

Gene silencing in bread wheat (*Triticum aestivum* L.)
following a biolistics approach

by

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Submitted in partial fulfilment of the requirements for the degree

Magister Scientiae

In the Faculty of Natural and Agricultural science

Department of Genetics

University of the Stellenbosch

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February 2014

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DECLARATION

By submitting this thesis/dissertation electronically, I Nadia Mitilda Fisher, hereby declare that the entirety of the work contained therein is my own, original work, and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

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Table of Content

Table of content	III
Acknowledgements	VII
List of Abbreviations	VIII
List of Figures	XI
List of Tables	XIV

Chapter 1

Introduction

1.1 Abstract	2
1.2 Introduction	3
1.3 Preface	6
1.4 References	7

Chapter 2

Literature Review: The link between hexaploid wheat (*Triticum aestivum* L.) and the Russian wheat aphid (*Diuraphis noxia*)

2.1 <i>Wheat</i>	10
2.1.1 Triticeae origins	10
2.1.2 Speciation	10
2.1.3 Triticeae production	11
2.1.4 The consequences of insufficient wheat production	12
2.2 <i>Russian wheat aphid</i>	12
2.2.1 Distribution	12
2.2.2 Identification of RWA resistant wheat cultivars	12
2.3 <i>Host-pest interaction</i>	13
2.3.1 Feeding	13
2.3.2 Symptoms of infestation	13
2.3.3 Host response in resistant plants	14
2.3.4 Wheat Russian wheat aphid interaction	14
2.3.5 Hypersensitive response	16
2.3.5.1 Genes involved in the hypersensitive response	16
2.3.5.1.1 Ascorbate peroxidase	18
2.3.5.1.2 Glutathione S-transferase	20
2.4 <i>Genomic approaches for gene identification</i>	25

2.4.1 Genetic markers	25
2.4.1.1 Molecular markers	26
2.4.1.2 Expressed sequence tags	26
2.4.2 Fluorescent <i>in situ</i> hybridization	26
2.4.3 Mapping	27
2.4.4 Reverse genetic approaches	28
2.4.4.1. Gene silencing	28
2.4.5 Plant transformation	30
2.4.5.1 Agrobacterium mediated transformation	30
2.4.5.2 Particle bombardment	33
2.4.5.3 Promoters	34
2.4.5.4 Reporter and selectable genes	35
2.4.5.5 Current status of wheat transformation	36
2.5 References	37

Chapter 3

Silencing of *APX* and *GSTF6b* in wheat to elucidate their function in RWA defence

3.1 Abstract	48
3.2 Introduction	49
3.3 Research Methodology	51
3.3.1 Isolating targeted genes out of wheat	51
3.3.1.1 Plant material	51
3.3.1.2 Designing gene specific primers	51
3.3.1.3 Treatment of equipment to remove RNase activity	52
3.3.1.4 RNA extraction	52
3.3.1.5 Polymerase chain reaction	52
3.3.1.6 Sequencing and confirmation of identity	53
3.3.2 Gene isolation from plasmids	53
3.3.2.1 Isolation and blunting of DNA	53
3.3.2.2 Phosphorylation of DNA	54
3.3.3 Construction of the silencing cassette	55
3.3.3.1 Preparing transformation vectors	55
3.3.3.2 Cloning	56
3.3.3.3 Fragment orientation determination	57
3.3.3.4 Fragment plasmid purification: DNA isolation	57
3.3.4 Tissue culturing	58
3.3.4.1 Growth conditions	58

3.3.4.2 Harvesting	58
3.3.4.3 Tissue culture and transformation via particle bombardment	58
3.3.5 Infestation procedures	59
3.3.6 Quantitative real time polymerase chain reaction	59
<i>3.4 Results</i>	61
3.4.1 Data assembly	61
3.4.2 RNA extraction	64
3.4.3 Primer screening	65
3.4.4 Confirmation of silencing construct	66
3.4.5 Tissue culture and transformation via particle bombardment	69
3.4.6 Aphid infestation analysis	75
3.4.7 Quantifying expression levels of transgenes	77
<i>3.5 Discussion</i>	82
3.5.1 The isolation of genes from the wheat genome	82
3.5.2 Establishing a transformation platform	82
3.5.2.1 Medium	83
3.5.2.2 Promoters	84
3.5.2.3 Transformation efficiency and difficulties associated with wheat transformation via particle bombardment	84
3.5.2.4 Particle bombardment as a relevant technique for knockout studies	84
3.5.3 qPCR analysis of R ₀ plants	85
3.5.3.1 qPCR analysis of Gamtoos S-pubi- <i>GSTFb</i> before and after infestation	86
3.5.3.2 qPCR analysis of Gamtoos R-pubi- <i>GSTFb</i> before and after infestation	86
3.5.4 Aphid cereal interaction	87
3.5.4.1 Phenotype of R ₀ transformants	87
<i>3.6 References</i>	89
<i>3.7 Appendix A</i>	96

Chapter 4

***In situ* hybridization of APX and GSTF6b in Hexaploid wheat**

4.1 Abstract	102
4.2 Introduction	103
4.3 Research Methodology	105
4.3.1 Plant material	105
4.3.2 DNA extraction	105
4.3.3 Amplification	105
4.3.4 Probe preparation	106

4.3.5 Chromosome squashes	106
4.3.5.1 Sample collection for chromosome squashes	106
4.3.5.1.1 Root sampling and fixation	106
4.3.5.1.2 Embryo sampling and fixation	106
4.3.5.2 Squashes	107
4.3.5.3 Hybridization	107
4.3.5.4 Slide preparation	108
4.3.6 Sample collection for chromosome suspension	108
4.3.6.1 Root sampling	108
4.3.6.2 Cells in metaphase	108
4.3.6.3 Fixation	108
4.3.6.4 Chromosome suspension	109
<i>4.4 Results</i>	110
4.4.1 Optimization of chromosome spreads using the squashing technique	110
4.4.2 Analysis of chromosome spreads using suspension	112
4.4.3 FISH analysis using chromosome suspension	113
<i>4.5 Discussion</i>	115
4.5.1 Optimal cultivars for cell cycle synchronization and accumulation of metaphase cells	115
4.5.2 Chromosome spreads vs chromosome suspensions	115
4.5.3 Limitations of microscopes used	115
4.5.4 FISH analysis	115
<i>4.6 References</i>	117
<i>4.7 Appendix B</i>	119
Chapter 5	
Summary	122

ACKNOWLEDGEMENTS

I would like to acknowledge the following people and organizations for their support, advice and patience throughout the duration of this study.

- Professor Anna-Maria Botha- Oberholster whose expertise and vast knowledge made this study a reality. I appreciate your assistance in writing reports i.e. grant proposals, scholarships and the writing of this thesis. Words cannot express my gratitude.
- Doctor van der Vyver very special thank you for all your assistance in a variety of different aspects i.e. teaching me the tissue culture technique, editing of this thesis and attending the 2nd Conference of Cereal Biotechnology and Breeding Hungary conference with me. Treasured memories I will never forget.
- Doctor van Eck for your fresh perspectives, out of the box thinking and valued contributions.
- Colleagues in the Cereal Genomic Research Group: Francois Burger, Thia Schultz, Anandi Bierman, Kenneth Mbwangi, Laura de Jager, Wynand Cloete and Marlon Le Roux (assisting with the tissue culturing part of the project)
- Department of Genetics and the Institute of Plant Biotechnology for providing infrastructure.
- Winter Cereal Trust (WCT) and the National Research Foundation of South Africa (NRF) for financial assistance for this project.
- Plant Pathology: Lindy Rose and Meagen Vermeulen thank you for your assistance with the microscope work.
- To my parents Stanford and Christina Fisher with love and admiration I dedicate this thesis to you. Thank you for always supporting my life goals no questions asked you're always in my corner. Thank you for your endless motivations.
- To my brother (Enver Fisher) and sisters (Astrid, Crystal, Christine, Nicole and niece Santina) thank you for your words of encouragements and love.
- The Love of my life Charlton Smith thank you for your constant love and patience but most importantly your silent prayers.

“Ek is tot alles instaat deur hom wat my die krag skenk”-Fillipense 4:13

List of abbreviations

Act I	Rice actin 1
Adh I	Alcohol dehydrogenase I
APX	Ascorbate peroxidase
ASA	Ascorbate
ASGA	Antisense mediated gene silencing
Avr	Avirulence
BAC	Bacterial artificial chromosome
BLAST	Basic Local Alignment Search Tool
Bp	Base pair (s)
BAP	Benzylaminopurine
°C	Degree Celsius
CaMV 35S	Cauliflower mosaic virus 35 S
cAPX	Cytosolic APXs
chAPX	Chloroplastic APX
Cm	Centimetre (s)
<i>Dn</i>	<i>Diuraphis noxia</i>
DNA	Deoxyribonucleic acid
DEPC	Diethyl pyrocarbonate
E. coli	Escherichia coli
EDTA	Ethylenediamine tetra acetic acid
ESTs	Expressed Sequence Tags
ET	Ethylene
Et al.	<i>Et alli</i> (and others)
EtOH	Ethanol
F	Phi class
FISH	Fluorescent <i>in situ</i> hybridization
G	Gram (s)
GFP	Green fluorescent protein
<i>GST</i>	Glutathione S-transferase

<i>GSTF6b</i>	Glutathione S-transferase phi class
GPOX	Glutathione peroxidise
GT	Glutathione transferases
GUS	β -glucuronidase
H ₂ O ₂	Hydrogen peroxide
H	Hours
HR	Hypersensitive response
IPTG	Isopropylthio- β -D-galactopyranoside
ITMI	International <i>Triticeae</i> mapping initiative
JA	Jasmonic acid
LB	Luria Broth
MAS	Marker Assisted Selection
MDHA	Monodehydroascorbate
MEGA	Molecular Evolutionary Genetics Analysis
Min	Minute/Minutes
MS	Murashige and Skoog medium
NCBI	National Centre for Biotechnology Information
Ng	Nanogram (s)
NILs	Near isogenic lines
O ₂	Superoxide
P	Probability
PCR	Polymerase chain reaction
PEG	Polyethylene Glycol
PTGS	Post transcriptional gene silencing
qRT-PCR	Quantitative real time PCR
R	Resistant
ROS	Reactive Oxygen Species
RPM	Revolutions Per Minute
RWA	Russian Wheat Aphid
S	Susceptible

SA	Salicylic acid
sAPX	Stromal ascorbate
ScBv	Sugarcane bacilliform badna virus
Sec	Second (s)
SEQ	Sequence
siRNA	Small interfering RNA
SOC	Super Optimum Catabolite
SSC	Saline-sodium citrate
TAE	Tris/Acetate/EDTA
tAPX	Thylakoid bound ascorbate peroxide
T ₀	Transgenic parent
T ₁	First generation transgenic plants
V	Volt (s)
<i>Vir</i>	Virulence
V/V	Volume/volume
U	Tau class GST
Ubi 1	Maize ubiquitin
μl	Microlitre (s)
μg	Microgram (s)
USA	United States of America
2,4 D	2,4 Dichlorophenoxyacetic acid
3' RACE	Rapid amplification of 3' cDNA ends

List of Figures

Figure 2.1: Map illustrating the domestication of Bread wheat in the Fertile Crescent (Feuillet et al. 2007)

Figure 2.2: A schematic representation of the origin of *Triticum aestivum* L due to multiple events of duplication (Feuillet et al. 2007)

Figure 2.3: Schematic representation of Flor's gene for gene model (Hammond-Kosack & Parker 2003)

Figure 2.4: Schematic representative plant signalling pathways involved in aphid feeding (Botha et al. 2005).

Figure 2.5: An illustration of the catalytic reaction which converts H₂O₂ into water (Chen et al. 2006)

Figure 2.6: Phylogenetic tree showing the different types of APX (Ishikawa & Shigeru 2008)

Figure 2.7: Phylogenetic tree showing the different classes of GSTs found both humans and plants (Dixon et al. 2002)

Figure 2.8: Pie charts illustrating: A: the occurrence of plant GSTs in maize, rice and soyabean and B: The expression of the different GST classes in maize, rice and soyabean (Frova 2003)

Figure 2.9: A schematic representation of the distribution of GST classes in the *Arabidopsis thaliana* genome (Dixon et al. 2002)

Figure 2.10: Classification system of plants GST which originated from mammalian nomenclature (Dixon et al. 2002)

Figure 2.11: Schematic representation summarising the function of GSTs in plants (Dixon et al. 2002)

Figure 2.12: Schematic representation illustrating the mechanism involve in PTGS

Figure 2.13: *Agrobacterium* mediated DNA transformation (Tzfira & Citovsky 2006)

Figure 2.14: Schematic representation comparing *Agrobacterium* and Particle bombardment transformation methods

Figure 2.15: Schematic representation of transformation vectors. a) pUbi-510 vector containing the transgene. b) pEmuKN co-bombardment vector containing NPTII as the selection gene

Figure 3.3.1: Schematic representation of transformation vectors. a) pUbi-510 vector containing the transgene. b) pEmuKN co-bombardment vector containing NPTII as the selection gene.

Figure 3.4.1: Alignment between seven hits for GST (forward primer)

Figure 3.4.2: Alignment between seven hits for GST (reverse primer)

Figure 3.4.3: A-C Showing isolated RNA of 6 samples

Figure 3.4.5: Colony PCR of cloned APX and GSTF6b fragments resolved on a 1.5% agarose gel

Figure 3.4.6: pUBI- 510 plasmids containing APX and GSTF6b respectively visualised on a 1.5% agarose gel

Figure 3.4.7: Sequence alignment of APX to clones 7 and 8

Figure 3.4.8: Clustal W alignment of the sequence of clones 8, 9, 12 and 13 to the reference sequence used to obtain this gene fragment from wheat.

Figure 3.4.9: Immature embryos isolated from wheat inflorescence. (A) Represents inflorescence of cultivar Gamtoos-R (Dn7) at the beginning of anthesis. (B) Shows the entire seed before the embryo has been isolated. (C) Representing an isolated embryo that is still intact 11 to 14 days after anthesis

Figure 3.4.10: Different stages of tissue culturing: (A) Callus tissue on induction A medium for 14 days. (B-D) Shows embryonic tissue on regeneration medium for 10 days; (B) and (C) represents embryonic tissue differentiating into plantlets, i.e. Gamtoos-S (Dn0) and Gamtoos-R (Dn7) cultivars respectively; while (D) shows non-differentiating calli; (E) Plantlet on shoot elongation medium for 14 days in a 90 mm pedri dish; (F) Plantlet on shoot elongation medium for 14–20 days in a tissue culture flask.

Figure 3.4.11: Different stages of plantlets on shoot elongation medium: (A and B) Plant transformed with construct pUBi-GST; (C) Plant transformed with construct pUBi-APX; and (D) Control (tissue culture derived plant).

Figure 3.4.12: Hardening off: (A) transformed Gamtoos-S (Dn0) pUBi-GST; (B) tissue culture derived control Gamtoos-R (Dn7); and (C) transformed Gamtoos-S (Dn0) pUBi-APX.

Figure 3.4.13: (A) Hardening off: Left: transformed Gamtoos-S (Dn0) pUBi-GST, Right: tissue culture derived control Gamtoos-R (Dn7); (B) Hardening off: Left: transformed Gamtoos-S (Dn0) pUBi-APX, Right: tissue culture derived control Gamtoos-R (Dn7).

Figure 3.4.14: (A) Aphid infestation: Left: tissue culture derived control Gamtoos-R (Dn7), Right: transformed Gamtoos-S (Dn0) pUBi-GST; (B) Aphid infestation: Left: tissue culture derived control Gamtoos-R (Dn7), Right: transformed Gamtoos-S (Dn0) pUBi-APX.

Figure 3.4.15: Symptoms due to infestation: (A) Gamtoos-S (Dn0), (B) Gamtoos-R (Dn7), (C) tissue culture derived control Gamtoos-R (Dn7), (D) transformed Gamtoos-S (Dn0) pUBi-GSTF6b, (E) transformed Gamtoos-S (Dn0) pUBi-APX.

Figure 3.4.16: Relative APX expression in uninfested and infested transgenic Gamtoos-S (Dn0) pUBi-APX wheat significantly different at $p < 0.05$.

Figure 3.4.17: Relative GSTF6b expression in uninfested and infested transgenic Gamtoos-S (Dn0) pUBi-GST6b wheat

Figure 3.4.18: Relative GSTF6b expression in the transgenic Gamtoos-R (Dn0) pUBi-GSTF6b wheat

Figure 3.7.2: a) Showing all the different equipment required for a particle bombardment machine. b) The green button used to accelerate the particle (DNA) in the explant (callus tissue).

Figure 4.3.1: Diagrammatic representation in preparation of metaphase chromosomes from root tips (Mirzaghaderi 2010).

