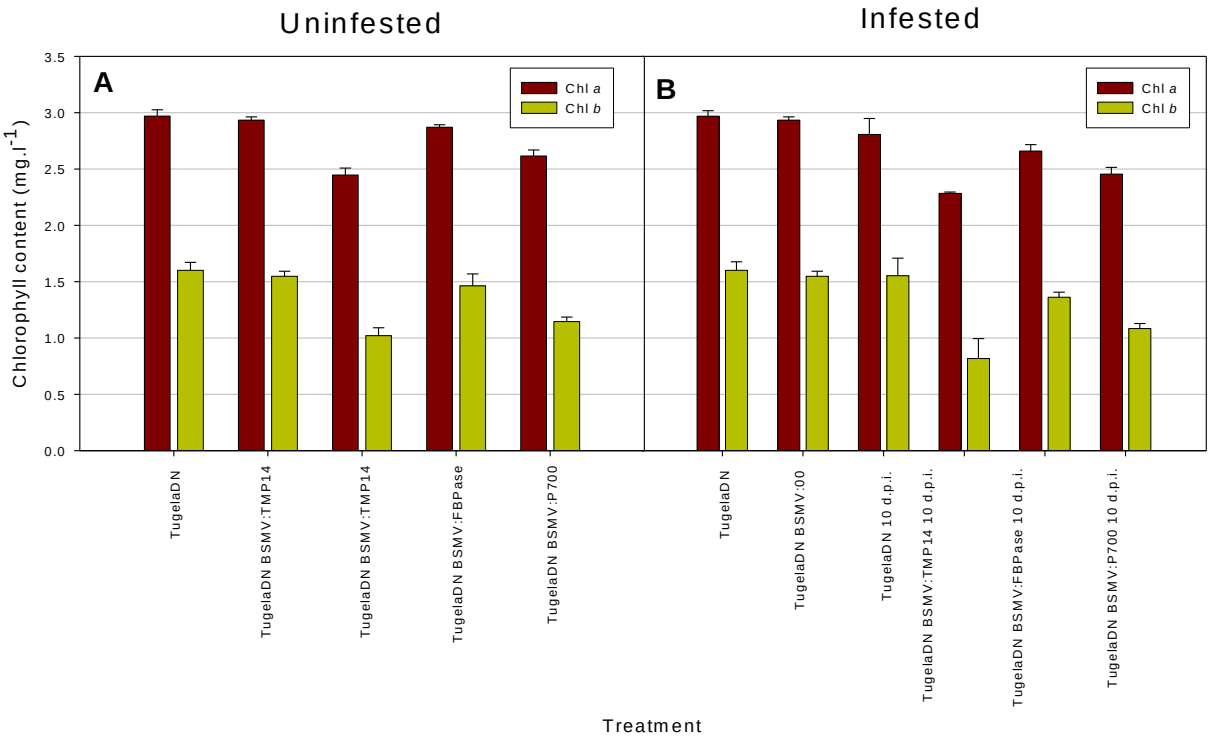


Figure 42: Chlorophyll measurements of silenced TugelaDN plants indicated as $mg.l^{-1}$. **(A)** Uninfested TugelaDN plants treated with silencing constructs; **(B)** Infested TugelaDN plants treated with silencing constructs.

Chlorophyll measurements were made to determine if TMP14, FBPase and P700 silencing decreases Chlorophyll content in the leaves of TugelaDN plants (Figure 42). It indeed indicated a significant decrease in chlorophyll *a* and *b* with the BSMV:TMP14 and BSMV:P700 plants and white streaks could even be observed on the leaves of BSMV:TMP14 plants (not shown). However no decreased chlorophyll content was observed with the FBPase silenced plants. The BSMV:00 viral constructs caused no change in chlorophyll content compared TugelaDN plants. The chlorophyll content of the infested samples indicated that the combination of aphid feeding and silencing constructs only caused the chlorophyll to decline slightly more compared with the TugelaDN plants only infected with the silencing constructs (Figure 42). The chlorophyll levels indicate that the plant is still healthy enough for analyzing aphid fecundities.

Tugela *Dn2*

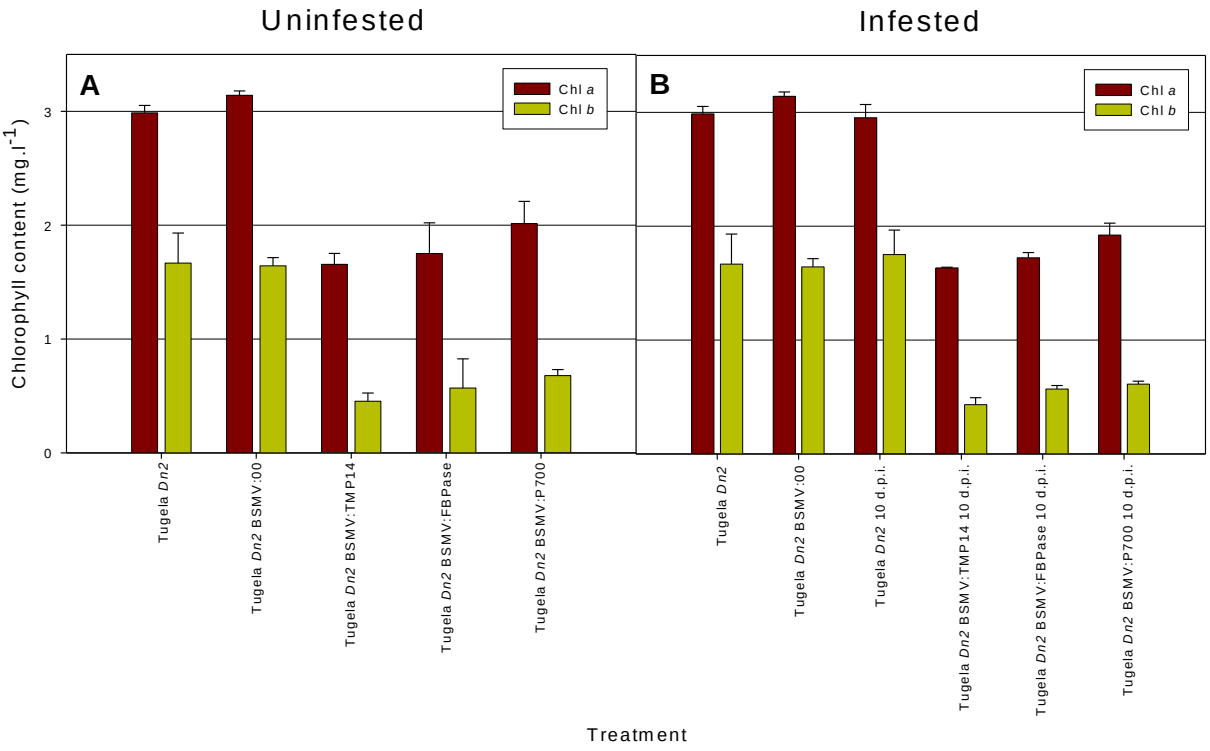


Figure 43: Chlorophyll measurements of silenced Tugela *Dn2* plants indicated as mg.l^{-1}. **(A)** Uninfested Tugela *Dn2* plants treated with silencing constructs; **(B)** Infested Tugela *Dn2* plants treated with silencing constructs.

Chlorophyll extractions indicate (Figure 43) a significant decrease in chlorophyl *a* and *b* with TMP14, FBPase and p700 silencing. No significant change in chlorophyll content was observed in BSMV:00 infected plants. Aphid feeding on Tugela *Dn2* does not cause the chlorophyll to decrease but the silencing of the three genes in combination with aphid feeding cause a significant decrease compared to the infested Tugela *Dn2* plants (10 d.p.i.).

Gamtoos *Dn7*

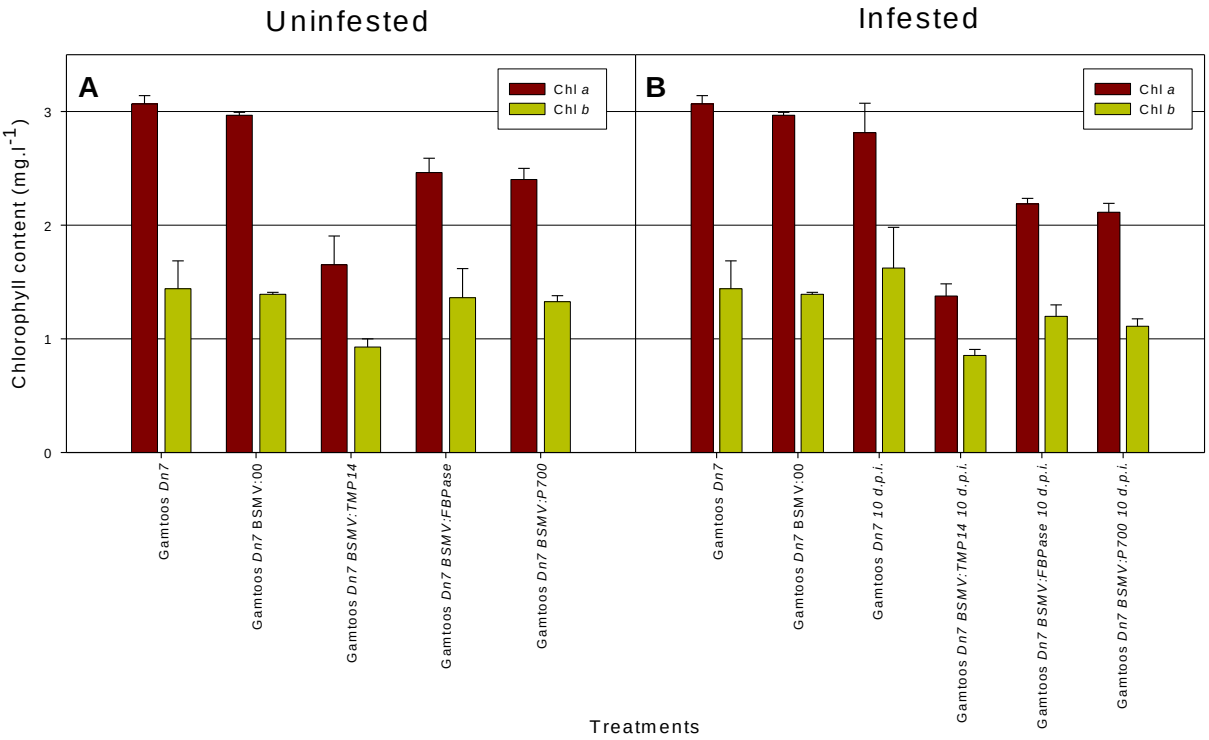


Figure 44: Chlorophyll measurements of silenced Gamtoos *Dn7* plants indicated as mg.l^{-1}. **(A)** Uninfested Gamtoos *Dn7* plants treated with silencing constructs; **(B)** Infested Gamtoos *Dn7* plants treated with silencing constructs.

A significant decrease in the chlorophyll *a* and *b* levels were observed in the TMP14, FBPase and P700 silenced plants, but the levels for FBPase and P700 silenced plants did not decline as much as TMP14 silenced plants (Figure 44). The decrease observed in BSMV:00 plants was not statistically significant.

The chlorophyll *a* content of Gamtoos *Dn7* decreased from ± 3.05 mg.l^{-1} to ± 2.8 mg.l^{-1} after 10 days of RWA feeding, but in plants where FBPase and P700 were silenced it decreased to ± 2.2 mg.l^{-1}. The silencing of TMP14 caused the chlorophyll *a* to decrease to 1.4 mg.l^{-1}.

2.3.8. Aphid fecundity and health of plants after silencing of selected genes

Aphid fecundity and the health of the infected plants were documented. While, plant health was assessed to establish whether the plants had any phenotypic symptoms because of the BSMV infection, aphid fecundity measurements were made to indicate whether the silencing of the selected genes decreased RWA resistance.