List of Tables

Table 2.1: The Advantages and Disadvantages of *Agrobacterium* mediated transformation vs particle bombardment

Table 3.3.1: Primers generated using the Geneious software

Table 3.3.2: Primers used for PCR amplification in the isolation of *APX* and *GSTF6b* genes from pGem-T Easy plasmids

Table 3.3.3: Primers used for determining relative expression levels of targeted genes

Table 3.4.1: Bioinformatic survey on *APX* in wheat obtained from NCBI

Table 3.4.2: Bioinformatic survey on *GST* in wheat obtained from NCBI

Table 3.4.3: Spectrophotometer measurements of isolated total RNA and cDNA

Table 3.4.4: Identity and E-values of cloned *APX* fragments

Table 3.4.5: Identity and E-values of cloned *GSTF6b* fragments

Table 3.4.6: Comparing mortality rate of isolated embryos — cultivars Gamtoos-S (*Dn0*) and Gamtoos-R (*Dn7*) — bombarded with constructs pUBI-*GSTF6b* and pUBI-*APX* when cultured on delayed selection

Table 3.4.7: A comparison between the mortality rate of isolated embryos — cultivars Gamtoos-S (*Dn0*) and Gamtoos-R (*Dn7*) — bombarded with construct pUBI-*GSTF6b* when cultured on early selection medium

Table 3.4.8: Spectrophotometer measurements of isolated total RNA from unfested and infested plant material

Table 3.7.1: Different tissue culturing mediums for Delayed selection

Table 3.7.2: Different tissue culturing mediums for Early selection

Table 3.7.3: Components of the precipitation mix used for bombardment

Table 4.3.1: Primers used to amplify fragments to label probes

Table 4.6.1: Fluorescent *in situ* reagents 1

Table 4.6.2: Fluorescent *in situ* reagents 2

Table 4.6.3: Fluorescent *in situ* reagents 3

Table 4.6.4: Fluorescent *in situ* reagents 4

Chapter 1

Introduction

1.1. Abstract

Global food security is hampered by a variety of insects/pest and plant diseases. In wheat, the Russian wheat aphid (RWA) is a significant pest problem in many areas of the world. Wheat has developed defensive mechanisms against the RWA over time which are activated upon feeding. One such mechanism is the hypersensitive response (HR) which is effective against phloem-feeding insects i.e. *D. noxia* (*Diuraphis noxia*, Kurdjumov, RWA). In this study, two genes associated with the hypersensitive response i.e. ascorbate peroxidase (*APX*) and glutathione S transferase (*GSTF6b*) were investigated to elucidate their function in the defensive mechanism of wheat using a reverse genetic approach i.e. particle bombardment. This study has succeeded in the established of a tissue culture and transformation system which generated three genetically modified wheat plants with decreased resistance to RWA feeding due to gene silencing. The establishment of this system enabled to test the association of defensive related genes in wheat to RWA resistance. Expression analysis performed on obtained transgenics before and after RWA infestation revealed that the silenced plants were more susceptible to RWA feeding. Chlorosis was observed in the Gamtoos-S-*APX* transgenic plant which is an indicator of oxidative damage to the photosynthetic machinery of the plant. Decreased *GSTF6b* transcripts was found in the transgenic Gamtoos-S-*GSTF6b* and transgenic Gamtoos-R-*GSTF6b* transgenic plants but no visible symptoms of infestation was observed in these two plants. Resistance breeding could be strengthened by developing broad spectrum resistance plants by incorporating wheat defensive related genes with known function into the breeding programs. The use of this transformation system will allow rapid identification and introduction of agronomically important genes by upregulating these genes to enhance bread wheat against aphid infestation.

1.2. Introduction

Bread wheat (*Triticum aestivum* L.) is the third most widely grown and consumed crop globally after maize and rice. Due to the rapid growing world population, the demand for wheat production will increase by 40%, which exceeds what is currently being produced (Abdul *et al.* 2010). The ever increasing human population is reducing the arable land available for wheat production; however, this is not the only factor hampering wheat production. Other factors that impact wheat production include the prevailing environment and biotic threats such as pathogens and pests.

The Russian wheat aphid (*Diuraphis noxia* Kurdjumov, RWA) is a serious pest of wheat and barley (*Hordeum vulgare* L.) (Gutsche *et al.* 2009). Since its initial discovery in 1978 in South Africa (Walters *et al.* 1980) and 1986 in the United States (Gutsche *et al.* 2009), crop losses worth billions of dollars have been reported (Marasas *et al.* 2005, Gutsche *et al.* 2009). This invasive pest species is occurring in most wheat producing areas except Australia (Marasas *et al.* 2005).

The leaf whorl and new leaves which are rich in phloem mineral nutrients, amino compounds and carbohydrates, (seem to be the preferential feeding site for *D. noxia*) (Gutsche *et al.* 2009). Typical symptoms of damage in susceptible plants include chlorotic leaf streaking, leaf rolling, head trapping and stunted growth (Botha *et al.* 2005, Gutsche *et al.* 2009). Leaf rolling is one of the detrimental aphid symptoms to wheat, resulting in reduced photosynthetic area for the host plant but in return provides an optimal environment for aphid reproduction (Gutsche *et al.* 2009).

A variety of aphid management strategies have been employed, which include the use of pesticides and also biological control (Prinsloo *et al.* 2002), adjusting crop planting time and the use of resistant cultivars (Merrill *et al.* 2009). Resistance breeding seems to be the most attractive control method of *D. noxia* which will eliminate the need for insecticide treatment. Eleven genetic resistance genes are available for bread wheat i.e. *Dn1*, *Dn2*, *Dn3*, *Dn4*, *Dn5*, *Dn6*, *Dn7*, *Dn8*, *Dn9*, *Dnx*, and *Dny* (Botha *et al.* 2010). However, effectiveness of these genes is

compromised by the emergence of new biotypes. At the moment aphids are controlled by a combination of resistance cultivars and the use of pesticides.

The major goal for plant scientist is to breed plants with increased and durable resistance to withstand pest invasion (Gurr & Rushton 2005). For many years increased resistance was bred using non-transgenic strategies such as marker assisted selection. This technique has proven to work well but is short lived due to the emergence of new aphid biotypes (Gurr & Rushton 2005).

An alternative to this approach is transgenic strategies whereby a single gene can be overexpressed to increase resistance of a particular plant defence response (Gurr & Rushton 2005). Genetic manipulation is starting to revolutionise cell biology, enabling researchers to investigate genetically controlled traits (Jones *et al.* 2005). This is one of the most vital tools available for modern plant breeding to generate crops with desired traits (Bhalla 2006).

In the present study, the aim was to confirm the function of genes involved in RWA resistance to improve pest resistance of wheat. The objectives were firstly, to elucidate and quantify the response of genes involved in the defensive pathway against RWA feeding following a transgenic approach using particle bombardment. Secondly, to establish the distribution, abundance and location of *APX* and *GSTF6b* in the wheat genome by performing fluorescent *in situ* hybridization (FISH).

Chapter 2 of this dissertation summarizes the relationship between wheat (*Triticum aestivum* L.) and the Russian wheat aphid (*Diuraphis noxia*, Kurdjumov), and focusses on what has been accomplished by other researchers in the field of wheat breeding by employing a variety of techniques to improve pest resistance of wheat.

Chapter 3 focuses on the establishment of a tissue culture and transformation procedure, which were used to determine any association of ROS scavenging enzymes, i.e. ascorbate peroxidase (*APX*) and glutathione S-transferase (*GSTF6b*), and wheat resistance to RWA.

Three transgenic plants with decreased transcript amounts of ROS scavenging enzymes were generated. From the transgenic plants (T_0) first generation plants (T_1) were obtained which will be examined in future studies.

Chapter 4 examines different methods of preparing chromosome spreads needed to determine the location of selected ROS scavenging enzymes, i.e. ascorbate peroxidase (*APX*) and glutathione S-transferase (*GSTF6b*), in the wheat genome using fluorescent *in situ* hybridization.

Chapter 5 consists of a summary describing the significance of this study.

Appendix A contains detailed protocols used for tissue culturing of immature embryos as well as particle bombardment procedures.

Appendix B contains the composition of buffers used for chromosome squashes and suspensions preparations

1.3. Preface

The findings obtained and presented in this dissertation are the results of a study undertaken between January 2012 to December 2013 in the Department of Genetics, University of Stellenbosch, under the supervision of Prof AM Botha-Oberholster, Dr C van der Vyver and Dr L van Eck.

Conference posters presented:

- 2012: **Local Poster presentation Stellenbosch, South Africa**
'Transformation of selected wheat cultivars to elucidate gene function'; N.M. Fisher*, C van der Vyver, L van Eck and A-M. Botha. South African Genetics and South African Society for Bioinformatics and Computational Biology SAGS / SASBCB conference presentations.
- 2013: **International Poster presentation Hungary, Budapest**
'Transformation of bread wheat to enhance aphid resistance'; N.M. Fisher*, C van der Vyver, L van Eck and A-M. Botha. 2nd Conference of Cereal Biotechnology and Breeding.

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Chapter 2

Literature review:

The link between hexaploid wheat (*Triticum aestivum* L.) and the Russian wheat aphid (*Diuraphis noxia*)

2.1. Wheat

2.1.1. Triticeae origins

One of the most widely grown crops across the world is wheat, which provides essential proteins and carbohydrates to the human diet (Feuillet *et al.* 2007). Wheat has been used as a main calorie source for mankind since the start of agriculture 10,000 years ago in the Mediterranean's Fertile Crescent, domesticated by the world's original (founder) farmers as shown in Figure 2.1 (Feuillet *et al.* 2007).

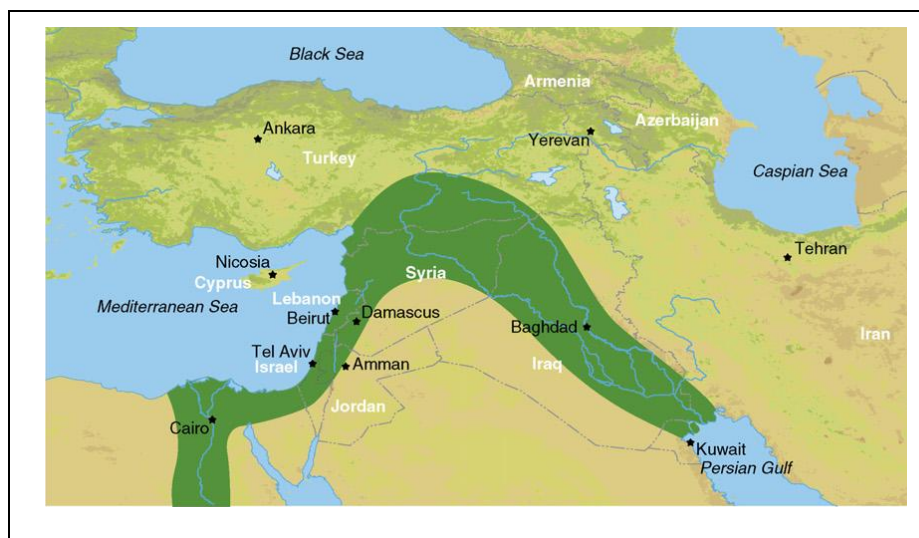


FIGURE 2.1: Map illustrating the domestication of Bread wheat in the Fertile Crescent (Feuillet *et al.* 2007).

2.1.2. Speciation

Wheat belongs to the tribe *Triticeae*, which contains more than 500 species in 26 genera (Feuillet *et al.* 2007). Species include: barley, rye and wheat (*Triticum aestivum* L.) formerly known as *Triticum spelta* (Gupta 1985). They originated from one common ancestor which taxonomically belongs to the *Poaceae* family (Feuillet *et al.* 2007). The *Triticeae* species (wheat, barley and rye) account for more than one third of cereal production globally and are believed to have diverged from each other more than 50 million to 70 million years ago (Gill *et al.* 2004).

The *Triticeae* species is the best known allopolyploid complex that exists, with bread wheat being an allohexaploid which was recreated artificially by McFadden and Sears in 1946 (Gupta 1985). Bread wheat possesses a genomic composition of AABBDD that originated from hybridization events between three distinct species (Fig 2.2) (He *et al.* 2003). All three genomes are morphologically similar with seven pairs of homeologous chromosomes totaling 42 (Cuadrado *et al.* 2008). Wheat has a genome size of 16,000 Mb, of which more than 80% comprises repetitive DNA (Cuadrado *et al.* 2008; Brenchley *et al.* 2012).

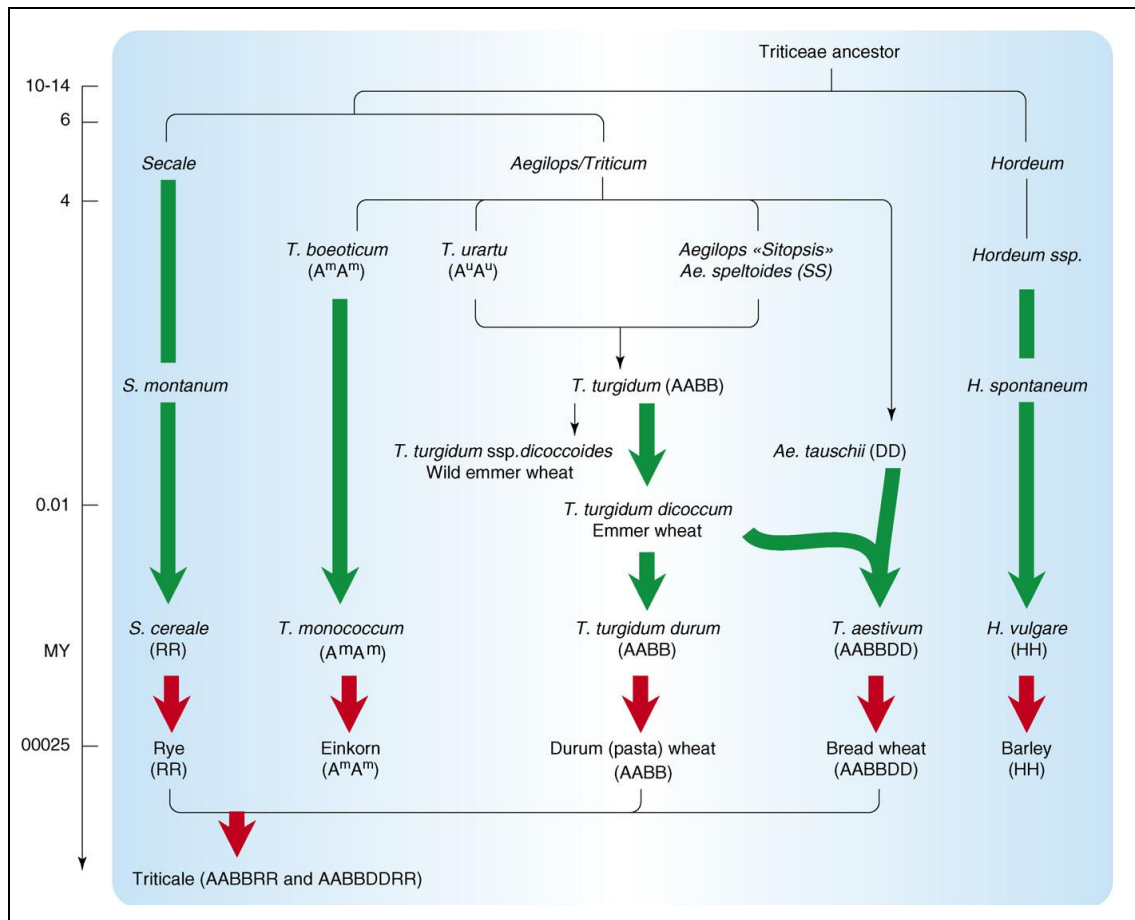


FIGURE 2.2 A schematic representation of the origin of *Triticum aestivum* L. due to multiple events of duplication (Feuillet *et al.* 2007).

2.1.3. Triticeae production

The total production of wheat in South Africa is not sufficient to meet the demands. During the 2010/2011 season, the demand was approximately 3,237 million tons (including human and

animals), with only 2,097 million tons produced locally. Therefore, the remaining 1,500 million tons were imported (Department of Agriculture, Forestry and Fisheries, South Africa 2011).

2.1.4. The consequences of insufficient wheat production

Global wheat production loss is caused by multiple factors affecting the sustainability of crop yield and quality. Factors include both abiotic and biotic threats, such as pathogens and pests, which greatly impact wheat production (Feuillet *et al.* 2007).

These affect food security directly, which negatively impacts both farmer's income and the global human population (Lucas 2010). Reports revealed that the 7 billionth child was born in October 2011 ([www.msnbc.msn.com world news](http://www.msnbc.msn.com/world/news)). With an global population estimated to reach nine billion by 2050 (Goff & Salmeron 2004), crop improvement strategies must be employed to meet the demands of the ever-increasing human population (Dwivedi *et al.* 2005).

2.2. Russian wheat aphid (RWA)

2.2.1. Distribution

The Russian wheat aphid (*Diuraphis noxia* Kurdjumov, RWA) was first identified in 1978 in South Africa (Walters *et al.* 1980), while it was reported in the United States in 1986 (Webster 1990). It then migrated from Western Asia to Africa and from Africa to South America and Mexico (Smith *et al.* 1991). The distribution of the RWA has become a serious threat to major wheat producing countries across the world, excluding Australia, causing extensive crop damage resulting in an estimated yield loss of 92% in some susceptible cultivars (Marasas *et al.* 2005).

2.2.2. Identification of RWA resistant wheat cultivars

An ideal solution to the RWA pest problem is the development of resistant cultivars. In 1987 two hexaploid wheat lines (SA 1684/PI 137739 and SA 2199/PI 262660) with signs of RWA resistance were identified (Du Toit 1987). It was found that resistance was controlled by a single dominant gene, which was independently inherited (Du Toit 1987). To date, a total of 11

resistance genes (*Dn1*, *Dn2*, *Dn3*, *Dn4*, *Dn5*, *Dn6*, *Dn7*, *Dn8*, *Dn9*, *Dnx*, and *Dny*) have been identified in wheat and its relatives (Botha *et al.* 2010). These genes are located on the *Triticeae* homoeologous groups 1 and 7 (Liu *et al.* 2001; Smith & Boyko 2007).

2.3. Host-pest interaction

There is an ongoing evolutionary battle of adaptation and counter adaptation between host resistance (Wheat) and aphid virulence (Russian wheat aphid) resulting in the emergence of new biotypes allowing them to overcome plant resistance, putting pressure on the plant in turn to withstand invasion (Botha 2013). Thus mechanisms must be identified by which RWA feeding can either be prevented or effectively controlled so as to minimize the damage to wheat (Dwivedi *et al.* 2005; Smith & Clement 2012).

2.3.1. Feeding

Aphids feed specifically on the phloem of their host by penetrating through intercellular tissues with their stylet mouth piece (Tjallingii 2006). They prefer to feed at the base of the youngest leaves of the host, which is a rich source of phloem containing essential nutrients, carbohydrates and amino acids (Franzen *et al.* 2007).

2.3.2. Symptoms of infestation

Over time wheat has developed natural defense mechanisms against the RWA, which is activated upon feeding (Zaayman *et al.* 2009). During feeding, *D. noxia* injects a phytotoxin into its host which causes toxicoses (Smith *et al.* 1991). The phytotoxin induces chloroplast breakdown, resulting in an 85% chlorophyll loss (chlorosis) in infested susceptible cultivars (Smith *et al.* 1991; Botha *et al.* 2006).

Visible symptoms of chlorosis are observed such as white and yellow longitudinal streaking of the leaves (Botha *et al.* 2006; Franzen *et al.* 2007), as well as a 25-50% reduction in yield (Smith *et al.* 1991). Other symptoms associated with feeding on susceptible plants include, head trapping, stunted growth and leaf rolling (Fouché *et al.* 1984).

2.3.3. Host response in resistant plants

Three types of defensive strategies can be deployed by wheat in response to RWA feeding, namely antibiosis, tolerance and antixenosis (Painter 1958). Antibiosis induces an immediate response by denying the aphids feeding on the host (Painter 1958). Tolerant plants have the ability to cope with aphid feeding implying that the plant can produce enough food for itself as well as the aphid. Antixenotic plants can be defined as being a non-preference host, thus they do not provide the essential foods (nutrients) and shelter for the aphids to survive (Painter 1958).

Each defensive response is associated with a different type of *Diuraphis noxia* (*Dn*) resistance gene. Antibiosis, tolerance and antixenosis are associated with the *Dn1*, *Dn2* and *Dn5* resistance genes respectively; these responses reduce aphid fitness on resistant cultivars (Botha *et al.* 2005).

2.3.4. Wheat-Russian wheat aphid interaction

Plants in general have to endure a variety of environmental challenges (Dangl & Jones 2001) although they are equipped with structural barriers that enable them to defend themselves (i.e. passive protection) against their natural enemies i.e. fungi, bacteria, viruses and nematodes (Gurr & Rushton 2005). These biotic stresses in turn resulted in plants that evolved sophisticated mechanisms to prevent or minimize such attacks (i.e. adaptive protection) (Dangl & Jones 2001). Upon aphid feeding on the host plant, different processes are activated which can either be directly or indirectly recognized by the pest/pathogen (Gurr & Rushton 2005). The first one is the gene for gene hypothesis model which was first described by Flor 1971 (Botha *et al.* 2005), and supported by Lapitan *et al.* (2007) for RWA-wheat and the second hypothesis being the guard hypothesis.

Starting with the gene for gene model, plants have surveillance systems in place that constantly monitor pest/pathogen plant invasion. Components of this system include a pair of genes which are divided into the resistance genes (R), which originates in the plant, and parallel with this is the avirulence gene (Avr) which originates in the pest/pathogen (Botha *et al.* 2005).

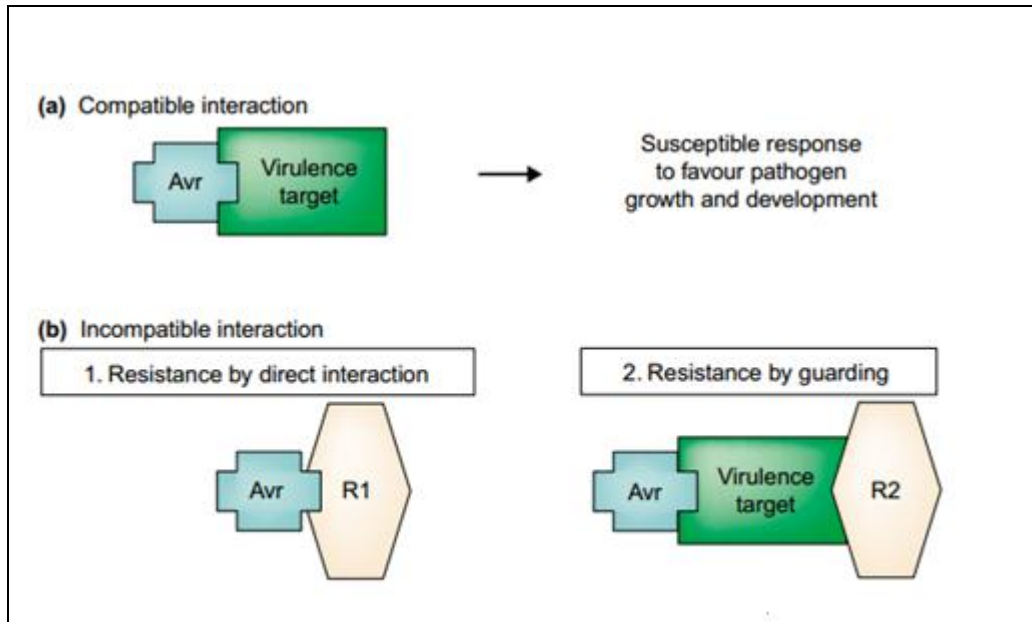


FIGURE 2.3: Schematic representation of Flor's gene for gene model (Hammond-Kosack & Parker 2003).

This model consists of two types of interactions i.e. the compatible and the incompatible. With the compatible interaction (Figure 2.3a) the pathogen/pest overcomes the host defensive response and as a result symptoms of infestation/disease are observed (Botha *et al.* 2005), whilst with the incompatible interaction (Figure 2.3b) disease occurrence is prevented by the resistance gene which restrict pathogen/pest invasion (Botha *et al.* 2005).

The guard hypothesis model was originally described by Van der Biezen and Jones (1998) who worked with *Pseudomonas syringae* AvrPto on tomatoes. They concluded that different resistance pathways are activated against the same pathogen ascribed to the fact that there is no correlation between a particular protein structure and pathogen (Van der Biezen & Jones 1998). The guard model for disease resistance proposes that the R proteins interact (i.e. guard) with a known protein (i.e. guardee), which is the target of the Avr protein, resulting in the activation of resistance due to interference (van der Hoorn & Kamoun 2008).

2.3.5. Hypersensitive Response

When aphids feed on the host plant, eliciting agents (i.e. oral secretion and oviposition) causes the activation of a cascade of physiological and biochemical responses (Figure 2.4). In the absence of recognition, susceptibility of the host occurs, which eventually will lead to plant death (Botha *et al.* 2005). In resistant plants, defence related genes and signalling pathways are activated which include, hypersensitive response (HR), jasmonic acid (JA) and salicylic (SA) (Botha *et al.* 2005). The hypersensitive response is a protective mechanism that the plant activates to reduce damage at the specific tissue site (Pieterse & Loon 1999).

Various studies performed on HR reflect plant wall strengthening occurs in the resistant wheat cultivars which minimize aphid damage (Botha *et al.* 2005). Activation of the HR results in the production of reactive oxygen species (ROS), which in turn results in an increase in oxidative enzymes i.e. peroxidase, polyphenol oxidases and lipoxygenases. The salicylic acid pathway regulates the expression of pathogen responsive genes (i.e. glutathione S transferase and proteinase inhibitors) while the jasmonic acid pathway regulates wound responsive genes (Botha *et al.* 2005). It is suggested by Botha *et al.* (2005) that these two pathways occur parallel in order to enhance resistance against aphids.

2.3.5.1. Genes involved in the hypersensitive response

Ascorbate peroxidase (APX) and glutathione S-transferase (GST) are antioxidants that play a major role in the removal of reactive oxygen species (ROS) generated by products of photorespiration, photosynthesis and the fatty acid-oxidation pathway (Danna *et al.* 2003). ROS includes compounds such as superoxide and hydrogen peroxide (H₂O₂).

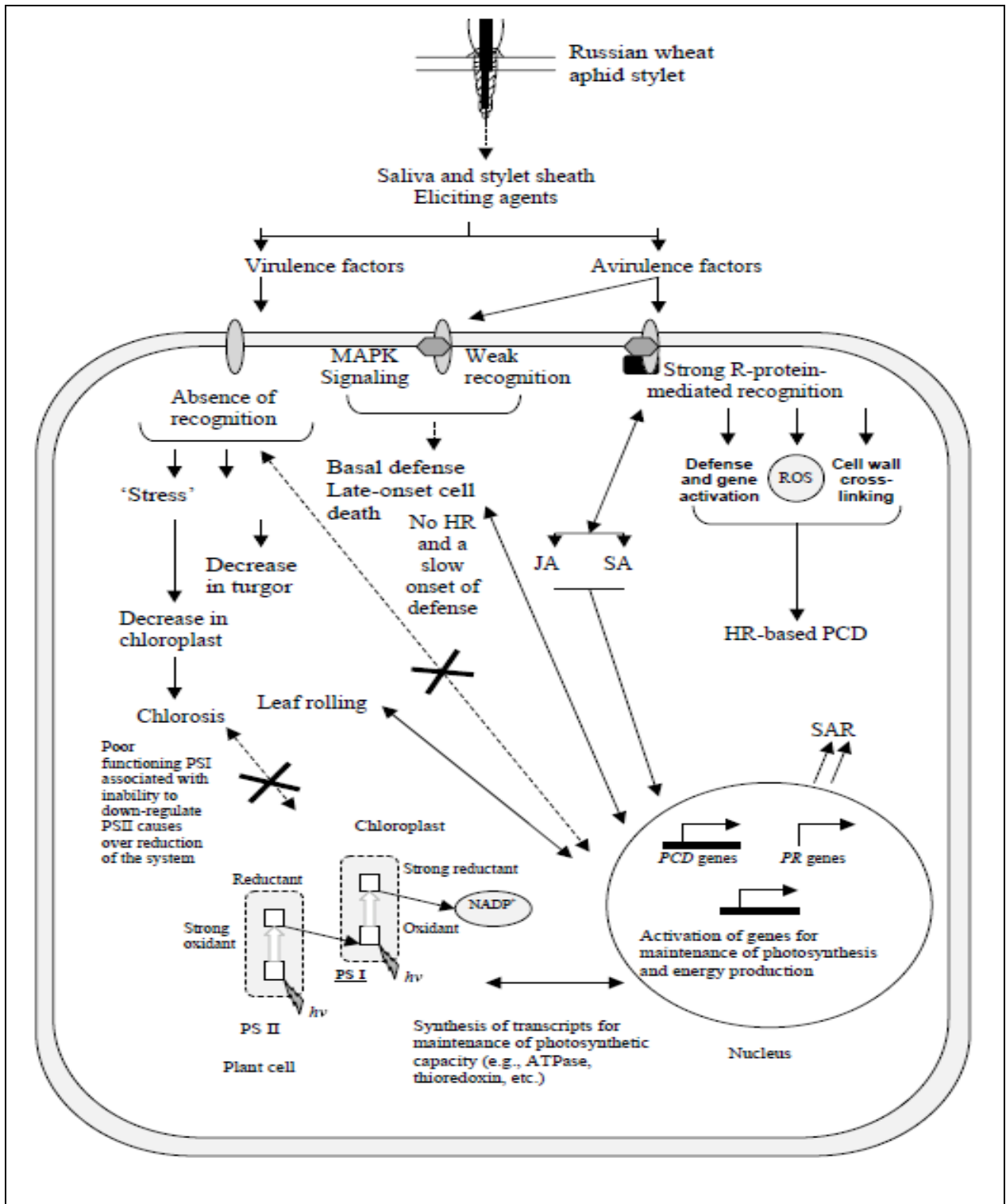


FIGURE 2.4: Schematic representation of the plant signalling pathways involved in aphid feeding (Botha *et al.* 2005).

Overproduction of these components results in the state referred to as oxidative stress, the ROS producing organelle in plants have been found to be the chloroplast (Ishikawa & Shigeru 2008). ROS production is elevated by both biotic and abiotic threats that cause severe cell damage (Danna *et al.* 2003). To combat ROS impairments, photosynthesizing organisms developed a defensive system (Ishikawa & Shigeru 2008), which activates ROS scavenging mechanisms (APX and GST), to ensure plant survival and productivity (Danna *et al.* 2003), and preventing peroxide damage (Dalton *et al.* 1986).

2.3.5.1.1. Ascorbate peroxidase

In eukaryotes, ascorbate (Asa) is frequently found, and in plants this is the most abundant antioxidant (Foyer & Noctor 2009). Ascorbate is transported via the phloem of plants with a dual function for both protection of the systemic plant transport system and for the plant-insect interactions (Foyer & Noctor 2009). Ascorbate adds nutritional value to the human diet, it is a water soluble redox compound and has a multitude of functions including involvement in the electron transport, control of the cell cycle, metabolism, plant pathogen response and abiotic stress (Ishikawa & Shigeru 2008).

Ascorbate is more effective when coupled to the redox system referred to as Asa/glutathione cycle, which is responsible for maintaining a balanced H_2O_2 level (Ishikawa & Shigeru 2008). Asa peroxidase (APX) is a scavenging enzyme with a primary function of protecting the chloroplast and other cells from hydrogen peroxide damage (Murtaza *et al.* 2011).

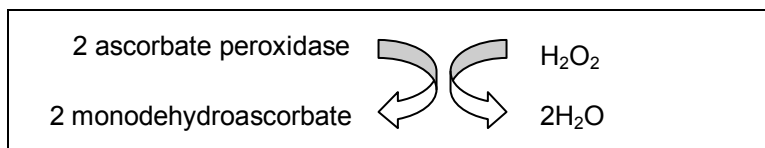


FIGURE 2.5: An illustration of the catalytic reaction which converts H_2O_2 into water (Chen *et al.* 2006).

APX reduces H_2O_2 to water by using Asa as an electron donor, resulting in the production of monodehydroascorbate (MDHA), as illustrated in Figure 2.5, generated during photosynthesis (Danna *et al.* 2003, Ishikawa & Shigeru 2008).

Three types of APX isoenzymes have been identified based on their respective subcellular location namely, cytosolic, chloroplastic and glyoxysomal APXs (Fig 2.6). According to Danna *et al.* (2003), cytosolic APXs (cAPX) is associated with pathogen response, whereas glyoxysomal APXs is responsible for removing the H₂O₂ which is generated by the fatty acid-oxidation pathway and photorespiration (Danna *et al.* 2003). Chloroplastic APX (chAPX) is a combination of both the thylakoid-bound (tAPX) and stromal (sAPX) isoforms. Plant susceptibility is enhanced upon stress responses due to an impaired function of APX, while stress protection is enhanced when APX is overexpressed (Foyer & Noctor 2009).

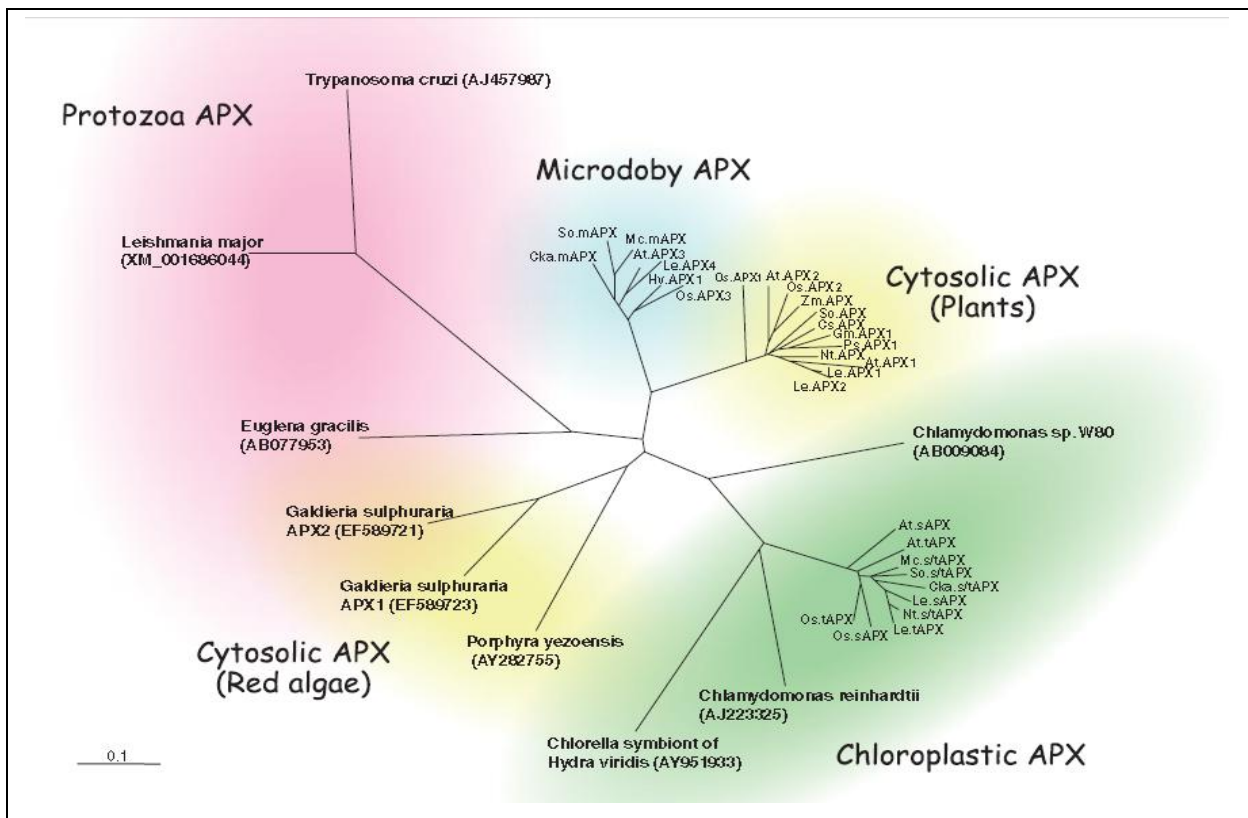


FIGURE 2.6: Phylogenetic tree showing the different types of APX (Ishikawa & Shigeru 2008).

A study performed by Danna *et al.* (2003) compared a mutated *Triticum aestivum* L. (bread wheat) line with partly decreased ascorbate concentration to the wild type. Results revealed significantly reduced amounts of tAPX activity in plants, which directly correlated with impairment of photosynthesis (Danna *et al.* 2003). They found that when these mutant plants

where grown at high light intensity, both biomass accumulation and photosynthetic activity were significantly reduced, suggesting the necessity of tAPX and the role it plays in photosynthesis (Danna *et al.* 2003). With this study, three homeologous *tAPX* genes were identified, namely *TaAPX-6A*, *TaAPX-6B*, and *TaAPX-6D*, which are expressed in hexaploid wheat (*Triticum aestivum* L.) mapped on chromosome 6.

2.3.5.1.2. Glutathione S-transferase

Glutathione transferases are one of the largest groups of ancient gene families with multifunctional proteins. According to literature, these proteins have been associated with a wide range of responses which are believed to have evolved from gene duplication events (Frova 2003).