Tugela

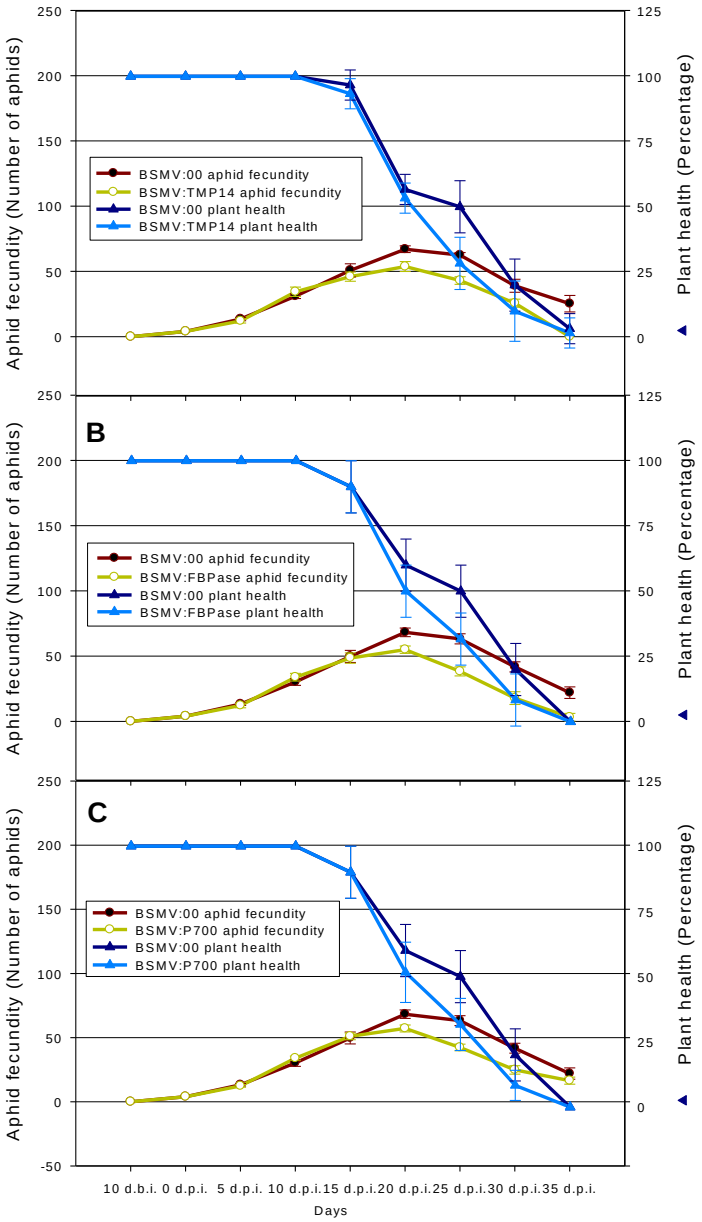


Figure 45: Aphid fecundity and plant health of Tugela plants. Amounts are demonstrated over a 45 day-period. 'd.b.i.' indicates days before infestation and 'd.p.i.' indicate days post infestation.

Aphid fecundity (Figure 2.32) increases up to a maximum of 68.25 aphids per Tugela plant at 20 d.p.i. where after it declined as the feeding pressure causes the plants to lose health and die. The quick death of the Tugela plants and the symptoms which include leaf curling and chlorotic streaking is a clear indication of RWA susceptibility. Silencing the three genes did not have any influence on the aphid fecundities up to the 10th day where after it started to decline more compared to the BSMV:00 plants. The plant health also started to decline after the 10th day (Figure 45). Plants infested with BSMV:PDS and uninfested plants died around 30 d.p.i. and the aphid fecundity of the infected plants corresponded with BSMV:00 plants infested with aphids (not shown).

TugelaDN

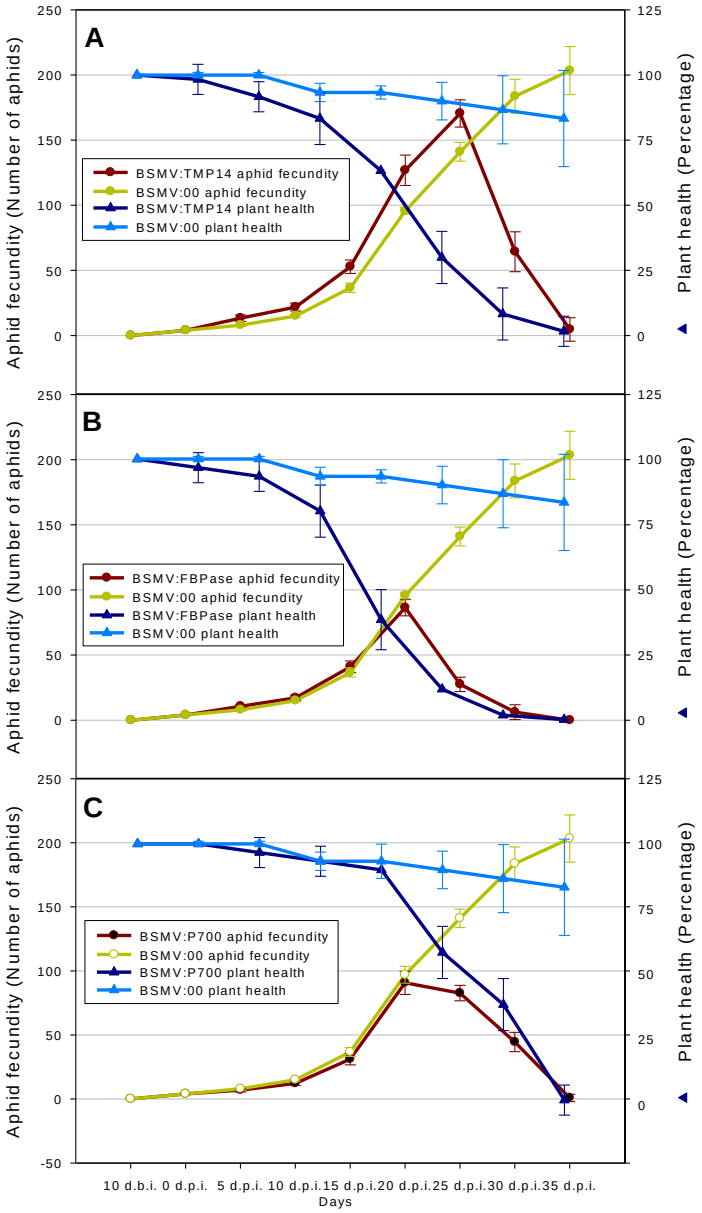


Figure 46: Aphid fecundity and plant health of TugelaDN plants. Amounts are demonstrated over a 45 day-period. 'd.b.i.' indicates days before infestation and 'd.p.i.' indicate days post infestation.

The aphid fecundity of the BSMV:00 control plants rise to a maximum of 203 at 35 d.p.i. and the plant's health does not decline significantly which indicate resistance to the RWA (Figure 46). The necrotic lesions on the leaves supported the plants resistance. The silencing of the three genes in TugelaDN caused a higher aphid fecundity for BSMV:TMP14 and BSMV:FBPase plants indicating an increased sucseptibility up to 10 d.p.i. and for BSMV:P700 plants the aphid fecundity was lower indicating increased resistance compared to the BSMV:00 plants (Figure 46). The silencing of TMP14 reduces the TugelaDN line's resistance to a greater extent compared to FBPase silenced plants that does not have a significant influence on resistance. The health of the BSMV:00 plants decrease slightly after 20 days as aphid fecundity rises above 50 aphids per plant. The plant health of the gene silenced plants diminished untill they died as aphid fecundity increase indicating a reduction in resistance. It took BSMV:P700 plants 35 d.p.i. to die compared to 30 d.p.i for the BSMV:TMP14 and BSMV:FBPase plants. Plants infested and uninfested BSMV:PDS plants died around 30 d.p.i. and the aphid fecundity of the infected ones corresponded with BSMV:00 plants infected with aphids (not shown).

Tugela *Dn2*

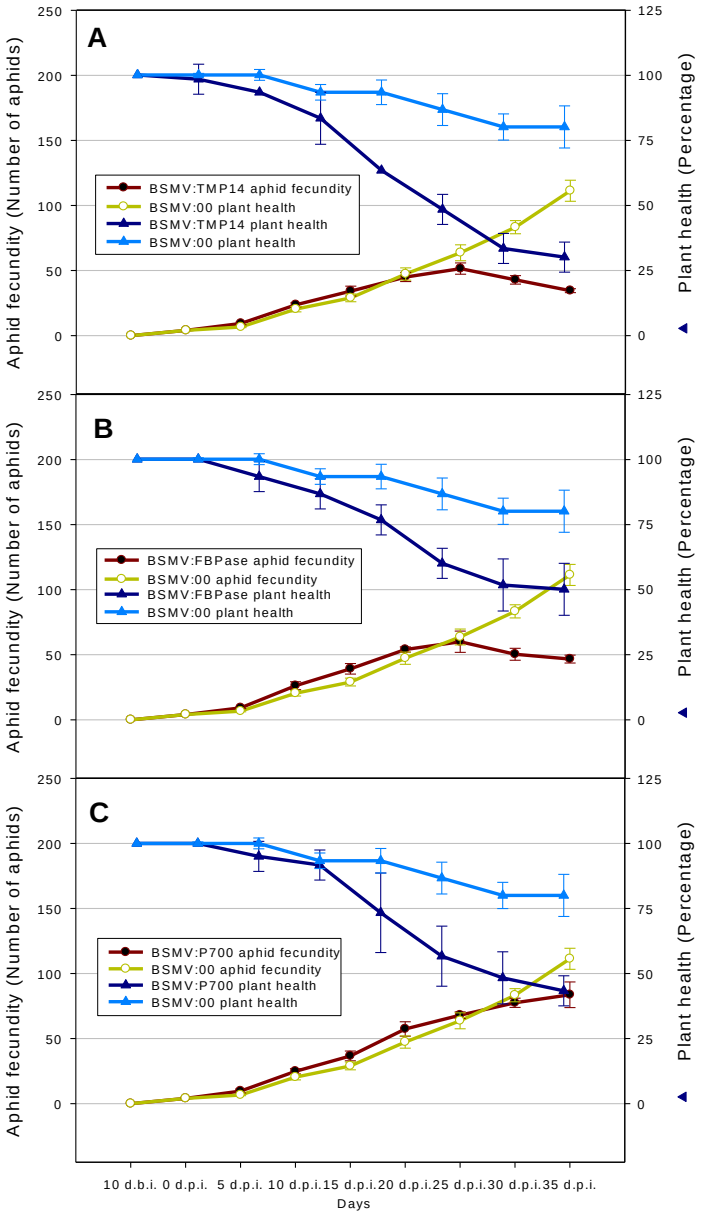


Figure 47: Aphid fecundity and plant health of Tugela *Dn2* plants. Amounts are demonstrated over a 45 day-period. 'd.b.i.' indicates days before infestation and 'd.p.i.' indicate days post infestation.

The silencing of the three genes in the Tugela *Dn2* line caused higher aphid fecundities compared to BSMV:00 plants (Figure 47). The gene silenced plants does not die within the 35 days, but health decreases. Infected and uninfected BSMV:PDS plants died around 30 d.p.i. and the aphid fecundity of the infected ones corresponded with BSMV:00 plants infested with aphids. The health of the BSMV:00 plants decrease more compared to the TugelaDN lines and the aphid fecundity rises to 110 aphids per plant compared to 200 of the TugelaDN lines.

Gamtoos *Dn7*

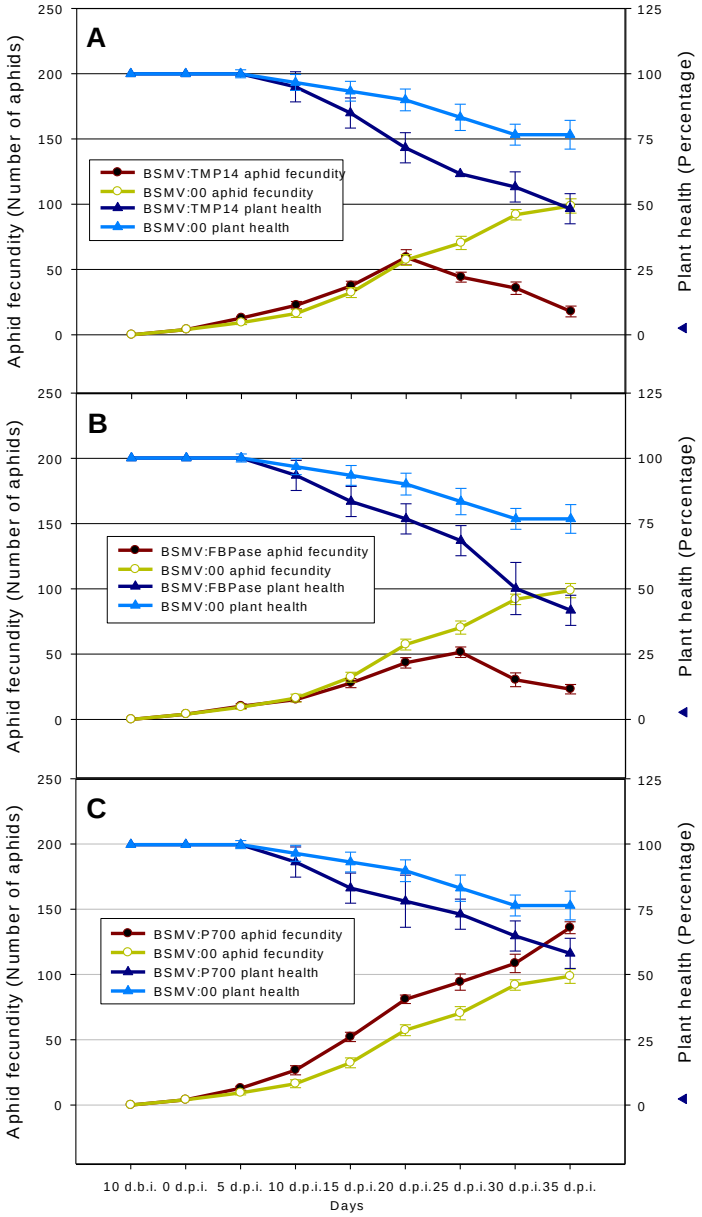


Figure 48: Aphid fecundity and plant health of Gamtoos *Dn7*. Amounts are demonstrated over a 45 day-period. 'd.b.i.' indicates days before infestation and 'd.p.i.' indicate days post infestation.