The distributions of these proteins are diverse, ranging from bacteria to mammals. In mammals, GSTs are primary involved in the detoxification of chemicals and chemotherapeutic agents. While in bacteria they are involved in the degrading of recalcitrant chemicals and antibiotic resistance reactions (Frova 2003).

In plants, glutathione transferases primarily detoxify herbicides (Frova 2003). In essence, the function between the three organisms is essentially the same, emphasizing the role GST plays in the detoxification pathway preventing/minimizing oxidative stress in all living organisms. They were discovered first in the 1960s in animals due to their importance in the metabolism and detoxification of drugs (Dixon *et al.* 2002). The first plant GST activity was later reported in the 1980s in maize, which was capable of detoxifying the herbicide atrazine preventing crop damage by this herbicide (Dixon *et al.* 2002).

To date, a total of 11 classes of GSTs have been identified, characterized and described (Fig 2.7). Eight of these classes represent mammalian GSTs, which include: Alpha, Kappa, Mu, Pi, Theta, Sigma, Zeta and Omega (Frova 2003). In plants, there are at least five distinct classes of GSTs namely, Alpha, Mu, Tetra, Phi (F) GSTFs and Tau (U) GSTUs (Sheehan *et al.* 2001). Limited sequence similarities exist between different GST classes, but their overall structure seems to be

conserved among the classes (Dixon *et al.* 2002). Phi and Tau are plant specific GSTs while Zeta, Theta and Lambda classes are universally found in mammals and plants and have similar functions (Frova 2003).

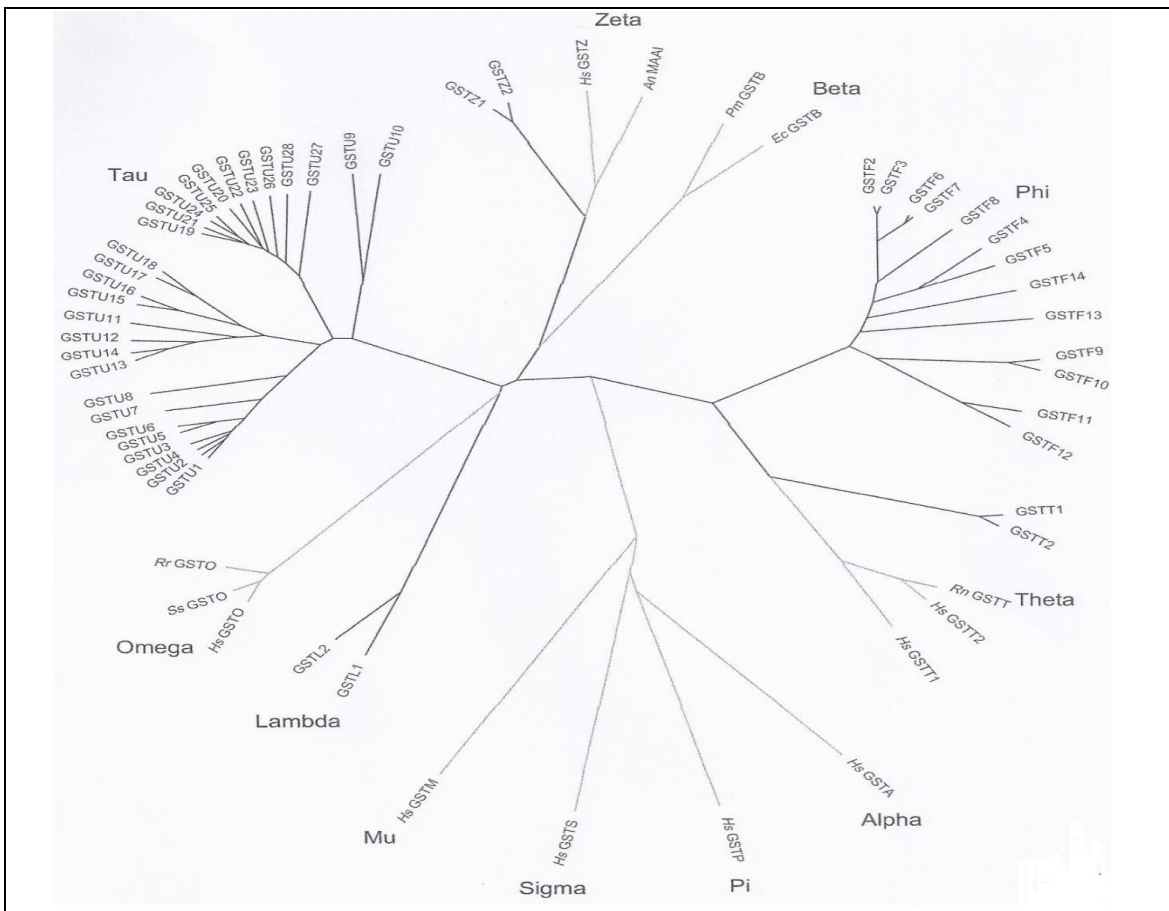


FIGURE 2.7: Phylogenetic tree showing the different classes of GSTs found both humans and plants (Dixon *et al.* 2002).

The Phi and Tau classes are the most abundant plant specific GSTs (Figure 2.8). The Phi class is primarily involved in the detoxification of herbicides, and possesses two introns at conserved positions within the gene. The Phi class represents many of the first plant GSTs upon its initial discovery (Frova 2003). Tau class is associated with multiple endogenous and exogenous stresses, which are triggered by a variety of stimuli such as pathogen attack, wounding, metal

toxicity, oxidative and temperature stress. Characteristic of the Tau class is that it contains a single intron at a conserved position in the gene, while the phi class contains two (Frova 2003).

The third GST class, the Zeta GSTs, are activated in plant senescence and induced by ethylene. The Zeta class was first discovered in carnations and contain of eight to nine introns in their genes and are not only related to plants but also to mammalian and fungal species (Frova 2003). Focusing on the last two classes, namely the Theta and the Lambda, these are found in a variety of species such as Arabidopsis, rice and mammalian GSTs. These genes comprise of six to eight introns differing among species (Frova 2003).

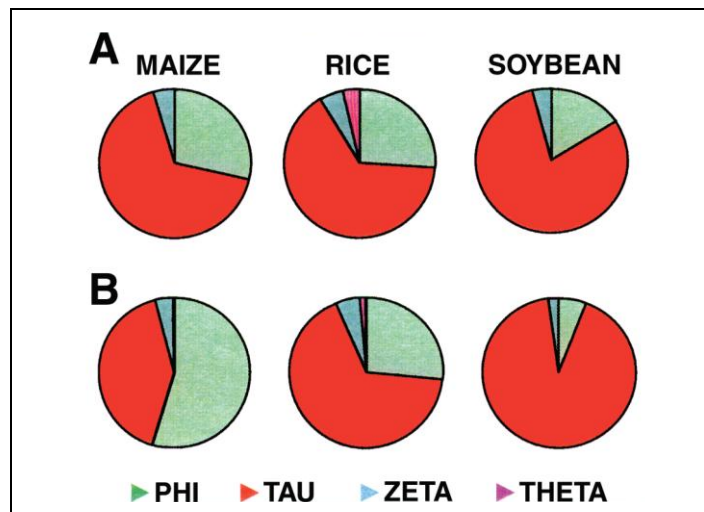


FIGURE 2.8: Pie charts illustrating: A: the occurrence of plant GSTs in maize, rice and soybean and B: The expression of the different GST classes in maize, rice and soybean (Frova 2003).

Since only a few plant species had been sequenced, it is difficult to study most species extensively. In an attempt to better understand the organization of plant GSTs, *Arabidopsis thaliana* was used as a model plant due to the availability of the full genome sequence (Dixon *et al.* 2002). In total, the Arabidopsis genome has 48 GST genes of which phi and tau are the most abundant classes (Fig 2.9).

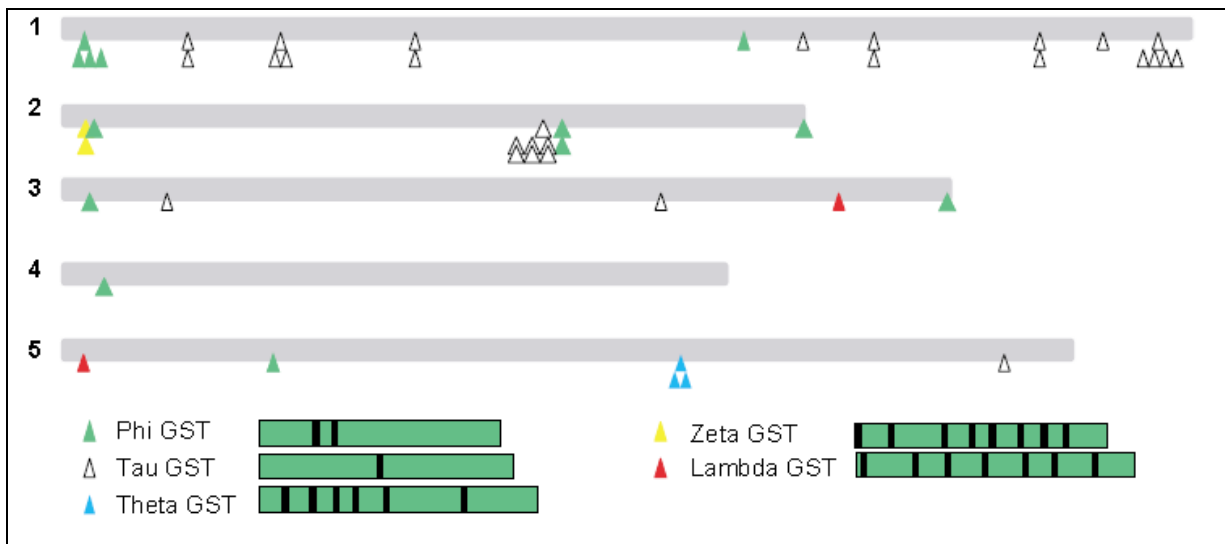


FIGURE 2.9: A schematic representation of the distribution of GST classes in the *Arabidopsis thaliana* genome (Dixon *et al.* 2002).

Due to the significant number of described classes of GSTs, a classification system was established. The system was first applied to animal species and is now adapted for application in plant species. The nomenclature used in plants is given in Figure 2.10, the first two letters giving the organism's name, followed by the class of GSTs and lastly the numbering system representing the GST protein subunits (Dixon *et al.* 2002).

Structural information is available for some plant GSTs, especially for the phi class in *Arabidopsis*, maize, rice, soyabean and wheat (Frova 2003). Cummins *et al.* (2003) showed that wheat possesses a phi class family of GSTs, similar to that of the maize GSTFs, based on their amino acid sequences and detoxifying activity.

Wheat contains six GSTF polypeptides: TaGSTF1, TaGSTF2, TaGSTF3, TaGSTF4, TaGSTF5 and TaGSTF6 (Cummins *et al.* 2003), while maize only contains three orthologs denoted as *ZmGSTF1*, *ZmGSTF2* and *ZmGSTF3* (Cummins *et al.* 2003). In plants these enzymes exhibit a broad spectrum of functions (Fig 2.11), including both catalytic and non-catalytic roles (Dixon *et*

al. 2002). In general, GSTs are flavonoid-binding proteins, which are enhanced under stress, causing accumulation of protective phenolic compounds (Cummins *et al.* 2003).

In addition, wheat has also glutathione peroxidase (GPOX) activity, associated with *ZmGSTF2*-like sequences. *GSTFs* possessing GPOX activity are involved in counteracting oxidative stress activated by either environmental changes or herbicides (Cummins *et al.* 2003).

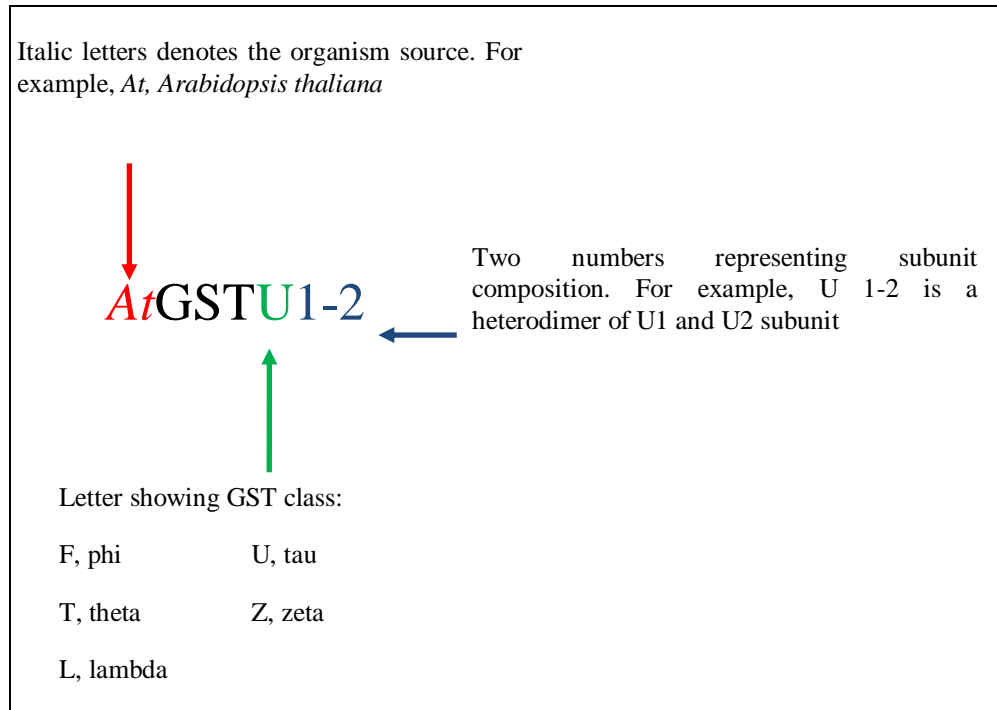


FIGURE 2.10: Classification system of plants GST which originated from mammalian nomenclature (Dixon *et al.* 2002).

They therefore concluded that the TaGSTFs in wheat, especially TaGSTF2 and TaGSTF3, contains similar antioxidant roles comparable to maize *ZmGSTF* (Cummins *et al.* 2003). Further analysis revealed that *TaGSTF6* of wheat seems to be a hybrid GST from *ZmGSTF1*-like and *ZmGSTF2*-like sequences producing the same enzymes as *ZmGSTF1* (Cummins *et al.* 2003). Studies by Cummins *et al.* (2003) also illustrated the potential involvement of GSTUs in the rapid metabolism of herbicides.

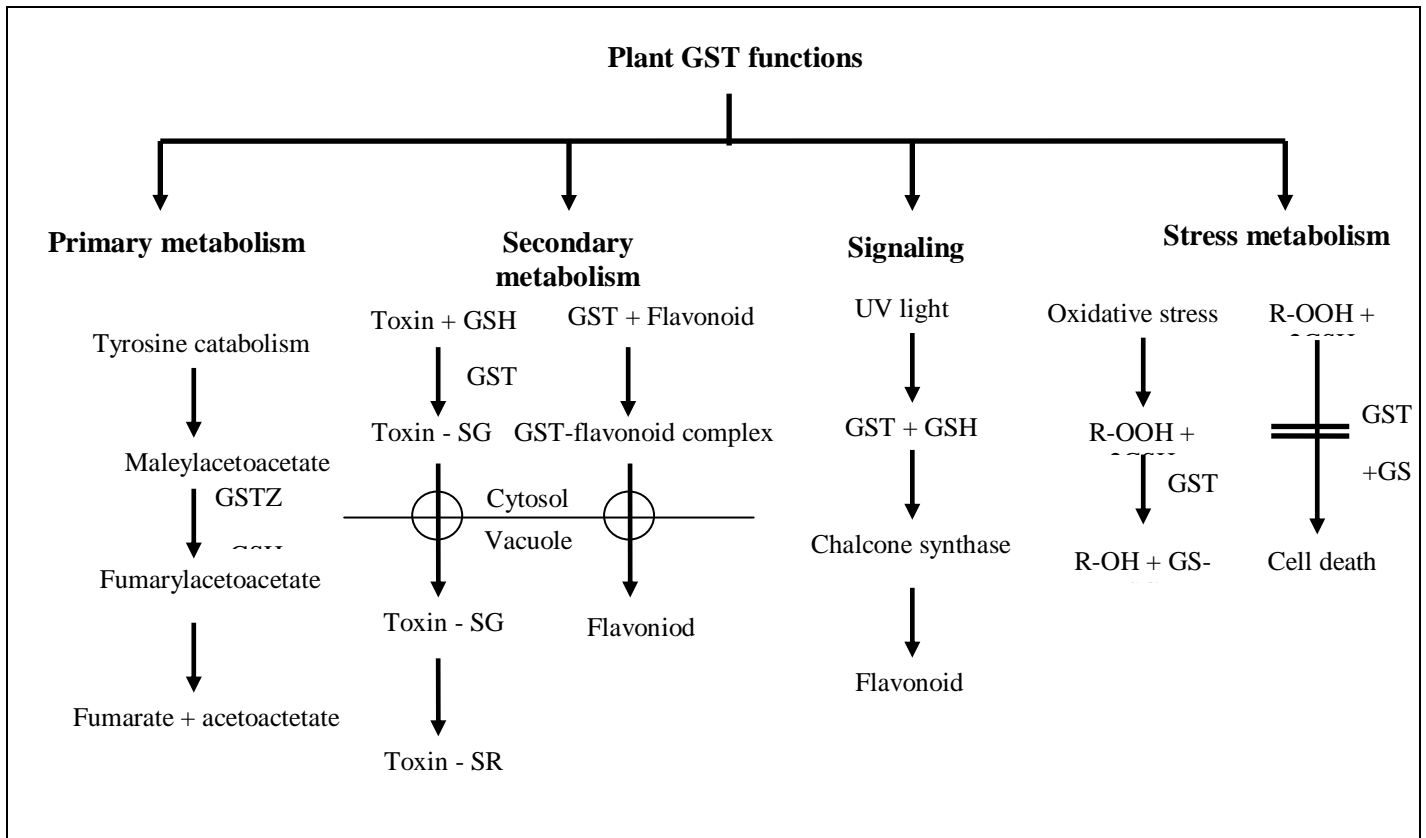


FIGURE 2.11: Schematic representation summarizing the function of GSTs in plants (Dixon *et al.* 2002).

2.4. Genomic Approaches for gene identification

Molecular tools hold promise for unravelling regulatory mechanisms and biological processes of the wheat genome, however, applying these tools is not without difficulty, due to the large genome size of bread wheat (Gupta *et al.* 2008). These approaches include: genetic markers, fluorescent *in situ* hybridization, mapping, gene expression and plant transformation.

2.4.1. Genetic Markers

In the search for genes and more specifically gene location, genetic markers serve as landmarks within areas on chromosomes. They flank gene rich areas, revealing/indicating the location of the gene (Kumar 1999). The implementation of genetic markers has accelerated the pace of traditional breeding. Since its discovery, many different types of genetic markers have been employed with a variety of applications (Botha *et al.* 2004).

Genetic markers are divided into three distinct classes, namely morphological, biochemical and molecular markers. Morphological markers are based on phenotypic traits or characters that are physically visible. Biochemical markers identify allelic variants of enzymes referred to as isoenzymes, while molecular markers identify variations within DNA sequences (Collard *et al.* 2005).

2.4.1.1. Molecular markers

Molecular markers allow precise, objective and rapid cultivar identification, which is useful for crop improvement (Zhu *et al.* 2011). They comprise of three distinct generations, but emphasis has shifted towards the third generation markers, which utilize EST (expressed sequence tag) and cDNA-AFLP/mRNA-AFLP technology. They score only the expressed region of the genome. The reason for the shift is mostly because of the large genome size and repetitive nature of the wheat genome (Gupta *et al.* 2003).

2.4.1.2. Expressed sequence tags markers

Structural and functional analysis of wheat enables researchers to unveil certain hidden “treasures” in the wheat genome. Even though the genome of bread wheat has been sequenced (Brenchley *et al.* 2012), understanding of its structure and function is still limited. Thus, alternative strategies must be implemented (Paolacci *et al.* 2009), such as ESTs that serve as effective tools to increase our understanding (Jia *et al.* 2009). There are as many as 1,315,749 *Triticum aestivum* ESTs freely available in the dEST (<http://www.ncbi.nlm.nih.gov>) database (Paolacci *et al.* 2009).

2.4.2. Fluorescent in situ hybridization

In the quest to understand genome function and structure, the gene location must first be identified, which is distributed between its inherited chromosome pairs (Cuadrado *et al.* 2008). *In situ* hybridization approaches employ labeled probes to identify sequence locations on chromosomes (Gupta *et al.* 2008). A variant of *in situ* hybridization is fluorescent *in situ* hybridization (FISH), which is a method of mapping DNA/RNA sequences to their respective physical locations on chromosomes.

During FISH, fluorescently labelled probes are used, which associate with areas of chromosomes that show high sequence similarities. FISH also provides information about the distribution and abundance of the hybridization signal. Hybridization signals are identified on chromosomes making use of fluorescence microscopy (Schwarzacher 2003). FISH analysis allows visualization of spatial distribution of sequences on chromosomes (Lécuyer *et al.* 2008).

2.4.3. Mapping

Mapping of molecular markers was initiated during the late 1980s, but started actively only in 1990 with the International *Triticeae* Mapping Initiative organization (ITMI), whereby they implemented the construction of molecular maps of the wheat genome (Gupta *et al.* 2008). According to Collard *et al.* (2005), a molecular map can be seen as a road map indicating the relative genetic position between the marker and the gene, while a physical map gives the actual location and distance on a chromosome.

The aim is to link the genetic map (which is constructed by molecular markers) to the physical position on a chromosome (Cuadrado *et al.* 2008). This will elucidate the structure and the evolutionary lineage of an organism. Physical mapping is becoming a major tool in genetics and more specifically plant and animal breeding (Ogundiwin *et al.* 2009).

One effective means of physical mapping is using map-based cloning. There are two aspects to map based cloning, firstly a DNA marker must be identified that is linked to the gene of interest, and secondly the gene of interest must be walked out of the genome (Collard *et al.* 2005). Chromosome walking is a sequence-based approach which is used to search for targeted genes of interest among billions of base pairs (Collard *et al.* 2005).

In order to achieve this, random markers are used to search for the targeted genes closer to one or more of the markers, resulting in a collection of overlapping cloned contigs (Collard *et al.* 2005). Once contigs using bacterial artificial chromosome (BAC) libraries are established, the target will be searched for within these contigs (Collard *et al.* 2005).

2.4.4. Reverse genetic approaches

Gene transfer technology enables researchers to examine host response to foreign gene introduction and elucidating the putative function of a specific gene (Serio *et al.* 2001). In order to identify or confirm gene function, two types of approaches can be applied, which include silencing (knockout/knockdown studies) and over expression of the gene of interest (Thomson 2002).

2.4.4.1. Gene silencing

Gene silencing allows gene identification as well as elucidating gene function by knocking out the targeted gene(s) (Scofield *et al.* 2005). The discovery of gene silencing enables scientists to investigate the role of specific gene products in plant growth and gene function (Shuai *et al.* 2003).

Due to the large genome size of wheat, mutational analysis is difficult and T-DNA transformation are also challenging. Therefore, methods such as virus induced gene silencing (VIGS), RNA interference (RNAi) and particle bombardment can be employed. VIGS is used to suppress endogenous gene expression which is a transient knockdown assay whereby plants are infected with recombinant virus vector carrying the gene of interest (Dinesh-Kumar *et al.* 2003).

All three above mentioned methods functions via a antisense mediated gene silencing (ASGA), which is a type of posttranscriptional gene silencing (PTGS) mechanism (Fire 1999). PTGS is a nucleotide sequence specific defence mechanism targeting both cellular and viral mRNAs, interfering with gene translation (Hamilton & Baulcombe 2006). Antisense silencing blocks the expression of genes by means of producing an artificial antisense single stranded sequence complementary to the sequence of the targeted gene of interest (Fire 1999). This results in the arrest of targeted gene translation confirming gene function through silencing of the gene of interest (Waterhouse *et al.* 1998).

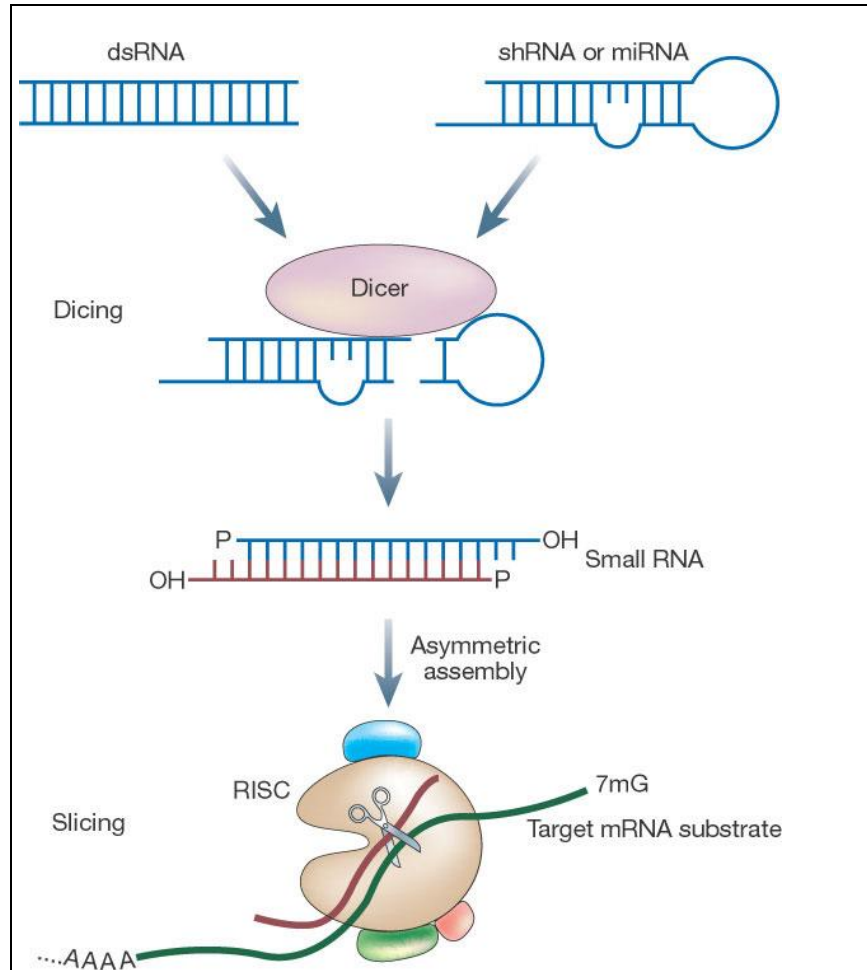


FIGURE 2.12: Schematic representation illustrating the mechanism involve in PTGS (Hannon & Rossi 2004).

The silencing process is triggered by dsRNA (Fig 2.12), which is digested into 21-23 nucleotides referred to as small interfering RNA (siRNA) (Fire 1999). siRNA is the product of the enzyme DICER which cleaves dsRNA. siRNA binds to a nuclease complex to form RNA induced silencing complex (RISC) (Fire 1999). RISC is activated once the siRNA unwinds which is an ATP dependent process. RISC activation results in the production of an antisense copy of the targeted RNA strand which is able to hybridize to the targeted mRNA, resulting in targeted mRNA cleavage and inhibiting gene expression (Fire 1999).

2.4.5. Plant transformation

Transformation of crops is a powerful tool, enabling researchers to identify genes as well as elucidating their function (Jones *et al.* 2005). It is a technique that allows the introduction of specific genes using vectors to shuttle gene constructs directly into different plant hosts, which results in the production of a genetically modified plant (Jones *et al.* 2005). It might be a quicker way of improving crops in comparison to conventional breeding strategies, provides essential knowledge about the function of the foreign gene introduced into its new host (Jones *et al.* 2005), and can add value to crop improvement (Thomson 2002). And thus can be used to improve the sustainability of crop yield and quality (He *et al.* 2010).

Plant transformation is a method of introducing a foreign gene, or a gene of interest, into a plant's genome (Thomson 2002). Effective and successful transformation is dependent upon three aspects which include: firstly the type of method used for transformation; secondly the employment of an appropriate promoter; and lastly the use of a genetic marker suitable for tracing the transformant. The two most widely used methods for transformation are use of *Agrobacterium tumefaciens* or biolistics, where the latter includes jet injection or microparticle bombardment (Furth 1997).

2.4.5.1. *Agrobacterium* mediated transformation

Agrobacterium tumefaciens is a soil borne plant pathogenic bacterium containing a Ti plasmid (tumour inducing plasmid), which is responsible for crown gall disease in plants (Jones *et al.* 2005). Dicots are more susceptible to *Agrobacterium* infections, while monocots are considered to be more resistant to change (Abdul *et al.* 2010). The Ti-plasmid is the major determinant of host range that could be transformed, rather than chromosomal genes (Gelvin 2003).

The Ti plasmid is essential for *Agrobacterium* transformation, due to its capability of transferring foreign DNA (T-DNA) into the nucleus of a host plant (Fig 2.13) (de la Riva *et al.* 1998). Although the plant could be transformed (i.e. containing the foreign DNA), the transformation process was subsequently accompanied by tumour formation (de la Riva *et al.* 1998). Since this was not ideal for transformation, other mechanisms had to be deployed to solve this problem. In the early

1980s, the tumour inducing plasmid of *Agrobacterium* was modified into a binary vector, disarming the *Ti* plasmid (Jones *et al.* 2005). This modified vector lacked the tumour inducing genes, whereby preventing the occurrence of the crown gall disease, and genes were further replaced with the genes of interest (Jones 2005).

Components of the binary vector system include the two plasmids, one plasmid containing the origin of replication and a variety of virulence (*vir*) genes, which includes *virA*, *virB*, *virC*, *virF*, *virH* and *virG* (Jones 2005). The second plasmid contains the T-DNA flanked by the left and right border sequence containing the gene of interest cloned into a multiple cloning site and a selectable marker to track transgene incorporation (Jones 2005). The *vir* genes and their proteins play an essential role in the transformation process, with *Agrobacterium* facilitating the transfer of DNA (T-DNA) into the host cells (Gelvin 2003).

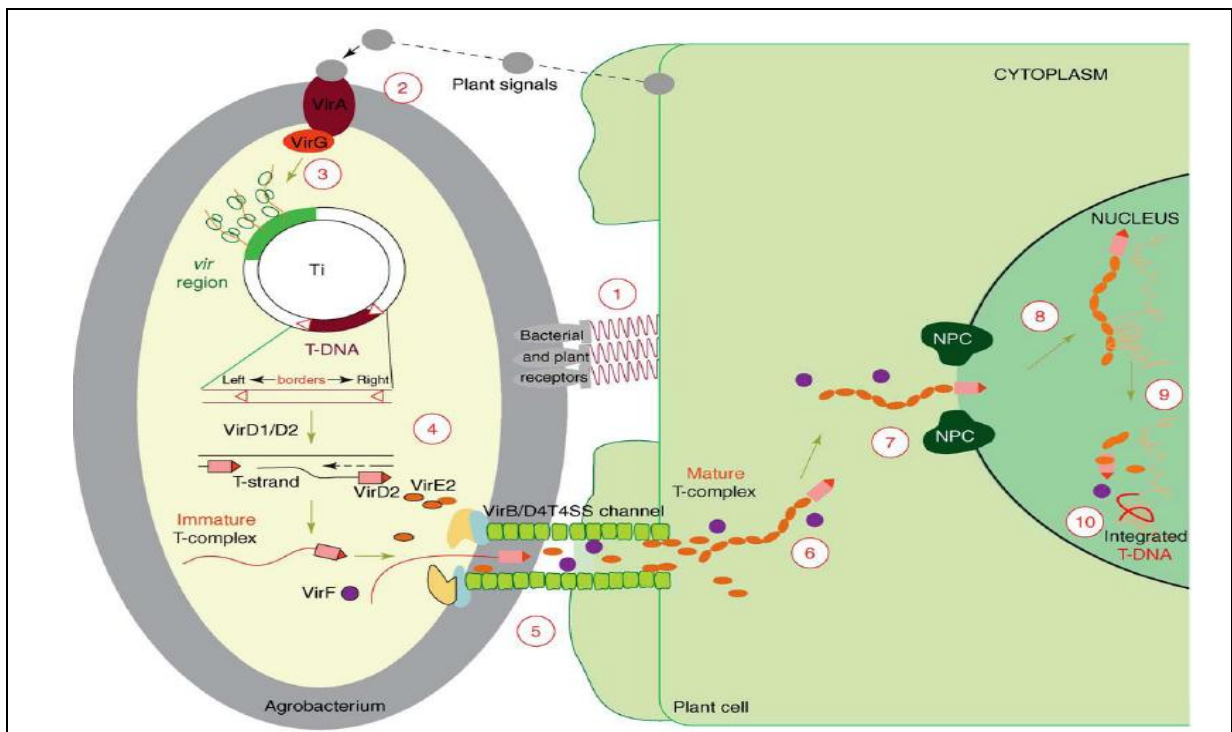


FIGURE 2.13: *Agrobacterium* mediated DNA transformation (Tzfira & Citovsky 2006).

Agrobacterium transformation is shown in Figure 2.13, starting with *virA*, which is a periplasmic antenna, having the ability to sense the presence of a particular phenolic plant compound activated upon wounding (Gelvin 2003). *VirB* proteins can form one of two channels, namely the membrane channel or the ATPase channel, which provides energy for transformation or export processes (Gelvin 2003). The *virC* and *virF* seem to be responsible for determining the range of plant species that could possibly be transformed resulting in the formation of gall tumours, whilst *virH* and *virG* are shown to be involved in hypervirulence of certain *Agrobacterium* strains (Gelvin 2003).

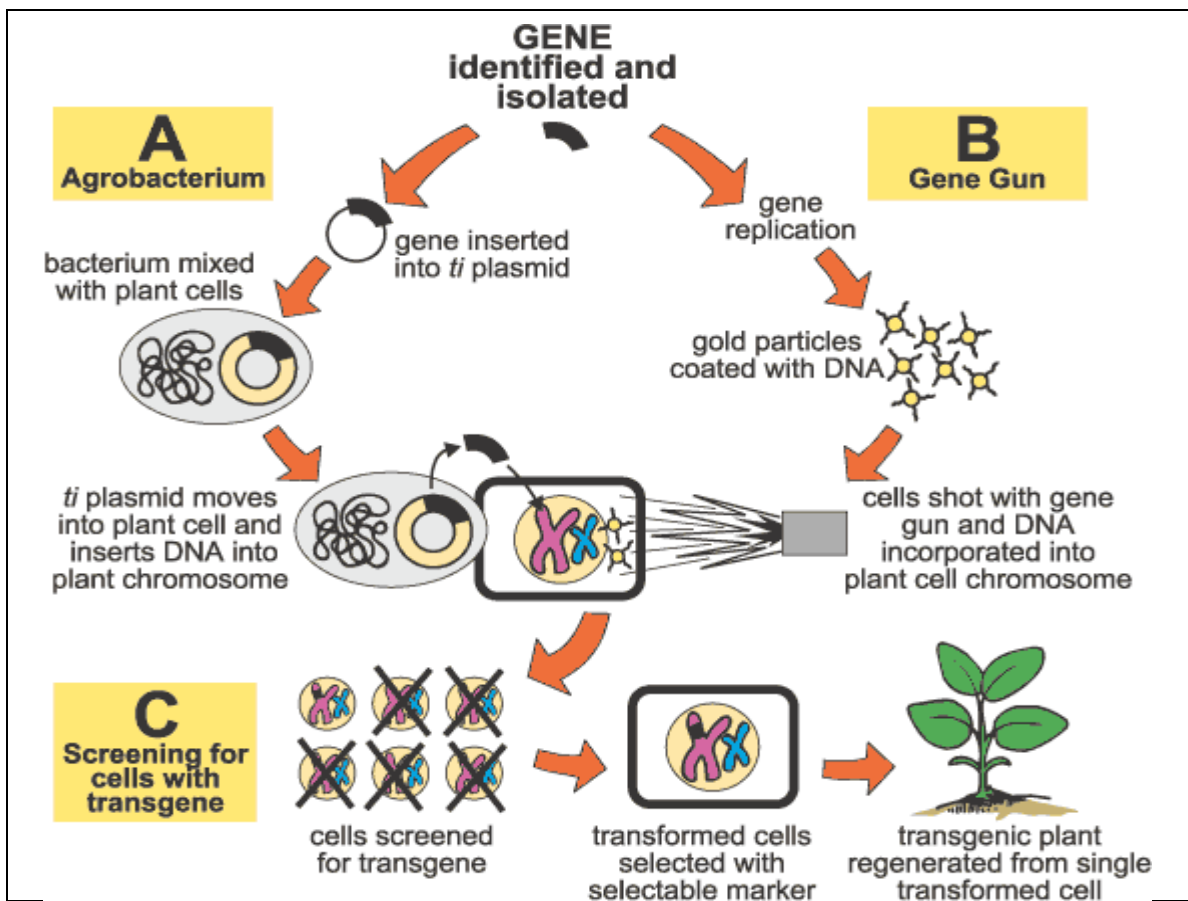


FIGURE 2.14: Schematic representation comparing *Agrobacterium* and particle bombardment transformation methods.