In the Gamtoos *Dn7* line the aphid fecundity of BSMV:P700 plants increased dramatically compared to the BSMV:00 plants indicating a loss in resistance (Figure 48). The health of the BSMV:P700 plants was not affected significantly and the plants survived longer than the TugelaDN and Tugela *Dn2* plant lines. In the BSMV:00 plants the aphid fecundity increased to less than 100 aphids per plant over the 35 day infestation period and the plant health also decreased slightly. Infected and uninfected BSMV:PDS plants died around 30 d.p.i. and the aphid fecundity of the infected ones corresponded with BSMV:00 plants infested with aphids.

2.3.9. Comparison of the different wheat lines

The response of the selected wheat lines to RWA feeding was measured and the data collected on 10 days post infestation are displayed in Table 10 and Figure 49.

Table 10: Comparison of the four wheat lines in the interaction with RWA feeding (10 d.p.i). The expression of TMP14, FBPase and P700 are displayed as either a positive or negative fold change.

	Tugela	TugelaDN	Tugela *Dn2*	Gamtoos *Dn7*
Chl *a* & *b* decline	Significant	None	None	None
Photosynthesis (Fv/Fm)	0.013	0.028	0.001	0.023
Aphid fecundity	30.3	15	20.33	16.33
TMP14 expression (fold change)	-2.6	+1.82	+1.3	+16.7
FBPase expression (fold change)	-5.28	-1.6	-1.39	+1.99
P700 expression (fold change)	-5.3	+1.4	+1.04	+1.54

The decline in chlorophyll content observed in TugelaDn, Tugela *Dn2* and Gamtoos *Dn7* due to aphid infestation was not significant compared to the significant decline observed in Tugela. Fluorescent studies indicated a decline in Fv/Fm readings of 0.028, 0.001 and 0.023 for the TugelaDN, Tugela *Dn2* and Gamtoos *Dn7* plants respectively when compared to a 0.013 decline observed in Tugela plants. The aphid fecundity of the Tugela wheat averaged 30.33 aphids per plant after 10 days of infestation, but with the resistant line TugelaDN it only increased to 15 aphids per plant. With Tugela *Dn2* and Gamtoos *Dn7* the aphid fecundity averaged 22.33 and 16.33 respectively. The expression of TMP14 was significantly up regulated with a fold change of 16.7 in Gamtoos *Dn7* plants infested with RWA's. TugelaDN and Tugela *Dn2* responded with a 1.82 and 1.3 fold change increase of TMP14 levels compared to a decrease of 2.6 fold change observed in Tugela plants. FBPase however was down regulated in TugelaDN and Tugela *Dn2* plants with a fold change of -1.6 and -1.39, respectively. With Gamtoos *Dn7* a fold change increase of 1.99 was observed in FBPase RNA levels compared the decrease of 5.28 fold change observed in Tugela plants. The fold change increase of P700 expression were 1.4, 1.04 and 1.54 in TugelaDN, Tugela *Dn2* and Gamtoos *Dn7* respectively compared to the decrease of 5.3 observed in Tugela plants (Table 10).

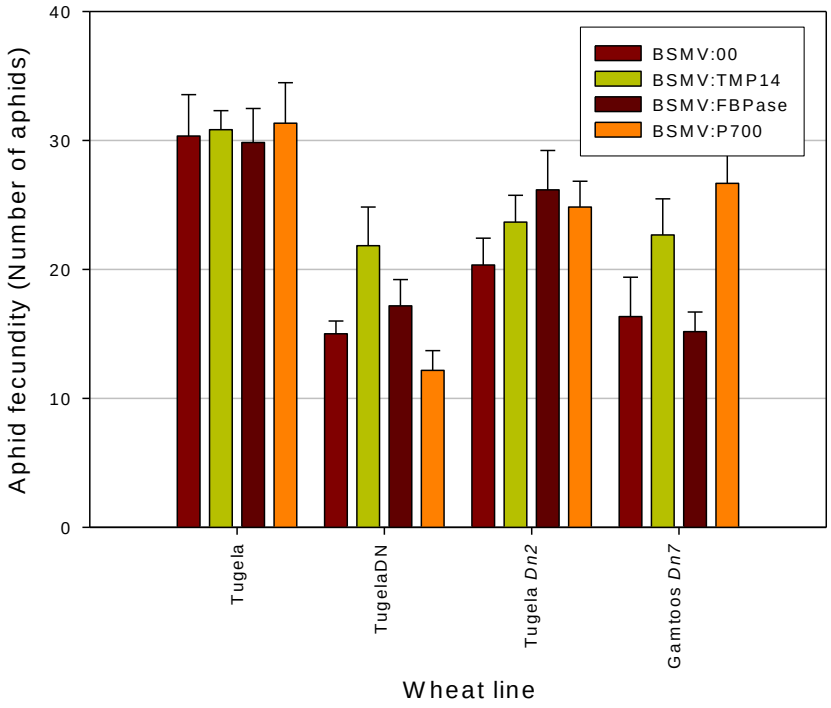


Figure 49: Summary of the aphid fecundities of the three wheat lines. Aphid fecundity is indicated as number of aphids per plant 10 d.p.i.

No significant decrease in aphid fecundity was observed (Figure 49) in the Tugela plants treated with the silencing constructs compared to the uninfested Tugela plants. Silencing with BSMV:TMP14 caused a significant increase in aphid fecundity when aphids are fed on the TugelaDN and Gamtoos *Dn7* BSMV:FBPase plants. Silencing with BSMV:FBPase caused an increase in aphid fecundity in the Tugela *Dn2* plants, but caused no significant increase in the fecundity of Gamtoos *Dn7* plants. The Tugela *Dn2* and Gamtoos *Dn7* plants responded with a significant increased aphid fecundity to the BSMV:P700 silencing, however with the silencing of BSMV:P700 in TugelaDN plants aphid fecundity decreased significantly (Figure 49).

2.4. Discussion

The RWA has devastating effects on the wheat crop production in South Africa and worldwide (Du Toit, 1992). Different resistant wheat lines have been bred over the years but they respond differently towards RWA feeding. Three different mechanisms of resistance were identified and classified as antixenosis, antibiosis and tolerance (Smith *et al.*, 1992). It was also found that photosynthesis does play a role in the defence response and to get a better understanding, representatives of these resistance mechanisms were selected for studies (Botha *et al.*, 2006a). The Gamtoos *Dn7*, TugelaDN and Tugela *Dn2* respectively represent the defence mechanisms mentioned and serve as good candidates for comparative studies. They are all resistant to the SA1 RWA biotype with the Tugela line being susceptible. Comparing these wheat lines in terms of photosynthesis and resistance towards the SA1 biotype will give us a better understanding of the resistance mechanisms. To analyse the role that differentially regulated genes have on the resistance of the resistant wheat lines, the VIGS system was optimized for silencing the specific genes.

2.4.1. First aim: Optimizing VIGS for wheat

To optimize the VIGS system for wheat plants, a virus with a host range that includes wheat to be used. Therefore the Barley stripe mosaic virus was obtained from Dr. Scofield who already manipulated the virus to serve as a vector for wheat. The virus is capable of spreading through the wheat plant and transcribes a specific exogenous RNA fragment (Scofield *et al.*, 2005). Photosynthesis related genes were successfully cloned into the vectors and RNA of the virus constructs was produced for infecting the wheat plants. Applying the virus RNA constructs to the wheat plants through rubbing was found to cause considerable damage to the leaves of the pant if it was done to vigorously. It was then standardized to rubbing the first and second leaves with the RNA buffer mixture only twice per leaf in a gentle manner. The virus was found to be optimally active for two weeks after infection (Haupt *et al.*, 2001; Hein *et al.*, 2005). Infection with the virus and infestation with RWA was thus done on the same time to optimally make use of the time span available. To re-infect the plants with the virus would cause too much variability in the active virus concentrations.

RNA extractions were done 10 days after infection rather than on 14 days to exclude the variation that will exist due to the virus losing optimal activity approaching the 14 days. Ten days also allowed enough time for effective silencing of the genes, for the aphids to multiply differentially due to the silenced genes and avoid the decrease of the plant's health affecting reproduction of the aphids. The RT-PCR analysis was done on the RNA obtained from uninfested plants, infested plants and plants infested as well as infected with the BSMV constructs. The results clearly indicated a decrease in the mRNA levels of the genes of interest. An average silencing of 40 % was observed for all the fragments used for silencing. The VIGS system was thus effectively applied to silence wheat genes in a transient manner. This technique could be used to silence any gene of interest, even genes that are essential for the plants survival.

2.4.2. Second aim: To determine the involvement of photosynthesis in RWA resistance

The commonly used wheat line Tugela that has a high grain yield (Tolmay *et al.*, 2006) was compared to the resistant near-isogenic line TugelaDN in terms of the effect RWA feeding has on photosynthesis. Aphid fecundity, the fluorescence profile, the chlorophyll content and the differential expression of selected photosynthesis related genes were brought into consideration. The aphid fecundity of the Tugela wheat averaged 30.33 aphids per plant after 10 days of infestation (Figure 45), but for the resistant TugelaDN aphid fecundity was less (15 aphids per plant) (Figure 46). The resistance mechanism of TugelaDN is clearly very effective in preventing RWA reproduction. It is hypothesised that RWA targets the chloroplasts of its host by the injected toxins and cause the breakage of the chloroplast in susceptible plants (Fouché *et al.*, 1984; Botha *et al.*, 2006a). This was clearly visible in the chlorophyll *a* and *b* content that decreased from 3.0 $mg.l^{-1}$ to 2.2 $mg.l^{-1}$ and from 1.6 $mg.l^{-1}$ to 1.1 $mg.l^{-1}$ respectively (Figure 2.27). The OJIP fluorescent profile and Fv/Fm readings also decrease drastically and it directly correlates with a reduction in the efficiency of photosynthesis. To confirm whether RWA feeding affect the expression of photosynthesis genes RNA samples of Tugela plants were extracted 10 days after infestation and analysed using RT-PCR (Figures 2.17, 2.18 and 2.19). It indicated a -2.6 fold reduction in TMP14 mRNA levels, a -5.28 fold reduction in FBPase mRNA

levels and a -5.4 fold reduction in P700 mRNA levels. The RWA toxins thus caused a significant decrease in the synthesis of photosynthesis related genes which had the result of decreased chlorophyll content (Botha *et al.*, 2006a). However, the toxins also caused the existing photosynthesis system to function at a lower rate evident in the OJIP kinetics.

The observations made for the resistant TugelaDN differs compared to its susceptible counterpart. There is only a slight change in chlorophyll content in TugelaDN plants submitted to RWA feeding. TugelaDN's resistance mechanism thus prevents the RWA's toxins from causing damage to its chloroplasts and rather than the expression of the genes being inhibited, they were induced. The recognition of RWA specific proteins cause signal transduction to the nucleus of the plant cells and the induction of TMP14 and P700 genes with 1.82 and 1.4 fold inductions respectively. The FBPase gene however was repressed with a fold change of -1.6. The photosynthesis machinery is thus maintained but the OJIP kinetic analysis indicates that the photosynthesis is compromised. The photosynthesis even had a greater decrease than observed in the susceptible Tugela plants. The reason for restricting photosynthesis might be to direct the energy to the formation of ROS rather than producing glucose for plant growth. By quickly producing ROS the plants stimulate the HR as an effective defence mechanism. Essential differences in the response to RWA feeding are observed in TugelaDN compared to Tugela on a biological, physiological and molecular level. This supports the hypothesis that photosynthesis does play a significant role in RWA resistance.