According to Gelvin (2003), the most stable temperatures for *Agrobacterium* strains are between 18 and 20°C, which have a significant effect on the transformation efficiency. This observation

suggests co-cultivating *Agrobacterium* with the plants cells at lower temperatures at the initial phase of transformation possibly resulting in a higher yield of transformed plants (Gelvin 2003).

2.4.5.2. Particle bombardment

Until the 1980s, there was no alternative method to *Agrobacterium* for transformation. Although multiple successes were achieved using *Agrobacterium*-mediated transformation, monocot transformation was still inefficient (Abdul *et al.* 2010). Thus other avenues had to be explored for monocot transformation.

Research started with protoplast cultures which gave rise to the technique known as particle bombardment (Abdul *et al.* 2010). Particle bombardment was the first acceleration device which allowed direct DNA delivery into a host plant and was reported by Sanford *et al.* in 1987 (Rao *et al.* 2009). The technique can be used for stable and transient transformation of plants. The process is initiated with the coating of gold or tungsten particles with plasmid DNA (Jones 2005). Plasmid DNA is accelerated into the host plant tissue at a high velocity using one of several acceleration systems (Furth 1997). This is an effective approach for distributing DNA over a wide area of the targeted tissue (Fig 2.14) (Jones 2005). Analysis of transgenic plants revealed that transformation using particle bombardment may result in one copy of the foreign DNA, but complex patterns can also be observed (Table 2.1) (Christou 1992).

TABLE 2.1: The advantages and disadvantages of *Agrobacterium*-mediated transformation vs particle bombardment

	Methods of transformation	
	<i>Agrobacterium</i> transformation	Particle bombardment
Genotype Specific	Yes	No
Vector requirement	Highly specific	Low specificity
Tissue penetration	Poor	Good
Number of transgene copies	Low	High
Host range	Limited	Broad
Insertion size	Large	Restricted
Efficiency	Host dependent (low/moderate)	Low

2.4.5.3. Promoters

One of the main factors affecting transformation frequency is the use of a suitable promoter. The promoter is the mechanism that controls where and when the gene will be expressed in a plant (Abdul *et al.* 2010). To date, the most commonly used promoters for monocot transformation are: CaMV 35S (Cauliflower mosaic virus 35S), Adh1 (alcohol dehydrogenase I), Act I (rice actin1), ubi 1 (maize ubiquitin) and ScBV (sugarcane bacilliform badna virus) (Abdul *et al.* 2010).

The pUbi-510 (Fig 2.15) and pEmUkN vectors were in this study. The pUbi-510 vector is controlled by two constitutive promoters causing gene expression throughout the entire lifecycle of a plant (Abdul *et al.* 2010). The promoters are the CaMV 35S promoter and the Ubi-1 promoter and a CaMV terminator site, which is responsible for informing the cellular machinery of a plant when the end sequence of a gene has been reached.

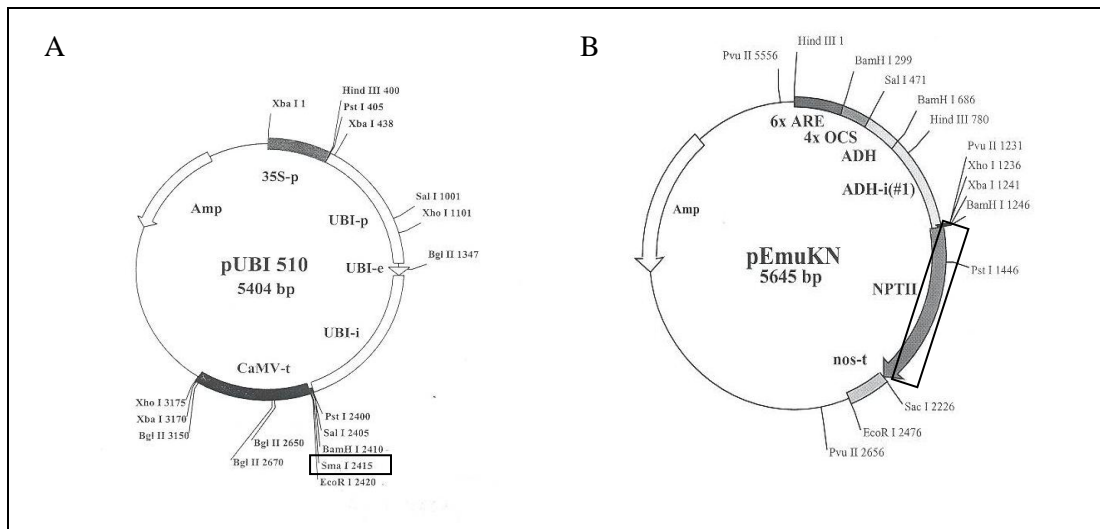


FIGURE 2.15: Schematic representation of transformation vectors. a) pUbi-510 vector containing the transgene. b) pEmuKN co-bombardment vector containing *nptII* as the selection gene.

2.4.5.4. Reporter and selectable genes

Reporter and selectable genes are useful to identify successful integration of the transgene into the explants of choice due to the low occurrence of transformation events (Abdul *et al.* 2010). These reporter genes are added to the gene construct which is used for transformation. There are two types of reporter gene systems which are screenable/scoreable and selectable marker gene systems.

Scoreable/screenable markers are gene products which can be easily detected through the application of reporter genes such as GUS (β -glucuronidase) and GFP (green fluorescent protein). These are also the most frequently used reporter genes for wheat, although others are available (Abdul *et al.* 2010). Selectable markers are gene sequences that act as genetic tags to identify transformed cells. Three types of selectable markers are frequently used which include: antibiotic resistance, herbicide resistance and metabolic markers (Abdul *et al.* 2010). The pEmuKN vector contains the *nptII* gene, which is an antibiotic resistance marker which serves as a selectable marker (Abdul *et al.* 2010). The *nptII* is one of the most widely used markers and was initially isolated from *Escherichia coli* (*E. coli*) (Abdul *et al.* 2010).

2.4.5.5. Current status of wheat transformation

Literature reports that the majority of wheat transformations were performed using microparticle bombardment (He *et al.* 2010). This technology is more frequently utilized due to its characteristics of being reproductive on any medium, unrestricted non-commercial use and distribution (He *et al.* 2010). However, when specifically looking at the regulatory processes involved in transformation, it is advantageous to use *Agrobacterium*-mediated transformation for dicots but not for monocots (He *et al.* 2010). However, transformation using *Agrobacterium* has made significant progress in certain wheat genotypes, which includes spring varieties such as Bobwhite, Fielder, Cadenza, and Veery 5 and a winter wheat variety such as Florida (He *et al.* 2010).

Factors influencing transformation efficiency include: type of *Agrobacterium* strain and vector used, as well as the medium composition, inoculation procedure, selective agent and the type of explant (He *et al.* 2010). To date, bread wheat transformation via *Agrobacterium* remains at a low frequency and is genotype dependent. He *et al.* (2010) suggested using durum wheat, which has been successfully transformed using *Agrobacterium*, as model system to further our understanding of important processes and elucidate gene function (He *et al.* 2010).

In this study, the regulation of candidate genes associated with the hypersensitive response were investigated. Firstly, using a reverse genetic approach to elucidate their gene function and secondly their distribution, abundance and location were determined by performing fluorescent *in situ* hybridization.

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Chapter 3

Silencing of *APX* and *GSTF6b* in wheat to elucidate their function in RWA defence

3.1. Abstract

Global wheat production loss is caused by multiple factors, such as abiotic and biotic stressors which greatly impacts wheat production and affects the sustainability of crop yield and quality of the end product. Russian wheat aphid (*Diuraphis noxia*, Kurdjumov, RWA) is such an economically important pest with a narrow host range feeding mostly on wheat and barley. Previously, a microarray study conducted on *Triticum aestivum* L. infested with *Diuraphis noxia* revealed the regulation of genes associated with the hypersensitive response; from these, two genes were selected for this study i.e. ascorbate peroxidase (*APX*) and glutathione S transferase (*GSTF6b*). Therefore, the focus of this chapter is to confirm the function of these hypersensitive related genes in RWA resistance using a reverse genetics approach. In order to achieve this, gene fragments for both *APX* and *GSTF6b* were isolated from wheat and, sequenced to confirm their identity. Gene fragments *APX* and *GSTF6b* were then cloned into the pUBI-510 transformation vector in antisense orientation, suitable to knockout their expression. Silencing constructs containing these gene fragments were then bombarded into 4-6 day old wheat embryogenic tissue. The embryogenic tissue was obtained from immature seeds of 11 to 16 day old anthesis Gamtoos-S (*Dn0*) and Gamtoos-R (*Dn7*) cultivars. Subculturing of embryogenic tissue was carried out on different mediums for different time periods. From 371 bombarded embryogenic tissues, 58 died on the shoot elongation medium and only three plantlets survived the entire selection process. To validate if the surviving plants were transgenic, qPCRs were conducted to confirm the presence of the transgene in the respective plants and to quantify the expression of the genes with/without RWA infestation. Nearly 50% and 70% reduction in *APX* and *GSTF6b* expression was observed in the respective plants when comparing with the control. After infestation, chlorosis was observed in the Gamtoos-S-*APX* transgenic plant while no distinguishable symptoms could be observed in the other infested material. This observation suggest probable oxidative damage to the photosynthetic machinery of the plant.

3.2. Introduction

The distribution of *Diuraphis noxia* (Russian wheat aphid, RWA) has become a serious threat to major wheat producing countries across the world, excluding Australia, causing extensive crop damage (Marasas *et al.* 2005). Thus, the major goal for wheat breeders are to breed crop plants with increased and durable resistance to withstand pest invasion (Gurr & Rushton 2005).

Even though for many years increased resistance was successfully achieved using non-transgenic strategies such as marker assisted breeding, pest resistance was short lived due to the emergence of new aphid biotypes. An alternative to conventional breeding approaches is transgenic strategies whereby a single gene can be overexpressed to increase resistance of a particular plant defence response (Gurr & Rushton 2005).

In wheat, difficulties with transformation are associated with the type of method implemented as well as gene delivery to generate regenerable explants and obtaining plantlets with the presence of a transgene. Parameters for successful wheat transformation include firstly the transfer of the gene of interest into the explant of choice; secondly the incorporation and expression of the foreign DNA into the host genome; and lastly the ability to recover fertile transgenic plants from transformed tissues. The major reason being ascribe to the high redunancy and dynamics of the wheat genome being 16000 Mb in size (Abdul *et al.* 2010, Brenchley *et al.* 2012).

The first transgenic wheat plant with an incorporated herbicide resistant gene was reported in 1992 by Vasil and coworkers paving the foundation for wheat transformation. In 1997, five years later the first transgenic wheat plants with male sterility were reported (De Block *et al.* 1997), followed in 1999, with wheat plants reported with improved tolerance to virusses (Stoger *et al.* 1999) and insects (Alpeter *et al.* 1999). Futhermore in 2003, transgenic wheat tolerance against the herbicide Roundup was produced using glutathione-S-transferases (Galovaic *et al.* 2010).

Glutathione transferases (GT), also known as glutathione-S-transferase (GST), is a large gene family consisting of 25-60 members which is a major phase II detoxification type of enzyme

(Frova 2003). Located predominantly in the cytosol, these enzymes have of a broad spectrum of functions, which includes both catalytic and non-catalytic roles (Dixon *et al.* 2002). There are eleven GST classes in total, of which at least five are plant classes namely: Alpha, Mu, Tetra, Phi (F) GSTFs and Tau (U) GSTUs (Sheehan 2001). The Phi and Tau classes are distinctly plant specific GSTs while the remaining three classes are commonly found in mammals and fungi. The Phi class is primary involved in the detoxification of herbicides, while the Tau class is associated with multiple endogenous and exogenous stresses, which are triggered by a variety of stimuli such as pathogen attack, wounding, metal toxicity, oxidative and temperature stress (Frova 2003).

Another gene involved in oxidative stress is ascorbate peroxidase. In plants, it is found that ascorbate plays an essential role in the developmental and growth regulation of plants as well as metabolism and in defence (Foyer & Noctor 2009). Ascorbate peroxidase (APX) is a heme-containing enzyme that plays a role in hydrogen peroxide (H₂O₂) detoxification. Three types of APX isoenzymes have been identified based on their respective subcellular location namely, cytosolic, chloroplastic and glyoxysomal APXs. According to Danna *et al.* (2003), cytosolic APX (cAPX) is associated with pathogen response, whereas glyoxysomal APX is responsible for removing the H₂O₂, which is generated by the fatty acid-oxidation pathway and photorespiration (Danna *et al.* 2003). Chloroplastic APX (chAPX) is a combination of both the thylakoid-bound (tAPX) and stromal (sAPX) isoforms. APX is responsible for removing or catalyzing H₂O₂ to 2 monodehydroascorbate (MDHA) to avoid oxidative stress (Danna *et al.* 2003).

When aphids feed on host plant, the first line of defense is rapid activation of cell death at the infested site, referred to hypersensitive response (HR) (Pieterse & Loon 1999). This is a defensive mechanism that the plant activates upon aphid feeding. Genes involved in this mechanism includes *APX* and *GSTF6b*, both play a major role in the removal of reactive oxygen species (ROS) generated as a byproduct of photorespiration and photosynthesis (Belkhadir *et al.* 2004). In essence, they are responsible for plant survival and productivity. Therefore, the objective of this part of the study was to elucidate and quantify the response of these genes involved in the defensive mechanism against Russian wheat aphid feeding following a transgenic approach using particle bombardment.

3.3. Research Methodology

3.3.1. Isolating targeted genes out of wheat

3.3.1.1. Plant material

Hexaploid germplasm of both winter and spring wheat cultivars was used in this study. The winter wheat cultivar TugelaDN was supplied by the Small Grain Institute, Bethlehem, South Africa. Resistant TugelaDN was bred by crossing Tugela and the RWA-resistant SA1684 (*Dn1*), the resulting population was backcrossed with Tugela producing near-isogenic lines (NILs) (Du Toit 1989). Spring wheat cultivars Gamtoos-S and the near isogenic line Gamtoos-R was also included into this study since Gamtoos-R is a fast growing international breeding line containing the *Dn7* resistance gene (Marais & du Toit 1993). The *Dn7* gene is a single dominant gene obtained from rye chromosome 1RS, which was combined with wheat giving rise to 1R/1B chromosome translocation (Anderson *et al.* 2003).

3.3.1.2. Designing gene specific primers

Available wheat sequences for *APX* and *GSTF6b* (Table 3.4.1) were obtained from the National Centre for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.gov>). Sequences were aligned with the CLUSTAL W function within the Geneious software package (Bio-matters Ltd) and MEGA 5.0 (Tamura *et al.* 2011), allowing primer design in the variable regions within the *APX* and *GSTF6b* genes using Primer 3 Software available in the Geneious 5.6 software (Bio-matters Ltd) (Table 3.3.1).

TABLE 3.3.1: Primers generated using the Geneious software

Primer #	Name	Sequence (5'-3')	Tm
AMO4-2012-F	APX fvd	GGCTTCCTGATGCTGGCCCAC	64.5°C
AMO4-2012-R	APX rvs	TTGGCGGCAACGAAGGGCTC	63.5°C
AMO5-2012-F	GST fvd	TACAAGAAGAACGAAGTGGACCTG	56.5°C
AMO5-2012-R	GST rvs	ACACACGCAAAGCGCAAAAA	57.0°C

3.3.1.3. Treatment of equipment to remove RNase activity

All equipment required for RNA isolation i.e. mortars and pestles, spatulas, tweezers and scissors were treated according to Sambrook *et al.* (1989) in 0.1% diethyl pyrocarbonate (DEPC) treated water for 24h in a fumehood, followed by enclosing treated equipment in foil which was autoclaved at 120°C, and then placed in a oven at 160°C till dried.

3.3.1.4. RNA Extraction

Plant material (*Tugela Dn1*) was collected at Welgevallen Experimental Farm, Stellenbosch, South Africa. The leaves were cut and cleaned with 70% Ethanol (EtOH). The plant material was flash frozen in liquid nitrogen (N₂) and stored at -80°C prior to usage. Mortars and pestles were used to grind frozen plant tissue in liquid nitrogen.

RNA was isolated using the Trizol extraction reagent (<http://www.invitrogen.com>), followed by DNase (<http://thermoscientific.com/fermentas>) treatment of the total RNA. Total RNA was purified and eluted in 30 µl nuclease-free water (<http://www.invitrogen.com>) using an RNeasy mini column protocol as described by the manufacturers (<http://www.Qiagen.com>). RNA quantification and purity of each sample was determined with a ND-1000 Nanodrop spectrophotometer (NanoDrop Technologies).

cDNA synthesis was performed using the BioRad iScript cDNA synthesis kit as described by the manufacturer (<http://www.Biorad>), starting with total RNA as template. The reaction was incubated in a Gene Amp 9700 (Applied Biosystem) thermocycler at three incubation steps, starting at 25°C for 5 min, followed by 42°C for 30 min and with final temperature of 85°C for 5 min before cooling to 4°C.

3.3.1.5. Polymerase chain reaction (PCR) Primer screening

Amplification reactions were performed with newly synthesized cDNA as template (50 ng) together with the following components: 0.2 mM dNTPs (Fermentas, Cat no. FERo181), 10 x buffer (Roche, Cat no. JMR-455) with a working stock of 1X, 2.5 mM MgCl₂ (Roche, Cat no.

JMR-456), 10 pM primers and 5 U SuperTherm *Taq* Polymerase (Crimson *Taq* DNA polymerase from Bio Labs, Cat no. JMR-801).

The Gene Amp 9700 (Applied Biosystem) thermocycler was used for incubation, with a hot start amplification at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, annealing temperature T_m °C (Table 3.3.1) for 30 s, and 72°C for 2 min, with final extension at 72°C for 7 min before cooling to 4°C. Amplification products were resolved by electrophoresis on 1.5% agarose gels (SeaKem® LE Agarose) at 80V (Bio-Rad Power Pac 1000) for 75 min in 1X Tris acetate (TAE) buffer (0.04 M Tris acetate and 0.001 M EDTA).

Before samples were loaded on a gel, the mixture containing sample, DNA ladder and GR green stain (<http://thermoscientific.com/fermentas>) were denatured at 98°C for 3 min in a thermocycler. The Multi Genius Bio Imaging system (SynGene) and UV light was used for visualisation which was followed by PCR product clean-up with the GeneJet PCR purification kit before as described by the manufacturers (<http://thermoscientific.com/fermentas>). The concentration and purity of samples were determined with the ND-1000 Nanodrop spectrophotometer (NanoDrop Technologies).

3.3.1.6. Sequencing and confirmation of identity

The obtained amplicons produced under 3.3.1.5 were sent for sequencing at the Central Analytical Facilities University of Stellenbosch with a PRISM® 3730X1 DNA analyser (Applied Biosystems Inc.). Sequencing required 10 µl of each sample together with 1.1 pM primer concentration for each primer pair (Table 3.3.1).

3.3.2. Gene isolation from plasmids

3.3.2.1. Isolation and Blunting of DNA

VIGS plasmids containing *APX* or *GSTF6b* (Schultz unpublished results) from -80°C glycerol stocks were grown on Luria Broth (LB) medium plates (10 g tryptone, 5 g yeast extract, 5 g NaCl and 15 g agar) containing 50 µl ampicillin (100 mg/µl) (Fermentas, Cat no. ZRA101-5) for

selection of antibiotic-resistant cells. Plates were incubated at 37°C overnight, followed by picking a single colony from incubated plates for each gene. Colonies were grown in 5 ml liquid LB medium (10 g tryptone, 5 g yeast extract and 5 g NaCl) with 50 µl ampicillin (100 mg/ml) (Fermentas, Cat no. ZRA101-5) at 37°C on a shaker at 150 revolutions per minute (rpm) overnight.

Amplification reactions were prepared as follows for both *APX* and *GSTF6b*: 5X Phusion buffer (Roche, Cat no. JMR 455), MgCl₂ (25 mM) (Roche Cat no. JMR 456), dNTPs (100 mM) (Fermentas, Cat no. FERO1081), forward primer (10 pM), reverse primer (10 pM) (Table 3.3.2), Phusion high fidelity *Taq* 5 U DNA polymerase from Finnzymes, Cat no. F-5305) and 0.5 µl of cultures in a total volume of 50 µl.

TABLE 3.3.2: Primers used for PCR amplification in the isolation of *APX* and *GSTF6b* genes from pGem-T Easy plasmids

Primer #	Name	Sequence (5'-3')	T _m
10/7	APX-L	CCACGTTTACCTGCTGAACA	55.4°C
10/8	APX-R	CGCAGCATCTGTAGGCAATA	55.1°C
10/17	GSTF6-L	CCTCAACCACTTCCCTACA	56.3°C
10/18	GSTF6-R	ATTTGCCTTATTCGCGACAC	53.8°C

The following cycling parameters were used: an initial hot start amplification at 98°C for 30 s, 25 amplification cycles consisting of denaturing at 98°C for 10 s, annealing at various temperatures (Table 3.3.2) for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 7 min before cooling to 4°C. Amplified product were visualized as described under section 3.3.1.5, followed by sequencing of obtained amplicons (refer to section 3.3.1.6).

3.3.2.2. Phosphorylation of DNA (*APX* and *GSTF6b*)

Blunt end DNA (300 ng/µl) under 3.3.2.1 was phosphorylated as with 10X T4 polynucleotide Kinase, ATP (10 mM) and T4 DNA polynucleotide Kinase (10 U). The reaction was incubated for 20 min at 37°C and 10 min at 75°C in a Gene Amp 9700 (Applied Biosystem) thermocycler which was followed by PCR cleanup with the GeneJet PCR purification kit as described by the

manufacturer (<http://thermoscientific.com/fermentas>) to purify the template for further analysis. Purity of samples was determined using the ND-1000 Nanodrop spectrophotometer (NanoDrop Technologies).

3.3.3. Construction of the silencing cassette

3.3.3.1. Preparing transformation vectors

A ubiquitin promoter transformation vector pUBI-510 and the co-bombarded vector pEmuKN were plated from -80°C glycerol stocks using looplast sticks on LB medium plates (10 g tryptone, 5 g yeast extract, 5 g NaCl and 15 g agar) containing 50 µl ampicillin (100 mg/ml) (Fermentas, Cat no. ZRA101-5). Plates containing transformation vectors were incubated at 37°C overnight. Followed by picking single colonies from the overnight transformants for both vectors and growing picked colonies in 50 ml liquid LB medium (10 g tryptone, 5 g yeast extract and 5 g NaCl) containing 50 µl ampicillin (100 mg/ml) (Fermentas, Cat no. ZRA101-5) at 37°C on a shaker at 150 rpm overnight. Larger quantities of DNA (1 ng/µl) of the co-bombarded vector was isolated using the GeneJet midiprep kit as described by the manufactures (Fermentas) and for the transformation vector smaller quantities of DNA was isolated with the GeneJet miniprep kit (Fermentas).

To construct a cassette for silencing, the transformation vector pUBI-510 (300 ng/µl), which is a circular vector (Figure 3.1A), was linearised with the *Sma*I restriction enzyme (5 U) in a 20 µl reaction containing 10X *Sma*I buffer and 14 µl millipore water. The reaction was incubated at 30°C for 2 hours and 65°C for 30 min.

Followed by dephosphorylation with shrimp alkaline phosphatase (1 U) and DNA was cleaned with a GeneJet PCR purification Kit (<http://thermoscientific.com/fermentas>). This was done to prevent the vector from re-circularizing. The reaction mixture was then incubated at 37°C for 10 min and for 5 min at 75°C which was followed by PCR clean-up using the GeneJet PCR purification kit as described by the manufacturers (<http://thermoscientific.com/fermentas>) to purify the template for further analysis.

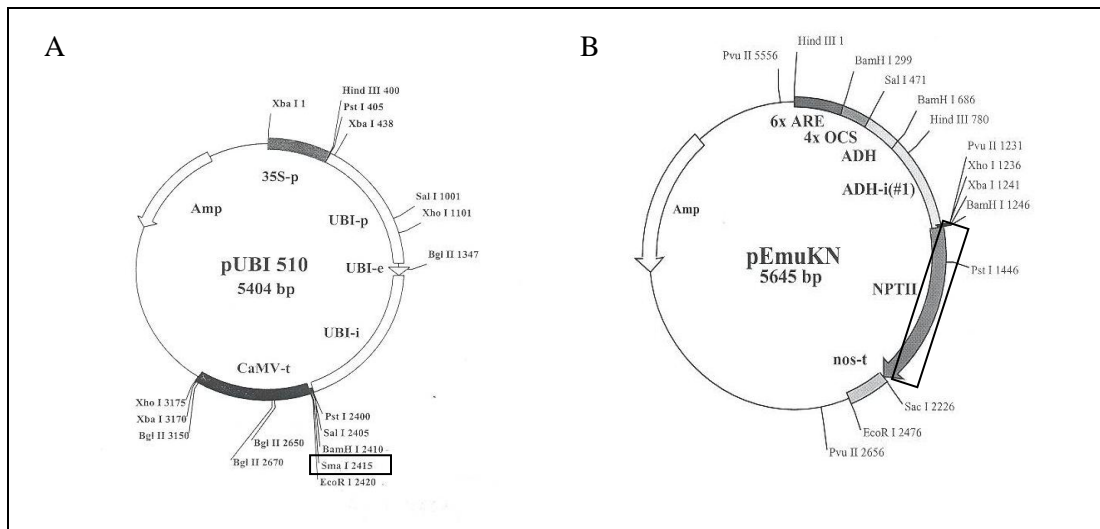


FIGURE 3.3.1: Schematic representation of transformation vectors. a) pUbi-510 vector containing the transgene. b) pEmuKN co-bombardment vector containing *nptII* as the selection gene.

3.3.3.2. Cloning

A ligation reaction was prepared with the amplification products (*APX* and *GSTF6b* from glycerol stocks) which were ligated into the linearised pUBI-510 transformation vector as follows: 10X T4 ligase buffer, T4 DNA ligase (5 U), pUBI-510 (300 ng), 50 ng amplified product, 50% (w/v) polyethylene glycol (PEG) 4000 and millipore water in a total volume of 10 μ l.

The ligation reaction was incubated at room temperature ($\sim 25^{\circ}\text{C}$) overnight. After incubation, the ligation reaction was mixed with 50 μ l *Escherichia coli* (*E. coli*) DH5 α competent cells. The reaction was then placed on ice for 20 min, followed by 42°C in a water bath for 45 s and the reaction was terminated by incubating for 2 min on ice. A volume of 950 μ l of super optimal broth with catabolite repression (SOC) medium (1 g tryptone, 0.25 g yeast extract, 0.5 g NaCl (1 M), 0.125 ml KCL (1 M), 0.5 ml $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (2 M) and 0.5 ml glucose (2 M)) was then added to the reaction and incubated for 1 h and 30 min at 37°C on the shaker at 150 rpm.

During the waiting period, 50 μ l ampicillin (100 mg/ml) (Fermentas, Cat no. ZRA101-5) was added to the LB agar plates (10 g tryptone, 5 g yeast extract, 5 g NaCl and 15 g agar). Thereafter, 100 μ l of each incubation reaction was added to the prepared LB medium plates. Plates were incubated overnight in a dark growth room at 37°C .

Single white colonies were picked from the plates and grown overnight in 50 ml liquid LB medium (10 g tryptone, 5 g yeast extract and 5 g NaCl) together with 50 µl ampicillin (100 mg/ml) (Fermentas, Cat no. ZRA101-5). This was followed by making glycerol stocks 15% (v/v) and colony PCRs for size selection were conducted.

Colony PCR reactions were prepared using 0.5 µl newly made glycerol stocks as template together with the following components: 10X buffer (Roche, Cat no. JMR 455), MgCl₂ (25 mM) (Roche Cat no. JMR 456), dNTPs (100 mM) (Fermentas, Cat no. FERO1081), forward primer (10 pM), reverse primer (10 pM) (Table 3.3.2), SuperTherm *Taq* Polymerase 5 U from Bio Labs (Cat no. JMR-801) in a total volume of 25 µl for 25 cycles.

The reaction was incubated in a Gene Amp 9700 thermo cycler, with a start amplification step at 98°C for 30 s, followed by 25 cycles of 98°C for 10 s, annealing at various temperatures (Table 3.3.2) for 30 s, and DNA extension at 72°C for 30 s, with final extension at 72°C for 7 min before cooling to 4°C. Visualisation and sequencing were performed as described under sections 3.3.1.5 and 3.3.1.6.

3.3.3.3. Fragment orientation determination

A standard amplification protocol was applied with a combination of vector specific and gene specific primers (Table 3.3.2). Amplification, visualisation and sequencing were performed as described in 3.3.1.5 and 3.3.1.6. CLC sequence viewer (<http://www.clcbio.com>), was applied to confirm the orientation of the cloned fragment after sequencing.

3.3.3.4. Fragment-plasmid purification: DNA isolation

After sequencing confirmation of the orientation of the cloned fragments was carried out with, both vectors (pUBI- *APX* and pUBI-*GSTF6b*) in culture were grown in 50 ml liquid LB medium (10 g tryptone, 5 g yeast extract and 5 g NaCl) with 50 µl ampicillin (100 mg/ml) (Fermentas, Cat no. ZRA101-5) and incubated at 37°C on a shaker at 150 rpm overnight. Larger quantities of DNA

(containing both gene and vector) (1 $\mu\text{g}/\mu\text{l}$) were isolated with the GeneJet midiprep kit as described by the manufacturers (<http://thermoscientific.com/fermentas>).

3.3.4. Tissue culturing

3.3.4.1. Growth conditions

Seeds from Gamtoos-S (*Dn0*) and its near isogenic line Gamtoos-R (*Dn7*) were planted at 4°C in potting soil. Germinated seeds (15 cm in length) were planted in 150 x 150 mm pots filled with sandy soil containing no nutrients in a greenhouse under controlled conditions (19-24°C) at the Welgevallen Experimental Farm, Stellenbosch, South Africa with natural day/night regimes. Nutrients (Multifeed™, South Africa) were supplied via an irrigation system, which waters the plants three times a day.

3.3.4.2. Harvesting

With the onset of anthesis, marking the first day of pollen formation, wheat ears were covered with white (50 x 197mm) flat bags preventing cross pollination between cultivars. Ears were collected between day 11 and 16 of anthesis. The next step was seed surface sterilisation which was done as follows: washing seeds in 25% sodium hypochlorite for 2 min then placing them in 70% ethanol for 15 min and afterwards rinsing seeds three times with autoclaved distilled water.

3.3.4.3. Tissue culture and transformation via particle bombardment

Embryos were isolated from immature seeds using pre-autoclaved forceps and a scalpel. Embryos were placed on Induction A medium (refer to appendix Table 3.7.1 and 3.7.2 for composition of the media) and sealed with parafilm tape for 4 to 6 days and incubated in a dark growth room at 26°C.

Cultured embryogenic embryos were bombarded with 5 mg tungsten particles (GradeM- 10, Bio-Rad # 165-2266) which were coated with the pUBI plasmids (1 $\mu\text{g}/\mu\text{l}$) either carrying *APX* or *GSTF6b*. The pUBI plasmids also had the *nptII* reporter gene. Bombardment was conducted using a helium driven particle bombardment chamber.

Bombarded embryos underwent a series of culturing and subculturing on different media and at different exposure periods as shown in Tables 3.7.1 and 3.7.2. Plantlets that survived the entire selection process were transferred to the soil, which consisted of sand, palm peat (Starke Ayres) /autoclaved potting soil and vermiculite (Rosarium). Plantlets were placed in a greenhouse with protective screens to prevent direct sunlight exposure for a period of three days.

3.3.5. Infestation procedures

For infestation about 20 Russian wheat aphids, biotype SA1, were used per leaf per plant. The entire plant was not exposed to aphid feeding, only one leaf of each plant was exposed (three transgenic plants and two controls plants i.e. one susceptible and one resistant). To ensure that only the one leaf would be infested, aphids were placed in 15 ml centrifuge tubes which were attached to the plant with the leaf inside the tube and then sealed with cotton. Leaf harvesting was done 48 h after infestation. Freshly cut leaf samples were immediately flash frozen in liquid nitrogen followed by storing them at -80°C till RNA was extracted.

3.3.6. Quantitative real time polymerase chain reaction (qRT-PCR)

To confirm if these plants were transgenic, quantitative real time PCR (qRT-PCR) analyses were performed to test the expression levels of the transgenes. RNA was extracted from uninfested and infested material (Gamtoos-S (*Dn0*) pUBi-APX, Gamtoos-S (*Dn0*) pUBi-GST, Gamtoos-R (*Dn7*) tissue culture control, Gamtoos-R (*Dn7*) and Gamtoos-S (*Dn0*), which was exposed to SA1 aphid feeding for a period of two days. Leaves were harvested after 2 days and frozen immediately in liquid nitrogen.

RNA was extracted using the Direct-zol RNA miniprep kit as described by the manufacturer (Zymo research), followed by DNaseI treatment (<http://thermoscientific.com/fermentas>). First strand cDNA synthesis was performed using the Transcriptor First Strand cDNA Synthesis kit (<http://www.roche-applied-science.com/Roche>). The reaction was incubated in the BioRad CFX96 Real time system C1000 thermal cycler.

Cycling parameters started with a initial incubation of 50°C for 2 min, and amplification starting at 95°C for 5 min followed by 50 cycles of 95°C for 10 s, and annealing with various temperatures (Table 3.3.3) for 30 s, and DNA extension at 72°C for 20 s. As a reference gene, 18S rRNA was used (Table 3.3.3). Samples were tested in replicas of three together with the serial dilution. The mathematical model Pfaffl (2004) was used to determine the relative quantification.

TABLE 3.3.3: Primers used for determining relative expression levels of targeted genes and annealing temperature T_m used

Primer #	Name	Sequence (5'-3')	T _m	Primer Purpose
11/14	APX3 q-L	GTTGTCCGATACGATGAAGC	53.5°C	qPCR
11/15	APX3 q-R	TAGTTCCCGGTCAGAGACG	55.9°C	qPCR
11/16	GST3 q-L	TGTACGAGTGCTCATCAACC	54.6°C	qPCR
11/17	GST3 q-R	CGAGGTAGTCGTGCTTGG	55.4°C	qPCR
11/102	18S-F	TGCCCTATCAACTTTCGATGG	54.9°C	qPCR
11/103	18S-R	TGGATGTGGTAGCCGTTTCTC	57°C	qPCR

3.4. Results

3.4.1. Data assembly

A combined total of 34 sequences were identified in the NCBI database (National Center for Bioinformatics) using a keyword search for both *APX* and *GST* in wheat. Of the 34 sequences, 10 were *APX* sequences and the remaining 24 were *GST* sequences (Table 3.4.1 and 3.4.2).