2.4.3. Third aim: To determine the different photosynthetic responses of the resistant wheat lines

Fluorescence studies were done to analyse the differences in photosynthetic capacity (Schreiber, 2002; Lazar, 2003) and chlorophyll measurements served as an indicator of chloroplast status (Haldrup *et al.*, 2003) before and during aphid feeding. Aphid fecundities were one of the parameters documented at 10 d.p.i. to represent the effectiveness of the resistance mechanisms (Mowry, 1994). The effect RWA feeding has on the four different wheat lines are summed up in Table 10 and are explained below in terms of the findings.

The chlorophyll measurements indicated that the susceptible Tugela wheat suffered intense chlorophyll decline which confirm previous findings of Botha *et al.,* (2006a). This is due to the chloroplasts that gets damaged by successful aphid feeding (Fouché *et al.*, 1984). The chlorophyll content of TugelaDN on the other hand only decreased minimally and could be due to the defence mechanism of TugelaDN that prevents chloroplasts degradation (Botha *et al.*, 2006a). Tugela *Dn2* relies on photosynthesis to tolerate the aphid infestation and thus the chlorophyll content does not decline much (Botha *et al.*, 2008). The chlorophyll content of Gamtoos *Dn7* again resembles that of TugelaDN (Lapitan *et al.*, 2009).

As the chloroplasts of the Tugela plants gets damaged by RWA feeding its photosynthetic capacity functions at a much lower level. This is clearly illustrated by the fluorometer readings documented 10 days after infestation, but TugelaDN's photosynthetic capacity shows an even greater decline than that of Tugela plants. Such a significant decline in photosynthesis without diminished chloroplasts is a common phenomenon found in other resistant plants that are infested with certain pathogens (Scharte *et al.*, 2005). The photosynthetic capacity of Tugela *Dn2* however undergoes almost no change. This is due to its mechanism of tolerating the aphid infestation, to make sure the normal energy supply from photosynthesis is maintained. The decline observed in the Gamtoos *Dn7* wheat under aphid infestation is almost as much as that of TugelaDN which indicates that the defence mechanisms used might be similar.

The rate of reproduction of aphids varies depending on the favourability of the feeding conditions and resistance mechanisms will have an adverse effect on it. Aphid fecundities illustrated that Tugela are clearly susceptible with 30.33 aphids per plant 10 days after infestation. The tolerance mechanism of Tugela *Dn2* is quite successful in decreasing the aphids per plant to 20.33 (Figure 47). Gamtoos *Dn7* managed to bring the aphids per plant down to 16.33 (Figure 48) with its antixenosis mechanism and TugelaDN was the most successful to bring it down to 15 aphids per plant (Figure 42) with the antibiosis mechanism. The different resistance mechanisms thus have different effects on aphid reproduction and also cause the plant's photosynthesis to

respond in a certain way. The extent of change in the photosynthetic machinery reveals the characteristic method of resistance.

2.4.4. Elucidating the roles of TMP14, FBPase and P700 in resistance

To obtain a better understanding of the significance of the photosynthesis related genes TMP14, FBPase and P700 in conferring resistance they were silenced using VIGS technology. Plants that have TMP14 or P700 silenced displayed an altered fluorescence profile and leaf chlorophyll content. The 'O' and 'J' stages in electron excitation stayed the same compared to normal plants but the 'I' and 'P' stages declined significantly. This is an indication that the photosystem I (PSI) complex in the photosynthesis system does not function properly. Due to both of these proteins having prominent functions in the PSI complex it provides confirmation that the genes are silenced. Their silencing also caused a significant decline in chlorophyll *a* and *b* content due to a drastic decrease in photosynthesis functioning (Figure 2.18-2.21). Chlorophyll synthesis will be inhibited as less is needed to serve the low photosynthesis capacity (Figure 2.23-2.26). Plants that have FBPase silenced do not display the same profile change (Figure 2.28-2.31) because the protein is not functional in the photosynthesis system but rather in the gluconeogenesis pathway (Plaxton, 1996). It is thus expected that when FBPase is silenced it will not strongly affect the fluorescence but slight changes can be observed as the downstream biochemical pathway is blocked (Tamoi *et al.*, 2006). A chlorophyll decline does occur but not to the same extent as observed for the other two genes.

Aphid fecundity differences in the wheat plants that are caused by silencing of the specific genes compared to plants that are not silenced are summed up in Figure 49. To prove that the aphid fecundity changes are not caused by the health of the plants that gets jeopardized by silencing, the photosynthesis related genes were also silenced in the susceptible Tugela plants. No major changes in the aphid fecundities could be observed and the test was accepted (Figure 45). Tugela plants under RWA feeding and infected with BSMV:PDS die at the same time (35 d.p.i.) as uninfected Tugela plants under RWA feeding (not shown). It is an indication that silencing PDS which negatively affects photosynthesis does not cause the plant to die earlier. It also does not affect aphid fecundity. Negatively affecting photosynthesis on its own thus does not cause a change in the state of resistance (not shown). The silencing of the other

three genes reveals a specific role of the gene in the defence mechanism rather than the damaged photosynthesis that cause a loss in resistance. The possible method of conveying resistance is explained for each resistant line in terms of the biological effects that could be observed due to the specific genes being silenced.

The aphid fecundities were measured at 10 d.p.i. This time interval was chosen as it showed the best indication of how the silencing of a gene affected aphid fecundity. It allowed enough time for the aphids to multiply differentially and avoid that the decrease of the plant's health affecting reproduction. Resistance of the TugelaDN, Tugela *Dn2* and Gamtoos *Dn7* are jeopardized when the TMP14, FBPase and P700 are silenced. This is evidenced by an increase of aphid fecundities (Figure 49). P700 and FBPase are exceptions to this because when P700 was silenced in TugelaDN and FBPase was silenced in Gamtoos *Dn7*, aphid fecundities decreased. It is an indication of increased resistance (Figure 49). All three these photosynthesis related genes are inhibited in Tugela plants but are induced in the resistant plants. This with the fluoremetric and chlorophyll extraction results indicated that these three genes' involvement in the defence mechanisms of these plants is promising. Data obtained in this study gives insight into the mechanisms which the resistant lines use to convey resistance. A hypothesis for each type of mechanism is provided below.

2.4.5. Hypothesis on the TugelaDN resistance mechanism

The TugelaDN line appears to have the most successful resistance mechanism against the SA1 RWA biotype (Figure 49). Upon recognition of the aphid in the resistant TugelaDN line, a photosynthetic decline beyond the level reached in susceptible controls are obtained (Figure 35). It is proposed that in an incompatible interaction the plant deliberately decrease its photosynthetic capacity and other assimilatory metabolism processes in order to initiate respiration and other processes needed for defence (Scharte *et al.*, 2005). The purpose of doing this might seem like suicide but it could be a desperate attempt on survival. Local suicide in the form of programmed (PCD) forms necrotic lesions through the hypersensitive response. It forms at the sight of feeding which prevents the aphids from further sucking the phloem (Botha *et al.*, 2006a). This defence mechanism, scavenges ROS for the use in the hypersensitive response (Botha *et al.*, 2006a). Increased ROS are obtained by intentionally blocking photosynthesis at the photosystem I complex which putatively drives the energy flow to the formation of the toxic ROS (Botha *et al.*, 2006a). This seemingly risky antibiotic defence mechanism is successful in keeping aphid numbers low as observed in Figure 49.

ROS have regulatory properties in stress conditions and is produced at various sources including NADPH-oxidase receiving electrons through ferredoxin (Fd). It is also scavenged by superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX) and peroxiredoxin (PrxR) (Apel and Hirt, 2004). Oxydative burst happens when production is induced and scavenging is inhibited which leads to HR and PCD (Allen *et al.*, 1999).

The initial and very rapid accumulation of H_2O_2 might induce genes involved in inducing a second and prolonged burst of H_2O_2 production (Baker and Orlandi, 1995). A substrate for H_2O_2 production is superoxide which is mainly formed in the Mehler reaction at PSI, either directly or via ferredoxin (Mehler, 1951). The flow normally progress from the excited electrons of P700 through Fd to the electron acceptor $NADP^+$. Under extreme conditions including high light intensity or low temperatures, electrons of PSI can similarly be transferred to oxygen, which results in the generation of ROS (Figure 51) (Baier and Dietz, 1999; Mittler *et al.*, 2004). This could be

exactly what happens when P700 is silenced as in the present study. It putatively minimizes the energy flow through Fd, forcing the direct energy flow to the increased production of ROS. An increased ROS pool enlarge the magnitude of HR and thereby increase the defence response (Mittler *et al.*, 2004). A stronger defence response can be observed as a decrease in aphid fecundity documented in Figure 46. The second possibility that can explain the increased resistance is the silencing of P700 cause the over reduction of the plastoquinol (PQ) pool which is capable of up or down regulation of genes in the chloroplast genome and the nucleus (Figure 50) (Pfannschmidt, 2003). These genes could be involved in relaying resistance. Due to the fact that we know that the resistance mechanism of TugelaDN scavenges ROS for the HR, the first possibility provides to be more likely. It might be possible that the second pathway is involved as it provides an explanation of how the PR proteins are induced.

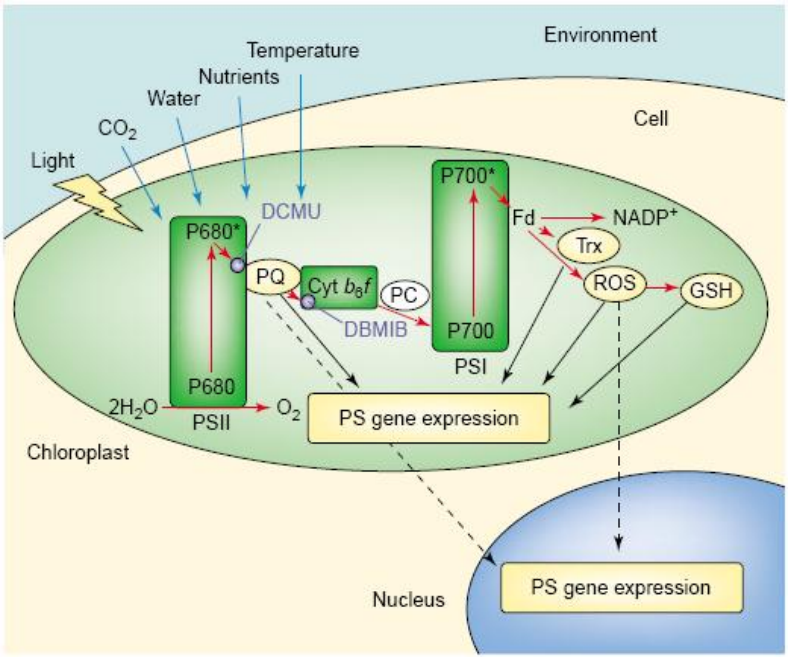


Figure 50: Photosynthesis regulating itself. Gene expression is regulated by an over reduced PQ pool due to an electron flow blockage within the photosynthesis electron transport. ROS also regulates gene expression, but happens due to an oxidative burst downstream of the photosynthesis electron transport (Pfannschmidt, 2003).

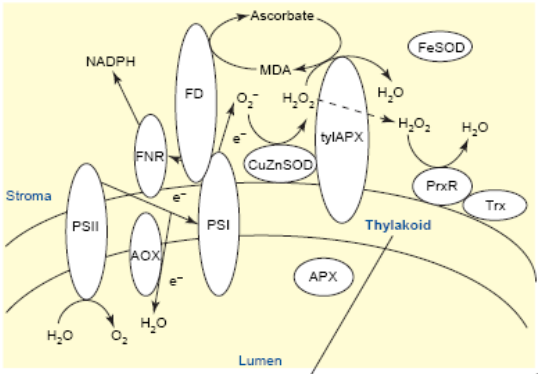


Figure 51: The maintenance of ROS equilibrium. ROS production can occur either indirectly through P700 excitation, Fd and NADPH oxydase or directly from the reduced PSI complex (Mittler *et al.*, 2004).

Once TMP14, which is characterized as one of the electron acceptors from the LHCII (Khrouchtchovaa *et al.*, 2005), is silenced aphid fecundity increases indicating a decline in resistance. If electrons cannot be accepted by TMP14 it cannot be passed on to the normal formation of ROS by the Mehler reaction through the Fd pathway. Less ROS means that the HR will not function optimally and thus aphid fecundity will rise. The silencing of TMP14 would lead to an over reduced PQ pool as no electrons can be passed on to the PS I (Pfannschmidt, 2003). This will cause an over reduced PQ pool which do not lead to an increase in resistance, but rather a decrease (Figure 46). It can up regulate the genes involved in maintaining the chloroplast rather than directly induce resistance genes (Pfannschmidt, 2003). Defence genes are thus more probably up regulated through ROS induction.

Silencing FBPase does not show a direct influence on resistance as the aphid fecundity stays more or less the same compared to the control plant. This indicates that the TugelaDN line's resistance is not dependent on carbon flux but mostly dependent on ROS regulation for HR and PR protein induction. Photosynthesis related genes are up regulated as shown in Figures 2.17 to 2.19 and can thereby maintain the chlorophyll levels in TugelaDN wheat lines upon RWA feeding (shown by Botha *et al.*, 2006a).

With an intentional decrease in photosynthesis the TugelaDN resistance mechanism prevents aphids from feeding through the use of the HR reaction. However at the same time it increases the production of photosynthesis related genes to maintain the

chloroplasts and photosynthesis. This could allow the plant to successfully recover after aphid feeding and be the determining factor of TugelaDN's resistance. Up regulation of these genes prevent PCD of tissue surrounding the necrotic lesions from dying from the ROS burst (Allen *et al.*, 1999). Thus photosynthesis would undergo photo inhibition but it will be maintained in the long term because of the up regulation of the genes.

2.4.6. Hypothesis on the Tugela *Dn2* resistance mechanism

Tugela *Dn2*'s response to RWA feeding is quite different from that of TugelaDN especially on the level of chlorophyll content and photosynthetic regulation. The Chlorophyll *a* and *b* content does not undergo any decline and the OJIP analysis indicates that photosynthesis is maintained at normal rates (Figure 43). The photosynthesis even increases slightly (Figure 43). The molecular changes in wheat plants under RWA feeding observed by Botha *et al.* (2006a) indicate an up regulation of genes that express proteins involved in the electron transport chain and the Calvin cycle. This supports the hypothesis that Tugela *Dn2*'s resistant mechanism is made possible by means of maintaining its photosynthetic capacity. This allow more energy in the form of carbon chains to be available for wound healing, mechanical support, compensatory growth, and general vigour and for making changes in photosynthetic partitioning (Pedigo, 1989; Smith, 1989). All the silencing trials of the three genes in the Tugela *Dn2* line had a negative impact on resistance evident in aphid fecundity that increased. The silencing of FBPase relays the biggest increase (Figure 49). An increased susceptibility occurs due to less carbon energy available to drive reactions necessary to tolerate RWA infestation (Figure 47). This proposes an opposite strategy than that found in the TugelaDN line and might not reduce the spreading of the RWA but could reduce the development of new biotypes. There is little pressure on the RWA to undergo mutational adaption to survive on the host as the host does not decrease fecundity or serve as a poor preference food source, but rather increase food production (Pedigo, 1989; Smith *et al.*, 1992; Botha *et al.*, 2006a).

2.4.7. Hypothesis on the Gamtoos *Dn7* resistance mechanism

With the highly effective resistant Gamtoos *Dn7* line, one observes HR and a decline in photosynthesis evident in the OJIP readings (Figure 35). The decline is not in the same degree as with the TugelaDN line showing that the Gamtoos *Dn7* line is not solely dependent on HR. With the Gamtoos *Dn7* line being more antixenotic than antibiotic, it has been found to use volatiles as its main weapon in defence (Castro *et al.*, 2005). If the Gamtoos *Dn7* line was highly dependent on HR the silencing of P700 would also increase resistance against RWA. Instead a major increase in susceptibility is observed which means that the regulation of P700 have a major role

in the antixenotic mechanism. This indicates that the Gamtoos *Dn7* line is to some extent dependent on carbon flux for the production of volatiles. This is confirmed with the silencing of TMP14 which increase susceptibility. The silencing of FBPase on the other hand shows a slight decrease in susceptibility. By silencing FBPase the gliconeogenesis pathway are blocked and the carbon flow will flow in the glycolytic direction as illustrated in Figure 52. With increased glycolysis glucose are converted to Acetyl Co-enzyme A or Glyceraldihyde-3-phosphate and pyruvate. These compounds can then be converted to various volatiles (Frey *et al.*, 1997). Silenced FPBase will thus benefit volatile production and thereby deter the aphids to a greater extent.

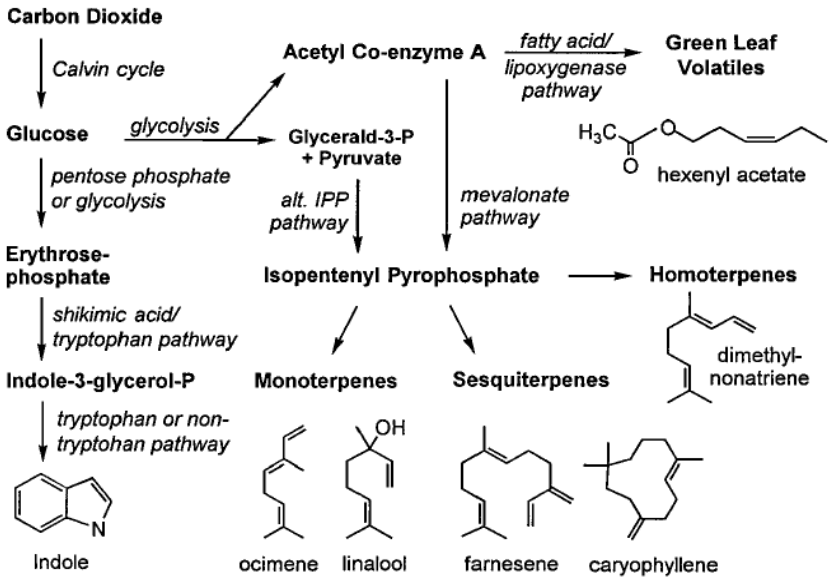


Figure 52: Biosynthetic pathways for the production of plant volatiles from the carbon source and the release thereof (Pare and Tumlinson, 1999). Indole, a product of the shikimic acid pathway, is formed from indole-3-glycerol-P either as an intermediate in Trp biosynthesis or by a Trp-independent pathway leading to a family of nitrogen-containing defence compounds (e.g. 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) (Frey *et al.*, 1997).

2.5. Conclusion

Photosynthesis as observed plays a key role in the defence mechanisms of RWA resistant wheat lines. The toxins injected by the RWA cause significant damage to the chloroplast of the susceptible Tugela wheat plant, however fail to do the same in the resistant wheat lines. The fluorescence readings also revealed a decrease in the photosynthetic rates of the susceptible Tugela and the lack thereof in the resistant Tugela *Dn2*. The photosynthetic rates of TugelaDN and Gamtoos *Dn7* wheat lines on the other hand decreased to a greater extent as a function of their defence mechanism and not as a cause of the aphid toxins. The expression of photosynthesis related genes, *TMP14*, *FBPase* and *P700* are inhibited in the Tugela plant which is caused by the injected RWA toxins. However, the resistant wheat lines recognise the RWA specific avirulence factors and up regulate the photosynthesis related genes to overcome the attack of the aphids. The regulation of these genes differs between the different defence mechanisms and by silencing them the differences were elucidated. The TugelaDN wheat essentially depends on the ROS produced by the photosynthesis reactions to furnish the hypersensitive reaction. The hypersensitive reaction cause necrotic lesions and effectively reduces RWA reproduction. Tugela *Dn2*'s tolerance mechanism depends on a fully functional photosynthesis system to provide carbon flux for maintenance reactions. Gamtoos *Dn7*'s defence mechanism depends on both the ROS scavenging and carbon flux. Combining the *Dn1* and *Dn7* genes in a single wheat line by using cross breeding could amplify the defence response. Both mechanisms restrain photosynthesis and the other assimilatory metabolism processes and will result in a stronger HR response as well as a stronger volatile production for resistance.

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Appendix

Appendix A: Gene sequences used for silencing

5’-GGACCCACGGGTCCGCGACTGGAGCACGAGGACACTGACATGGAC
TGAAGGAGTAGAATATCCGACCAACGCGCGCGCAAGTGGTGATGCTG
TGAGGGCGTACACTGACCGAAGGCATCGATCTCCATGGCCCCAACCG
TCGCCTCCCCAGCCACGGGCGCCGTCCCCTCGCCGATCGCGAGCGACC
TCGGGAAGGCCGCGCGCTCCGTCGGGCTCGGGCTCCCCGCGCTGCCGC
CGCTGCCCGGCCTCGCGTCCCACGGCCAGCCCCGCGTCGCCTCCTTCT
GTAAAAGGCTCGCGAGGAACGTGGTGTCAATGGCCGCCGGTGAGCCG
GCCGCGCCGCTGGCCGACAACGCCGAGCTTACAGAGTTCTTCAACGG
CTTGAAACAAGAGTGGGACAGAGTGGAGGACAAGTACGCGGTGACCA
CGCTCGCCGTCGCCGCCACGCTCGGCATGTGGAGCGCCGGCGGAGTA
GTATCGGCAATCGACAGGCTCCCCGTGGTTCCAGGTCTCATGGAGGTC
GTTGGCATTGGTTACAGCGGGTGGTTTGCGTACAAGAACCTGCTATTC
AAGCCCGACAGGAAAGCGTTCTTCGCTAAGGTCAGGAACATTTACGA
GGATATAATCAGCGGCTAGACGATGAAGCACTTGAAAAGGTGCATCC
CAGTTGAGAGAAGCACAAAGTACTCAAAACGGAGACGTGCAATTACT
GTATATCTGTACTATATATTTGAGCATAGCATCTAATATAATCCTTACT
CAATTGTATATCTAAATAAGTTGGACATATTGACGCAAAAAACTTTTG
ATGTGTTTAGCCCCCTTTTCCCAGATTTTCAACAAG-3’

Figure 53: Thylakoid membrane phosphoprotein (14 kDa) gene sequence used for silencing.

5’-GCCTGCGCTGCAAGCAAAGCCCGCAACACGTACCAACTCTCCTACG
AGCTGAGCGAGCGAACGGGAGAGAGGGTCGGCGTCGGAGAAGGAAG
ATGGATCACGCGGCGGACACGTTCCGGACGGACCTGATGACCATCAC
GCGGCACGTGCTGAACGAGCAGAGCCGGCACCCGGAGTCGCGCGGCG
ACCTCACCATCCTCCTCTCCCACATCGTCCTCGGCTGCAAGTTCGTCGC
CTCCGCCGTCAACAAGGCCGGCCTCGCCAAGCTCACCGGCCTCGCCGG
CGAGACCAACGTCCAGGGGGAGGAGCAGAAGAAGCTGGACGTGCTCT
CCAACGAGGTGTTCGTCAATGCCCTCGTCAGCAGCGGCCGCACCTGCG
TTCTTGTGTCCGAGGAGGACGAGAAGGCGACGTTCGTGGACCCTAAG
CTCCGTGGAAAGTACTGTGTCTGCTTTGACCCCCTGGATGGATCCTCC
AACATCGACTGCGGCGTCTCCATCGGAACGATCTTTGGGATCTACATG
ATCAAGAACCAAGACACCGTGACTCTGGAGGAAGTACTGCAGCCTGG
GAAGGACATGATTGCTGCCGGATACTGCATGTATGGGAGTTCCTGCAC
GCTTGTCCTGAGCACTGGAAATGGTGTCAACGGCTTCACGCTTGACCC
CTCTCTCGGGGAGTTCATAATGACTCATCCAGATATCAAGATACCGCC
GAAAGGAAAGATCTATTCGGTTAATGAAGGGAACGCCAAGAACTGGG
ACACGCCTACTGCAAAGTACGTGGAGAAGTGCAAGTACCCCACGGAT
GGTTCATCACCTAAATCCCTTAGATACATCGGCAGCATGGTTGCTGAT
GTGCACCGCACCTTGCTATACGGCGGCATATTTCTGTACCCCGCGGAC
AAGAAGAGCCCAAGCGGAAAGCTCCGTGTGATGTATGAGGTGTTCCC
CATGTCATTCCTGATGGAGGAGGCTGGAGGCCAGTCTTTCACAGGCAA
AGGACGGTCGCTCGACCTGATCCCCACCGACATCCACGAGAGATCGC
CGATATTCCTCGGCAGCAGCGACGACGTGGAGGAGATCAAGGCACTG
TACGCGGAGGAGGCCAAGAAGGCAGGGTCTGCATGATGATCGGCGGC
CGTCGCGTGTGAATCGATGGCGACCCATGGGCCTTTGCTGGCAATGTG
ATTCGTATGTCAGGCATTTCTTTTCTATACGTCCGTGTCATTCAGTGTA
ATATAGCGTGTGGAGGCCTCGAACAGCTTCATTCATGAGCAGCCAAG
GCTTCTGCCTCTCACAACAATCCACTGATGATGGCACGTAATATACCT
CCATAACTACATGTTTCC-3’

Figure 54: Fructose-1, 6-bisphosphatase gene sequence used for silencing.

5’-ATGAATGAACCGCCAGGAGCTCGTATGAGAGTTGGTTTGACTGCCC
TAACTATGGCAGAATATTTCCGAGATGTTAATAAGCAAGACGTGCTTC
TATTCATCGATAATATCTTTCGTTTTGTTCAAGCAGGATCGGAGGTATC
TGCCTTATTAGGGAGAATGCCCTCTGCAGTGGGTTATCAACCTACTCT
TAGTACAGAAATGGGTTCTTTGCAAGAAATAATTACTTCTACTAAAAA
GGGATCTATAACTTCGATCCAAGCGGTTTATGTACCTGCGGACGATTT
GACCGACCCTGCTCCTGCTACAACATTTGCACATTTGGATGCTACTAC
CGTACTTTCCAGAGGATTAGCTTCCAAAGGGATTTATCCTGCAGTAGA
TCCTTTAGATTCAACCTCAACTATGTTACAACCTCGGATCGTTGGCAA
CGAACATTATGAAACTGCGCAAAGAGTTAAGCAAACTTTACAACGTT
ACAAAGAACTTCAGGACATTATCGCAATTCTTGGGTTGGATGAATTAT
CGGAGGAGGATCGTTTAACTGTAGCAAGAGCACGAAAAATTGAGCGC
TTCTTATCACAACCGTTTTTTGTGGCAGAAGTTTTTACCGGTTCTCCGG
GAAATGGCCAAATTGGCGTATTACCAAACCACGCCCCCATTAACACA
GCTGTAGATATGGGTCCCTTGAGAATACGCCTCCTCAACGATCAATGG
TTAACGGCGGTTCTGTGGAGCGGTTTTGCCAGAATAGTTAATAATGAG
ATCATCATTTTAGGA-3’

Figure 55: P700 Chloroplast gene (ATP synthase regulator) gene sequence used for silencing.

Appendix B: Fluorometer readings

Chlorophyll *a* fluorescence of the wheat plants were measured using the OS-30p Chlorophyll Fluorometer from Opti-sciences (USA). For each parameter readings were taken from three plants to serve as biological repeats. The maximum quantum yield (Fv/Fm) that reveal the photosynthetic condition of the plants photosynthesis are indicated in table format. The statistical significance of the results is indicated as P-values.

Table 11: Fluorometer readings (Fv/Fm) of Tugela and T

Treatment	#1 repeat	#2 repeat	#3 repeat	#4 repeat	Average	Standard deviation	P-value
Tugela	0.756	0.757	0.761		0.758	0.002645751	0.19667358
Tugela BSMV:00	0.762	0.763	0.769		0.764666667	0.003785939	0.009852457
Tugela 10 d.p.i.	0.747	0.743	0.745		0.745	0.002	0.02469517
Tugela BSMV:TMP14	0.745	0.749	0.744	0.735	0.74325	0.005909033	0.029566209
Tugela BSMV:FBPase	0.752	0.748	0.757	0.745	0.7505	0.005196152	0.013596339
Tugela BSMV:P700	0.752	0.754	0.75	0.755	0.75275	0.002217356	0.057625934
Tugela BSMV:TMP14 10 d.p.i.	0.736	0.739	0.733	0.728	0.734	0.004690416	0.016355627
Tugela BSMV:FBPase 10 d.p.i.	0.74	0.739	0.748	0.732	0.73975	0.006551081	0.001555729
Tugela BSMV:P700 10 d.p.i.	0.732	0.74	0.741	0.738	0.73775	0.004031129	0.005891735
TugelaDN	0.756	0.77	0.77		0.765333333	0.008082904	0.196673582
TugelaDN BSMV:00	0.778	0.759	0.762		0.766333333	0.010214369	0.933035047
TugelaDN 10 d.p.i.	0.73	0.734	0.748		0.737333333	0.009451631	0.021401654
TugelaDN BSMV:TMP14	0.749	0.754	0.753		0.752	0.002645751	0.052441261
TugelaDN BSMV:FBPase	0.758	0.763	0.757		0.759333333	0.00321455	0.302514167
TugelaDN BSMV:P700	0.753	0.749	0.745		0.749	0.004	0.057190958
TugelaDN BSMV:TMP14 10 d.p.i.	0.729	0.73	0.733		0.730666667	0.002081666	0.033207082
TugelaDN BSMV:FBPase 10 d.p.i.	0.735	0.733	0.742		0.736666667	0.004725816	0.049906287
TugelaDN BSMV:P700 10 d.p.i.	0.723	0.729	0.727		0.726333333	0.00305505	0.034578416

Table 12: Fluorometer readings (Fv/Fm) of Tugela *Dn2* a

Treatment	#1 repeat	#2 repeat	#3 repeat	#4 repeat	Average	Standard deviation	P-value
Tugela *Dn2*	0.764	0.76	0.755		0.759666667	0.00450925	0.723499368
Tugela *Dn2* BSMV:00	0.751	0.748	0.764		0.754333333	0.008504901	0.534616218
Tugela *Dn2* 10 d.p.i.	0.755	0.763	0.757		0.758333333	0.004163332	0.761803466
Tugela *Dn2* BSMV:TMP14	0.746	0.742	0.745		0.744333333	0.002081666	0.028939239
Tugela *Dn2* BSMV:FBPase	0.748	0.746	0.763		0.752333333	0.009291573	0.440838575
Tugela *Dn2* BSMV:P700	0.737	0.739	0.743		0.739666667	0.00305505	0.044363035
Tugela *Dn2* BSMV:TMP14 10 d.p.i.	0.745	0.743	0.734		0.740666667	0.005859465	0.003673107
Tugela *Dn2* BSMV:FBPase 10 d.p.i.	0.749	0.753	0.752		0.751333333	0.002081666	0.142002221
Tugela *Dn2* BSMV:P700 10 d.p.i.	0.743	0.741	0.743		0.742333333	0.001154701	0.023893627
Gamtoos *Dn7*	0.764	0.77	0.78	0.769	0.77075	0.00670199	0.052441261
Gamtoos *Dn7* BSMV:00	0.768	0.775	0.772	0.767	0.7705	0.003696846	0.939046202
Gamtoos *Dn7* 10 d.p.i.	0.758	0.767	0.76	0.764	0.76225	0.004031129	0.0235435
Gamtoos *Dn7* BSMV:TMP14	0.76	0.756	0.763	0.758	0.75925	0.002986079	0.025735719
Gamtoos *Dn7* BSMV:FBPase	0.769	0.762	0.764	0.767	0.7655	0.003109126	0.231482548
Gamtoos *Dn7* BSMV:P700	0.745	0.749	0.744	0.748	0.7465	0.002380476	0.008660689
Gamtoos *Dn7* BSMV:TMP14 10 d.p.i.	0.738	0.756	0.755	0.755	0.751	0.008679478	0.009547608
Gamtoos *Dn7* BSMV:FBPase 10 d.p.i.	0.768	0.757	0.763	0.745	0.75825	0.009912114	0.087917955
Gamtoos *Dn7* BSMV:P700 10 d.p.i.	0.745	0.737	0.74	0.756	0.7445	0.008346656	0.024215195

Appendix C: Chlorophyll measurements

Chlorophyll *a* and *b* measurements are recorded in the tables below and indicate damage caused to the photosynthetic machinery. The statistical significance of the results is indicated as P-values.

Table 13: Chlorophyll *a* readings of Tugela and TugelaDN. Content is indicated as $mg.l^{-1}$.

Treatment	#1 replicate	#2 replicate	#3 replicate	Average	Standard deviation	P-value
Tugela	2.937	2.943	2.934	2.938	0.004582576	0.403508149
Tugela BSMV:00	2.953	2.992	2.979	2.974666667	0.019857828	0.071853881
Tugela 10 d.p.i.	2.007	2.047	2.267	2.107	0.14	0.00973256
Tugela BSMV:TMP14	1.503	1.506	1.501	1.503333333	0.002516611	7.01776E-07
Tugela BSMV:FBPase	1.722	1.724	1.724	1.723333333	0.001154701	4.59378E-06
Tugela BSMV:P700	1.774	1.776	1.773	1.774333333	0.001527525	2.29751E-06
Tugela BSMV:TMP14 10 d.p.i.	1.311	1.25	1.22	1.260333333	0.046371687	0.000250073
Tugela BSMV:FBPase 10 d.p.i.	1.38	1.421	1.46	1.420333333	0.040004166	0.000251186
Tugela BSMV:P700 10 d.p.i.	1.341	1.482	1.283	1.368666667	0.102344191	0.001291186
TugelaDN	2.959	3.03	2.919	2.969333333	0.056216842	0.403508149
TugelaDN BSMV:00	2.906	2.933	2.963	2.934	0.028513155	0.485594454
TugelaDN 10 d.p.i.	2.804	2.832	2.77	2.802	0.031048349	0.008396875
TugelaDN BSMV:TMP14	2.4997	2.3786	2.4613	2.446533333	0.061885728	0.014792871
TugelaDN BSMV:FBPase	2.8623	2.8547	2.896	2.871	0.021981583	0.154828194
TugelaDN BSMV:P700	2.6353	2.6553	2.5553	2.6153	0.052915026	0.001910197
TugelaDN BSMV:TMP14 10 d.p.i.	2.288	2.292	2.27	2.283333333	0.011718931	0.001518716
TugelaDN BSMV:FBPase 10 d.p.i.	2.667	2.712	2.6	2.659666667	0.056358969	0.000813568
TugelaDN BSMV:P700 10 d.p.i.	2.463	2.51	2.39	2.454333333	0.060467622	0.000365527

Table 14: Chlorophyll *a* readings of Tugela *Dn2* and Gan 

hyll content is indicated as mg.l^{-1}.

Treatment	#1 replicate	#2 replicate	#3 replicate	Average	Standard deviation	P-value
Tugela *Dn2*	2.988	2.952	2.952	2.964	0.02078461	0.178628769
Tugela *Dn2* BSMV:00	3.103	3.179	3.141	3.141	0.038	0.032825392
Tugela *Dn2* 10 d.p.i.	3.073	2.873	2.913	2.953	0.105830052	0.844369053
Tugela *Dn2* BSMV:TMP14	1.658	1.658	1.652	1.656	0.003464102	7.24701E-05
Tugela *Dn2* BSMV:FBPase	1.749	1.752	1.752	1.751	0.001732051	0.000114839
Tugela *Dn2* BSMV:P700	2.013	2.015	2.013	2.013666667	0.001154701	0.000168752
Tugela *Dn2* BSMV:TMP14 10 d.p.i.	1.628	1.633	1.64	1.633666667	0.006027714	0.000126608
Tugela *Dn2* BSMV:FBPase 10 d.p.i.	1.769	1.722	1.678	1.723	0.045508241	0.000183273
Tugela *Dn2* BSMV:P700 10 d.p.i.	1.823	2.032	1.91	1.921666667	0.104987301	0.004572497
Gamtoos *Dn7*	2.307	2.34	2.31	2.319	0.018248288	0.000174815
Gamtoos *Dn7* BSMV:00	2.987	2.976	2.938	2.967	0.02570992	0.000621784
Gamtoos *Dn7* 10 d.p.i.	2.72	3.034	2.688	2.814	0.191196234	0.038480816
Gamtoos *Dn7* BSMV:TMP14	0.947	0.955	0.954	0.952	0.004358899	4.40565E-05
Gamtoos *Dn7* BSMV:FBPase	1.764	1.764	1.76	1.762666667	0.002309401	0.000325449
Gamtoos *Dn7* BSMV:P700	1.701	1.702	1.701	1.701333333	0.00057735	0.000272779
Gamtoos *Dn7* BSMV:TMP14 10 d.p.i.	1.32	1.5	1.31	1.376666667	0.106926766	0.002951001
Gamtoos *Dn7* BSMV:FBPase 10 d.p.i.	2.22	2.21	2.13	2.186666667	0.049328829	0.038847979
Gamtoos *Dn7* BSMV:P700 10 d.p.i.	2.035	2.118	2.189	2.114	0.077077883	0.043867273

Table 15: Chlorophyll *b* readings of Tugela and TugelaDN tent is indicated as $mg.l^{-1}$.

Treatment	#1 replicate	#2 replicate	#3 replicate	Average	Standard deviation	P-value
Tugela	1.59	1.67	1.63	1.63	0.04	0.637083632
Tugela BSMV:00	1.48	1.35	1.53	1.453333333	0.092915732	0.132777441
Tugela 10 d.p.i.	0.87	1.27	1.07	1.07	0.2	0.026148319
Tugela BSMV:TMP14	0.501	0.504	0.507	0.504	0.003	0.000391239
Tugela BSMV:FBPase	0.595	0.599	0.6	0.598	0.002645751	0.000452578
Tugela BSMV:P700	0.57	0.576	0.574	0.573333333	0.00305505	0.00040855
Tugela BSMV:TMP14 10 d.p.i.	0.454	0.474	0.504	0.477333333	0.025166115	0.000359405
Tugela BSMV:FBPase 10 d.p.i.	0.55	0.484	0.532	0.522	0.034117444	0.001464064
Tugela BSMV:P700 10 d.p.i.	0.504	0.489	0.376	0.456333333	0.069973805	0.001712799
TugelaDN	1.5855	1.5387	1.6779	1.6007	0.07083389	0.637083632
TugelaDN BSMV:00	1.597	1.511	1.535	1.547666667	0.044377171	0.37090467
TugelaDN 10 d.p.i.	1.523	1.426	1.724	1.557666667	0.151994517	0.455416039
TugelaDN BSMV:TMP14	1.1007	0.9909	0.9705	1.0207	0.070028851	0.01279546
TugelaDN BSMV:FBPase	1.3523	1.5646	1.47	1.4623	0.10635925	0.235329783
TugelaDN BSMV:P700	1.184	1.106	1.148	1.146	0.039038443	0.007152819
TugelaDN BSMV:TMP14 10 d.p.i.	0.74	0.694	1.02	0.818	0.176442625	0.006296045
TugelaDN BSMV:FBPase 10 d.p.i.	1.312	1.396	1.378	1.362	0.044226689	0.039044391
TugelaDN BSMV:P700 10 d.p.i.	1.033	1.118	1.1	1.083666667	0.044792112	0.008763292

Table 16: Chlorophyll *b* readings of Tugela *Dn2* and Gan 

hyll content is indicated as mg.l^{-1}.

Treatment	#1 replicate	#2 replicate	#3 replicate	Average	Standard deviation	P-value
Tugela *Dn2*	1.451	1.456	1.457	1.454666667	0.00321455	0.014950183
Tugela *Dn2* BSMV:00	1.612	1.621	1.696	1.643	0.04611941	0.017652734
Tugela *Dn2* 10 d.p.i.	1.516	1.795	1.915	1.742	0.204711993	0.132223071
Tugela *Dn2* BSMV:TMP14	0.448	0.455	0.458	0.453666667	0.005131601	1.33067E-06
Tugela *Dn2* BSMV:FBPase	0.565	0.571	0.573	0.569666667	0.004163332	4.25591E-07
Tugela *Dn2* BSMV:P700	0.676	0.68	0.679	0.678333333	0.002081666	1.2905E-06
Tugela BSMV:TMP14 10 d.p.i.	0.39	0.405	0.502	0.432333333	0.060797478	0.00109065
Tugela BSMV:FBPase 10 d.p.i.	0.565	0.541	0.603	0.569666667	0.031262331	0.00039599
Tugela BSMV:P700 10 d.p.i.	0.626	0.584	0.629	0.613	0.025159491	0.000325613
Gamtoos *Dn7*	0.912	0.948	0.946	0.935333333	0.020231988	0.00039304
Gamtoos *Dn7* BSMV:00	1.378	1.387	1.41	1.391666667	0.016502525	0.000362099
Gamtoos *Dn7* 10 d.p.i.	1.318	1.915	1.64	1.624333333	0.298808188	0.051064103
Gamtoos *Dn7* BSMV:TMP14	0.224	0.227	0.232	0.227666667	0.004041452	0.000201176
Gamtoos *Dn7* BSMV:FBPase	0.558	0.562	0.564	0.561333333	0.00305505	0.000723665
Gamtoos *Dn7* BSMV:P700	0.623	0.629	0.628	0.626666667	0.00321455	0.001014227
Gamtoos *Dn7* BSMV:TMP14 10 d.p.i.	0.813	0.834	0.913	0.853333333	0.052728866	0.08102697
Gamtoos *Dn7* BSMV:FBPase 10 d.p.i.	1.292	1.21	1.09	1.197333333	0.101593963	0.061448212
Gamtoos *Dn7* BSMV:P700 10 d.p.i.	1.165	1.128	1.039	1.110666667	0.064763673	0.063062649

Appendix D: Aphid fecundities

Aphid fecundity measurements (aphids per plant) that reveal a change in wheat resistance to the RWA and the plant health are indicated in the tables below. The statistical significance of the results is indicated as P-values.

Table 17: Comparison of aphid fecundity of the four whe UNIVERSITEIT VAN PRETORIA UNIVERSITY OF PRETORIA YUNIBESITHI YA PRETORIA tments. Ten days post infestation are compared in the table

Treatment	#1 replicate	#2 replicate	#3 replicate	#4 replicate	#5 replicate	#6 replicate	Average	Standard deviation	P-value
Tugela BSMV:00	29	34	28				30.33333	3.21455	0.023704
Tugela BSMV:TMP14 10 d.p.i.	33	37	31	39	32	34	34.33333	3.076795	0.009852
Tugela BSMV:FBPase 10 d.p.i.	33	32	38	31	37	32	33.83333	2.926887	0.367544
Tugela BSMV:P700 10 d.p.i.	33	35	32	33	35	36	34	1.549193	0.095466
TugelaDN BSMV:00	15	14	16				15	1	0.023704
TugelaDN BSMV:TMP14 10 d.p.i.	24	23	20	21	24	18	21.83333	2.639444	0.047967
TugelaDN BSMV:FBPase 10 d.p.i.	17	15	20	19	17	15	17.16667	2.041241	0.118083
TugelaDN BSMV:P700 10 d.p.i.	11	12	13	11	14	12	12.16667	1.169045	0.035099
Tugela *Dn2* BSMV:00	22	18	21				20.33333	2.081666	0.079425
Tugela *Dn2* BSMV:TMP14 10 d.p.i.	25	24	20	24	26	23	23.66667	2.065591	0.318995
Tugela *Dn2* BSMV:FBPase 10 d.p.i.	25	29	23	30	24	26	26.16667	2.786874	0.201993
Tugela *Dn2* BSMV:P700 10 d.p.i.	28	22	24	27	23	25	24.83333	2.316607	0.039012
Gamtoos *Dn7* BSMV:00	13	19	17				16.33333	3.05505	0.011696
Gamtoos *Dn7* BSMV:TMP14 10 d.p.i.	26	18	24	23	24	21	22.66667	2.804758	0.258739
Gamtoos *Dn7* BSMV:FBPase 10 d.p.i.	16	11	15	18	16	15	15.16667	2.316607	0.539434
Gamtoos *Dn7* BSMV:P700 10 d.p.i.	22	26	29	31	24	28	26.66667	3.32666	0.023388

Table 18: Plant health and aphid fecundity of Tugela plants under treatr

Days	Tugela plant health	std dev	Tugela aphid fecundity	std dev	BSMV:00 plant health	std dev	BSMV:00 aphid fecundity	std dev	BSMV:TMP14 plant health	std dev	BSMV:TMP14 aphid fecundity	std dev
10 d.b.i.	100	0	0	0	100	0	0	0	100	0	0	0
0 d.p.i.	100	0	4	0	100	0	4	0	100	0	4	0
5 d.p.i.	100	0	13.5	1.290994	100	0	13.25	0.957427	100	0	12	1.603567
10 d.p.i.	100	0	31	1.825742	100	0	30.25	2.629956	100	0	34.375	3.583195
15 d.p.i.	96.66667	5.773503	50.75	5.057997	90	10	49.75	4.645787	93.33333	5.773503	46	3.545621
20 d.p.i.	56.66667	5.773503	67	2.581989	60	10	68.25	3.304038	53.33333	5.773503	53.75	3.807887
25 d.p.i.	50	10	62.5	1.914854	50	10	63.25	3.86221	28.33333	10	43	2.9277
30 d.p.i.	20	10	39	4.966555	20	10	41.75	3.86221	10	11.54701	25.625	3.067689
35 d.p.i.	3.333333	5.773503	25.25	6.344289	0	0	22	4.396969	1.666667	5.773503	0	0

Days	BSMV:FBPase plant health	std dev	BSMV:FBPase aphid fecundity	std dev	BSMV:P700 plant health	std dev	BSMV:P700 aphid fecundity	std dev
10 d.b.i.	100	0	0	0	100	0	0	0
0 d.p.i.	100	0	4	0	100	0	4	0
5 d.p.i.	100	0	12.16667	1.722401	100	0	12.33333	1.032796
10 d.p.i.	100	0	33.83333	2.926887	100	0	34	1.549193
15 d.p.i.	90	10	48.5	3.937004	90	10	51	2.607681
20 d.p.i.	50	10	55	2.828427	51.66667	11.54701	57.16667	2.71416
25 d.p.i.	31.66667	10	38.33333	3.444803	31.66667	10	42.33333	2.581989
30 d.p.i.	8.333333	10	17.83333	4.875107	8.333333	5.773503	24.83333	3.311596
35 d.p.i.	0	0	3.166667	2.926887	0	0	16.5	2.738613

Table 192**.14:** Plant health and aphid fecundity of TugelaDN plants unde

Days	TugelaDN plant health	std dev	TugelaDN Aphid fecundity	std dev	BSMV:00 plant health	std dev	BSMV:00 aphid fecundity	std dev	BSMV:TMP14 plant health	std dev	BSMV:TMP14 aphid fecundity	std dev
10 d.b.i.	100	0	0	0	100	0	0	0	100	0	0	0
0 d.p.i.	100	0	4	0	100	0	4	0	100	0	4	0
5 d.p.i.	100	0	8.333333	1.527525	100	0	8	1	98.33333	5.773503	13.33333	1.75119
10 d.p.i.	100	0	14.33333	2.081666	100	0	15	1	91.66667	5.773503	21.83333	2.639444
15 d.p.i.	96.66667	5.773503	34.33333	3.05505	93.33333	5.773503	36.66667	3.511885	83.33333	10	52.83333	3.81663
20 d.p.i.	93.33333	5.773503	100.3333	10.21437	93.33333	5.773503	95.66667	2.516611	63.33333	0	126.8333	11.61752
25 d.p.i.	83.33333	5.773503	153	4.582576	90	10	141	7.211103	30	10	170.5	11.94571
30 d.p.i.	76.66667	5.773503	181.3333	9.865766	86.66667	5.773503	183.6667	13.05118	8.333333	10	64.33333	12.19289
35 d.p.i.	70	0	190.3333	5.507571	83.33333	5.773503	203.3333	18.44813	1.666667	5.773503	4.666667	7.659417

Days	BSMV:FBPase plant health	std dev	BSMV:FBPase aphid fecundity	std dev	BSMV:P700 plant health	std dev	BSMV:P700 aphid fecundity	std dev
10 d.b.i.	100	0	0	0	100	0	0	0
0 d.p.i.	100	0	4	0	100	0	4	0
5 d.p.i.	96.66667	5.773503	10.66667	1.21106	100	0	7	1.095445
10 d.p.i.	93.33333	5.773503	17.16667	2.041241	96.66667	5.773503	12.16667	1.169045
15 d.p.i.	80	10	41	4.472136	93.33333	5.773503	31.16667	3.544949
20 d.p.i.	38.33333	11.54701	86.33333	6.250333	90	0	90.66667	10.76414
25 d.p.i.	11.66667	0	27.5	5.540758	58.33333	10	82.66667	5.853774
30 d.p.i.	1.666667	0	6.166667	5.671567	38.33333	10	44.5	5.822371
35 d.p.i.	0	0	0	0	1.666667	5.773503	0.833333	2.041241

Table 202.15: Plant health and aphid fecundity of Tugela *Dn2* plants unc

Days	Tugela *Dn2* plant health	std dev	Tugela *Dn2* Aphid fecundity	std dev	BSMV:00 plant health	std dev	BSMV:00 aphid fecundity	std dev	BSMV:TMP14 plant health	std dev	BSMV:TMP14 aphid fecundity	std dev
10 d.b.i.	100	0	0	0	100	0	0	0	100	0	0	0
0 d.p.i.	100	0	4	0	100	0	4	0	100	0	4	0
5 d.p.i.	100	0	7.666667	1.154701	100	0	6.666667	0.57735	98.33333	5.773503	9.333333	1.21106
10 d.p.i.	100	0	21	2.645751	100	0	20.33333	2.081666	93.33333	0	23.66667	2.065591
15 d.p.i.	96.66667	5.773503	28.66667	2.516611	93.33333	5.773503	29	3	83.33333	10	34.16667	3.430258
20 d.p.i.	93.33333	5.773503	45.33333	3.21455	93.33333	5.773503	47.33333	4.725816	63.33333	0	45	6.63325
25 d.p.i.	83.33333	5.773503	65.33333	4.041452	86.66667	5.773503	63.66667	6.110101	48.33333	5.773503	51.5	7.816649
30 d.p.i.	76.66667	5.773503	87.66667	5.033223	80	0	83.33333	5.033223	33.33333	5.773503	42.83333	7.678976
35 d.p.i.	70	0	107.6667	15.04438	80	0	111.3333	8.082904	30	5.773503	34.5	4.037326

Days	BSMV:FBPase plant health	std dev	BSMV:FBPase aphid fecundity	std dev	BSMV:P700 plant health	std dev	BSMV:P700 aphid fecundity	std dev
10 d.b.i.	100	0	0	0	100	0	0	0
0 d.p.i.	100	0	4	0	100	0	4	0
5 d.p.i.	100	0	9.166667	0.752773	100	0	9.666667	1.21106
10 d.p.i.	93.33333	5.773503	26.16667	2.786874	95	5.773503	24.83333	2.316607
15 d.p.i.	86.66667	5.773503	39.16667	3.430258	91.66667	5.773503	36.66667	4.457204
20 d.p.i.	76.66667	5.773503	54	5.09902	73.33333	15.27525	57.33333	7.659417
25 d.p.i.	60	5.773503	60	5.727128	56.66667	11.54701	68	5.621388
30 d.p.i.	51.66667	10	50.33333	6.592926	48.33333	10	77.5	5.822371
35 d.p.i.	50	10	46.66667	4.885352	43.33333	5.773503	83.66667	7.865537

Table 216: Plant health and aphid fecundity of Gamtoos *Dn7* plants und

Days	Gamtoos *Dn7* plant health	std dev	Gamtoos *Dn7* Aphid fecundity	std dev	BSMV:00 plant health	std dev	BSMV:00 aphid fecundity	std dev	BSMV:TMP14 plant health	std dev	BSMV:TMP14 aphid fecundity	std dev
10 d.b.i.	100	0	0	0	100	0	0	0	100	0	0	0
0 d.p.i.	100	0	4	0	100	0	4	0	100	0	4	0
5 d.p.i.	100	0	10	1	100	0	9.333333	1.527525	100	0	12.83333	1.169045
10 d.p.i.	93.33333	5.773503	15.66667	2.081666	96.66667	5.773503	16.33333	3.05505	95	5.773503	22.66667	2.804758
15 d.p.i.	86.66667	5.773503	29.33333	3.511885	93.33333	5.773503	32.33333	3.785939	85	5.773503	37.5	3.271085
20 d.p.i.	83.33333	5.773503	61.66667	3.21455	90	0	57.33333	4.163332	71.66667	5.773503	59.33333	5.609516
25 d.p.i.	83.33333	5.773503	73.33333	7.505553	83.33333	5.773503	70.33333	5.033223	61.66667	0	44.16667	3.125167
30 d.p.i.	76.66667	5.773503	89	3.605551	76.66667	5.773503	92	4	56.66667	5.773503	35.66667	5.680376
35 d.p.i.	73.33333	5.773503	99	11.78983	76.66667	5.773503	98.66667	5.507571	48.33333	5.773503	17.83333	3.920034

Days	BSMV:FBPase plant health	std dev	BSMV:FBPase aphid fecundity	std dev	BSMV:P700 plant health	std dev	BSMV:P700 aphid fecundity	std dev
10 d.b.i.	100	0	0	0	100	0	0	0
0 d.p.i.	100	0	4	0	100	0	4	0
5 d.p.i.	100	0	10.16667	1.722401	100	0	12.83333	1.169045
10 d.p.i.	93.33333	5.773503	15.16667	2.316607	93.33333	5.773503	26.66667	3.32666
15 d.p.i.	83.33333	5.773503	27.83333	4.665476	83.33333	5.773503	52.16667	3.430258
20 d.p.i.	76.66667	5.773503	43.33333	4.082483	78.33333	10	81	3.63318
25 d.p.i.	68.33333	5.773503	51.5	2.880972	73.33333	5.773503	94.16667	11.90658
30 d.p.i.	50	10	30.33333	4.501851	65	5.773503	108.5	7.893035
35 d.p.i.	41.66667	5.773503	23.16667	3.920034	58.33333	5.773503	135.8333	44.77685

Appendix E: RT-PCR results

The RT-PCR results of the TMP14, FBPase and P700 transcripts are indicated in the tables below. The statistical significance of the results is indicated as P-values.

Table 22**.17:** RT-PCR results for TMP14 expression.

Treatment	#1 repeat	#2 repeat	#3 repeat	#4 repeat	#5 repeat	Average	Standard deviation	P-value
Tugela	14.593	15.819	13.324			14.57866667	1.247561755	0.014341906
Tugela 10 d.p.i.	3.196	6.613	6.024	6.759	5.521	5.6226	1.443271388	0.015812279
TugelaDN	10.855	10.512	9.551	9.124		10.0105	0.808641866	0.014341906
TugelaDN 10 d.p.i.	19.063	19.43	18.648	15.77		18.22775	1.669350448	0.000678593
TugelaDN BSMV:TMP14 10 d.p.i. A	9.158	7.329	9			8.468333333	0.993961938	0.005159825
TugelaDN BSMV:TMP14 10 d.p.i. B	6.152	6.45	7.525			6.709	0.722213957	0.002418199
Tugela *Dn2*	17.55898714	14.1826453	14.45580382	16.49689542		15.67358292	1.626701516	0.602098533
Tugela *Dn2* 10 d.p.i.	19.91655384	25.20346383	20.4766179	18.49424166	19.77602811	20.77338107	2.580554391	0.083941028
Tugela *Dn2* BSMV:TMP14 10 d.p.i. A	3.714846487	3.611899304	2.883259142	2.588487358	3.642416743	3.288181807	0.516200747	8.16893E-05
Tugela *Dn2* BSMV:TMP14 10 d.p.i. B	3.82238284	3.001547381	3.285553928	2.533041897		3.160631511	0.539350509	0.001206201
Gamtoos *Dn7*	8.554793225	10.47183389	9.281457609	11.4525624	9.409967637	9.834122953	1.134466038	0.012698749
Gamtoos *Dn7* 10 d.p.i.	162.5555718	202.7636867	166.4822303	125.2792984	162.6671459	163.9495866	27.44051646	0.000243369
Gamtoos *Dn7* BSMV:TMP14 10 d.p.i. A	6.441864758	8.059473401	5.701793874			6.734377344	1.205751176	0.005046549
Gamtoos *Dn7* BSMV:TMP14 10 d.p.i. B	5.616592371	8.520317773	5.591724462	6.932951314		6.66539648	1.386251571	0.002039155

Table 23: RT-PCR results for FBPase expression.

Treatment	#1 repeat	#2 repeat	#3 repeat	#4 repeat	#5 repeat	Average	Standard deviation	P-value
Tugela	0.836346307	0.86829667	0.8087931	0.825341353		0.837812026	0.029778851	2.3713E-05
Tugela 10 d.p.i.	0.176150139	0.140108003	0.157392423			0.157883522	0.018026086	0.001270997
TugelaDN	0.486714379	0.48144199	0.451810888	0.45921871	0.355699105	0.446977014	0.053084308	2.3713E-05
TugelaDN 10 d.p.i.	0.263359626	0.288157462	0.180778491	0.258362313	0.322342499	0.262600078	0.052286816	0.010060804
TugelaDN BSMV:FBPase 10 d.p.i. A	0.410423273	0.355573742	0.324746562	0.456050176		0.386698438	0.058250359	0.013995694
TugelaDN BSMV:FBPase 10 d.p.i. B	0.120705783	0.094991381	0.14295161	0.156820786	0.125556467	0.128205205	0.023466589	0.010802327
Tugela *Dn2*	0.482243742	0.492523923	0.625928027	0.448794979	0.609687854	0.531835705	0.080335022	0.006261474
Tugela *Dn2* 10 d.p.i.	0.315742673	0.367348946	0.377123831	0.423040669	0.410245533	0.378700331	0.04200051	0.015375911
Tugela *Dn2* BSMV:FBPase 10 d.p.i. A	0.131545339	0.113531513	0.092031113	0.159510602		0.124154641	0.028574104	0.001485141
Tugela *Dn2* BSMV:FBPase 10 d.p.i. B	0.22480608	0.124107352	0.115363316	0.22241602		0.171673192	0.060086742	0.013793862
Gamtoos *Dn7*	0.473009674	0.663629378	0.478584146	0.452912231	0.334159237	0.480458933	0.11808137	0.003796041
Gamtoos *Dn7* 10 d.p.i.	1.07524301	1.104108375	0.778862398	0.867215701		0.956357371	0.158550578	0.005847904
Gamtoos *Dn7* BSMV:FBPase 10 d.p.i. A	0.317349277	0.240203774	0.186865617	0.26197391	0.253329627	0.251944441	0.046779798	0.001681987
Gamtoos *Dn7* BSMV:FBPase 10 d.p.i. B	0.277134193	0.181447757	0.265635993	0.242359618	0.179368036	0.229189119	0.046265389	0.004268047

Table 24: RT-PCR results for P700 expression.

Treatment	#1 repeat	#2 repeat	#3 repeat	#4 repeat	#5 repeat	Average	Standard deviation	P-value
Tugela	39.28735557	40.00325904	33.71855498			37.6697232	3.44048356	0.013415092
Tugela 10 d.p.i.	7.061091439	7.153434999	6.663090838			6.959205759	0.260566437	0.003556514
TugelaDN	19.83077573	19.76857418	20.07663145	18.71236894		19.59708757	0.604623081	0.013415092
TugelaDN 10 d.p.i.	29.05098734	32.24570186	31.85187872			31.04952264	1.741947666	0.007775258
TugelaDN BSMV:P700 10 d.p.i. A	1.060395764	1.16021903	1.445910194			1.222174996	0.200085607	0.00098979
TugelaDN BSMV:P700 10 d.p.i. B	0.589671562	0.547011719	0.974282139			0.70365514	0.235338469	0.001023614
Tugela *Dn2*	4.639284148	4.847963888	6.398854452	5.761488628		5.411897779	0.818789857	0.006059194
Tugela *Dn2* 10 d.p.i.	5.428454508	5.221493783	6.288525684			5.646157991	0.565849251	0.309598996
Tugela *Dn2* BSMV:P700 10 d.p.i. A	0.096707513	0.230047438	0.135631589	0.185137295		0.161880959	0.058092265	0.003916742
Tugela *Dn2* BSMV:P700 10 d.p.i. B	1.086684661	0.203626783	0.769948768	0.214177257	0.182638188	0.491415132	0.414413131	0.004693619
Gamtoos *Dn7*	11.07920667	12.63137898	15.46906643	22.20087141		15.34513087	4.918641309	0.016383397
Gamtoos *Dn7* 10 d.p.i.	28.70944439	23.29342244	18.72472655			23.57586446	4.9983475	0.12679951
Gamtoos *Dn7* BSMV:P700 10 d.p.i. A	0.130892706	0.281287889	0.107714935	0.140812651	0.155319471	0.163205531	0.06824661	0.01483184
Gamtoos *Dn7* BSMV:P700 10 d.p.i. B	0.065684286	0.200573079	0.196235025	0.182638188		0.161282645	0.064188557	0.015236895