TABLE 3.4.1: Bioinformatic survey on *APX* in wheat obtained from NCBI

Accession number	Gene Name	Chr. location	Gene length
AF532973.1	chromosome 6A thylakoid-bound ascorbate peroxidase mRNA, partial cds	6A	1306 bp
AF387739.1	Hanfeng 9703 leaf ascorbate peroxidase (<i>WAPXI</i>) mRNA, partial cds; nuclear gene for chloroplast product	Nc	405 bp
EF184291.1	thylakoid bound ascorbate peroxidase mRNA, complete cds	Nc	1315 bp
AY513261.1	C 306 thylakoid ascorbate peroxidase (<i>APX</i>) gene, complete cds	Nc	3160 bp
AY513262.1	C 306 thylakoid ascorbate peroxidase (<i>APX</i>) mRNA, complete cds	Nc	1392 bp
AY513263.1	HD 2687 thylakoid ascorbate peroxidase (<i>APX</i>) mRNA, partial cds	Nc	693 bp
AF532974.1	chromosome 6D putative ascorbate peroxidase mRNA, partial cds	6D	1208 bp
AF532972.1	chromosome 6BL thylakoid-bound ascorbate peroxidase mRNA, partial cds	6BL	1236 bp
FJ890988.1	ascorbate peroxidase (<i>PODI</i>) mRNA, partial cds	Nc	952 bp
EF555121.1	peroxisomal ascorbate peroxidase (<i>APX</i>) mRNA	Nc	893 bp

nc: not characterized

TABLE 3.4.2: Bioinformatic survey on *GST* in wheat obtained from NCBI

Accession number	Gene Name	Class	Gene length
AJ440796.1	<i>gstf1</i>	Class F	927 bp
AJ440791.1	<i>gstf2</i>	Class F	865 bp
AJ440792.1	<i>gstf3</i>	Class F	930 bp
AJ440793.1	<i>gstf4</i>	Class F	721 bp
AJ440794.1	<i>gstf5</i>	Class F	866 bp
AJ440795.1	<i>gstf6</i>	Class F	897 bp
AJ441055.1	<i>gstf6b</i>	Class F	904 bp
AY064479.1	<i>MRP1</i>	nc	2647 bp
AY064481.1	<i>GST 19E50</i>	nc	965 bp
AF479764.1	<i>GST 28E45</i>	nc	1018 bp
AY064480.1	<i>GST Cla47</i>	nc	901 bp
AF184059.1	<i>GST1</i>	nc	914 bp
AF002211.1	<i>GST (2)</i>	Zeta class	945 bp
AF109714.1	<i>GST (3)</i>	Zeta class	2947 bp
AF387085.1	<i>GST (4)</i>	nc	911 bp
AY377972.1	<i>Azar2 GST</i>	nc	384 bp
AF475124.1	<i>GST like protein</i>	nc	360 bp
EU584497.1	<i>Micheal GST</i>	nc	854 bp
FJ797431.1	<i>GHPOD</i>	nc	614 bp
X56004.1	<i>gstA2</i>	nc	3196 bp
EU823285.1	Soissons glutaredoxin	nc	512 bp
X56012.1	<i>gstA1</i>	nc	2178 bp
AF542185.1	Glutaredoxin	nc	757 bp
GQ494009.1	<i>DHAR</i>	nc	803 bp

For this study, we were particularly interested in the Phi (F) class GSTs since the Phi class is one of the most abundant plant-specific GSTs, which is primary involved in the detoxification (Frova 2003). Only 7 out of 24 sequences were phi class GSTs. Therefore, alignments were only performed on the 7 phi class sequences (Figure 3.4.1). Of these, only two of the sequences, *GSTF6* and *GSTF6b*, were phi class GSTs. Therefore, the forward primer for GST was designed over a deletion gap that occurred in 5 of the 7 sequences to specifically detect the *GSTF6b* sequence, which was used in this study (Figure 3.4.1).

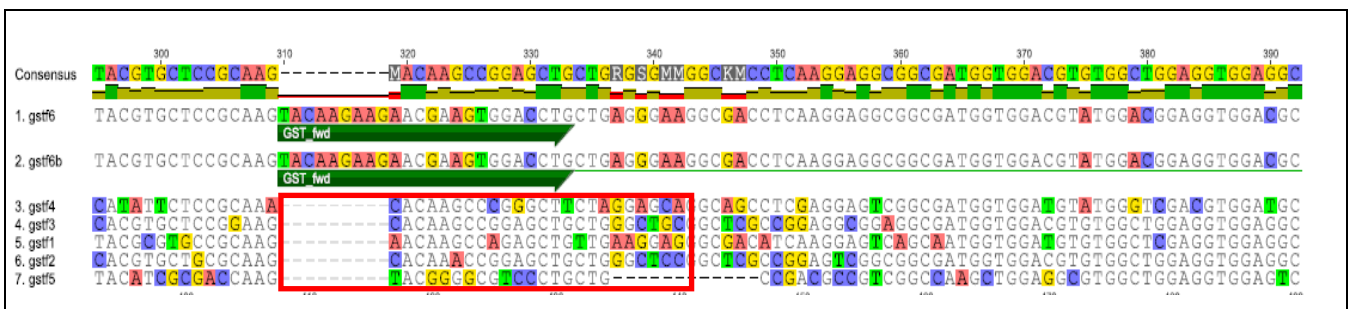


FIGURE 3.4.1: Alignment between seven hits for GST (forward primer).

For the reverse primer (Figure 3.4.2), there was no consensus between the phi class GSTs, all sequences differed from each other making it easier to design specific primers for only the *GSTF6b* fragment.

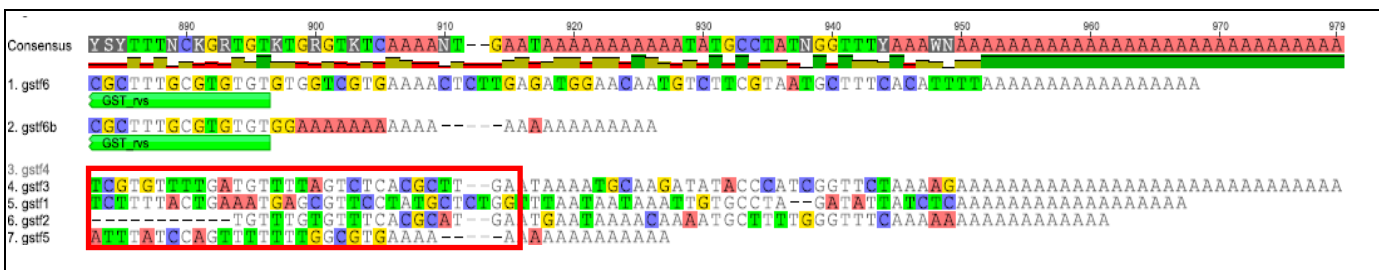


FIGURE 3.4.2: Alignment between seven hits for GST (reverse primer).

3.4.2. RNA Extraction

After RNA was extracted from the plant tissues the quality and concentrations of isolated RNA were verified on a 1.5% agarose gel (Figure 3.4.3) and also with a spectrophotometer (Table 3.4.2). The total RNA of samples 1, 4 and 6 (figure 3.4.3) was then used for cDNA synthesis. The quantity and quality of the obtained cDNA are shown in Table 3.4.3.

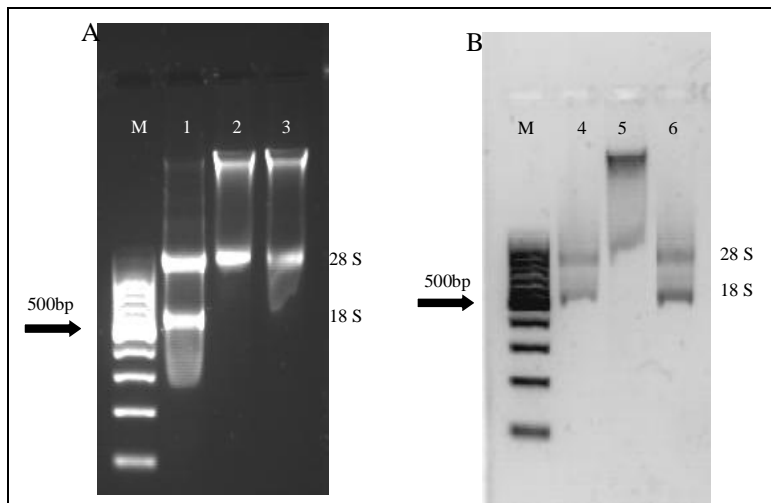


FIGURE 3.4.3: A-B showing isolated RNA of 6 samples from Tugela Dn1 leaf material.

The 260/280 absorbance ratio was used to validate the RNA quality. Ratio's range from 1.97-2.08 with samples 4 and 6 having the best quality ratio.

TABLE 3.4.3: Spectrophotometer measurements of isolated total RNA and cDNA

Genotype	[Total RNA]			[cDNA]		
	ng/μl	260/280	260/230	ng/μl	260/280	260/230
Tugela <i>Dn1</i> (1)	95.26	2.07	0.71	1027.84	1.83	2.20
Tugela <i>Dn1</i> (2)	57.34	1.97	1.61	60.32	1.87	0.14
Tugela <i>Dn1</i> (4)	188.20	2.08	2.22	97.13	1.20	0.46
Tugela <i>Dn1</i> (6)	110.05	2.08	2.08	960.93	1.88	2.26

3.4.3. Primer screening

In an attempt to isolate *APX* and *GSTF6b* from wheat, an amplicon of 600 bp was obtained for *APX* (Figure 3.4.4A) but no amplicon could be produced for *GSTF6b* (Figure 3.4.4B) with the designed primer pairs (Table 3.3.1). The obtained *APX* amplification product was sent for sequencing. However, after blast analysis the identity did not match that of the AF 387739.1 accession of *APX*. An alternative strategy was followed and the fragments were isolated using existing VIGS-constructs (Schultz *et al.* unpublished data) containing *APX* and *GSTF6b*. From these, fragments of 350 and 250 bp in size were obtained for *APX* and *GSTF6b* respectively, as shown in figure 3.4.4C and D.

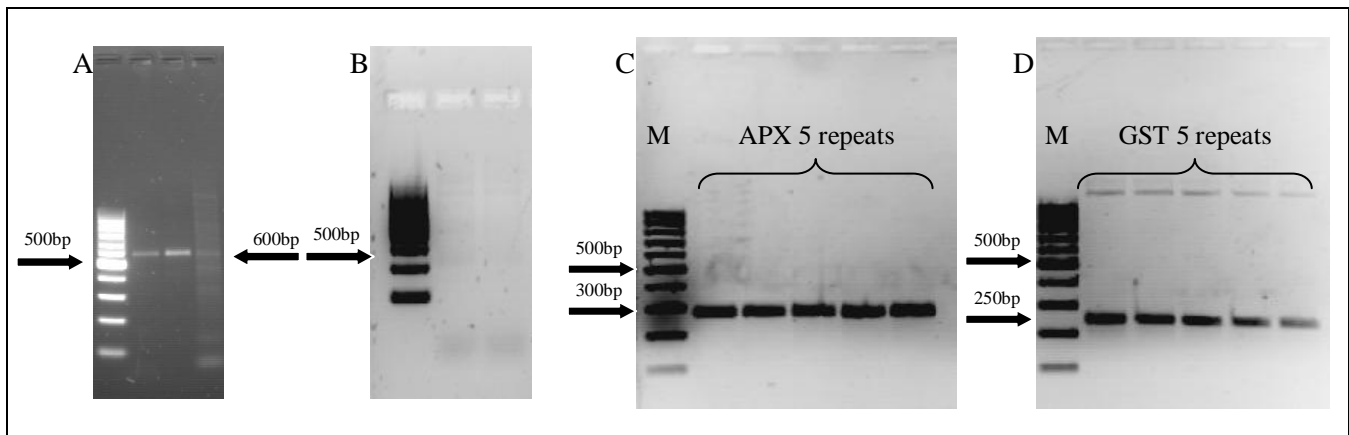


FIGURE 3.4.4: Amplification products resolved on a 1.5% agarose gel: a) primer pair AMO4-2012 (Table 3.3.1) for *APX*. b) primer pair for *GSTF6b* (Table 3.3.1). c) primer pair for *APX* (Table 3.3.2). d) primer pair for *GSTF6b* (Table 3.3.2).

3.4.4. Confirmation of silencing construct

Positive transformants were selected and used for colony PCR in order to determine the presence of the gene fragment of interest in the plasmid. Band fragments of approximately 350 bp for *APX* and 250 bp for *GSTF6b* (Figure 3.4.5) were found when amplified with primer pairs shown in Table 3.3.2.

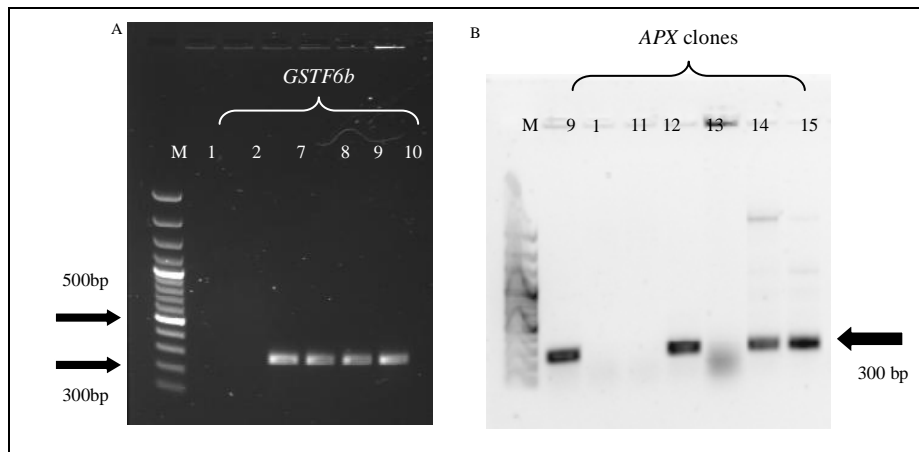


FIGURE 3.4.5: Colony PCR of cloned *APX* and *GSTF6b* fragments resolved on a 1.5% agarose gel.

Positive clones identified after conducting colony PCR analysis (Figure 3.4.5), were used in combination with vector specific forward primer labelled A21 (80 bp) and gene specific forward primer (Table 3.3.2) to determine the orientation of the silencing constructs. A total of four clones for *APX* and *GSTF6b* in the antisense orientation with band sizes ~ 400bp and 300 bp, respectively, were obtained (Figure 3.4.6).

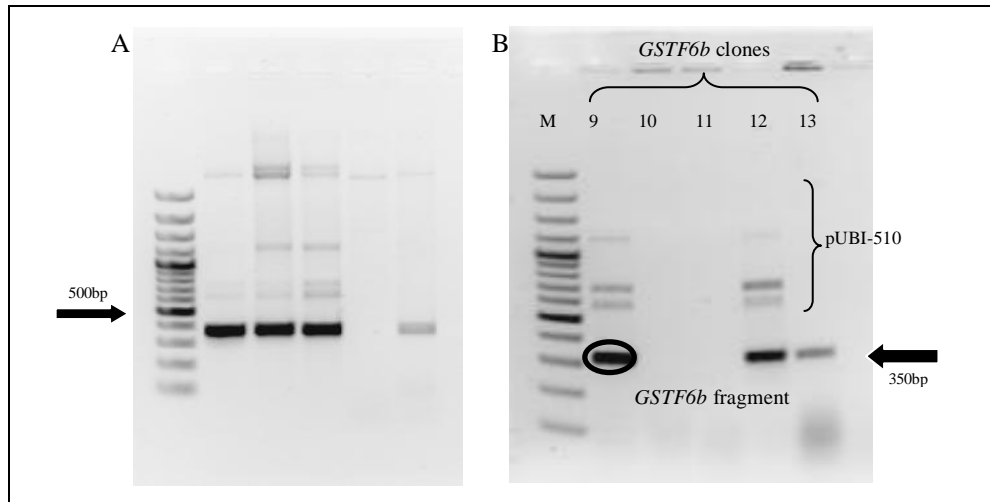


FIGURE 3.4.6: pUBI- 510 plasmids containing *APX* and *GSTF6b* respectively visualised on a 1.5% agarose gel.

Sequencing revealed that for *APX*, from a total of four clones, only two had the correct antisense identity when blasted against the NCBI database (Table 3.4.3) using BLAST N. Analysis of the *GSTF6b* sequences showed that two out of the four clones were in the right antisense orientation (Table 3.4.4 and Table 3.4.5).

Table 3.4.4: Identity and E-values of cloned *APX* fragments

Accession number	Gene	Description	E-value
AF387739.1	<i>APX</i> *	<i>Triticum aestivum</i> Hanfeng 9703 leaf ascorbate peroxidase (<i>WAPXI</i>)	2.00E-130
ABX60821.1	Clone1	<i>Triticum monococcum</i> epidermal p-coumarate 3-hydroxylase	1.00E-097
AK251450.1	Clone 2	<i>Hordeum vulgare</i> subsp. vulgare cDNA clone: FLbaf127j11	3.00E-068
AF387739.1	Clone 7	<i>Triticum aestivum</i> Hanfeng 9703 leaf ascorbate peroxidase (<i>WAPXI</i>)	2.00E-131
AF387739.1	Clone 8	<i>Triticum aestivum</i> Hanfeng 9703 leaf ascorbate peroxidase (<i>WAPXI</i>)	9.00E-115

*Reference sequence

Table 3.4.5: Identity and E-values of cloned *GSTF6b* fragments

Accession number	Gene	Description	E-value
AJ441055.1	<i>GSTF6*</i>	<i>Triticum aestivum</i> mRNA for glutathione transferase	5.00E-101
ABX60821.1	Clone 8	<i>Triticum monococcum</i> epidermal p-coumarate 3-hydroxylase	1.00E-097
AJ441055.1	Clone 9	<i>Triticum aestivum</i> mRNA for glutathione transferase	1.00E-100
AJ441055.1	Clone 12	<i>Triticum aestivum</i> mRNA for glutathione transferase	1.00E-100
AJ303373.1	Clone 13	<i>Triticum aestivum</i> mRNA for cytochrome P450 reductase (r2 gene)	5.00E-101

*Reference sequence

Alignments for clones 7 and 8, together with the sequence of the *APX* gene obtained from the NCBI, showed that clone 7 had a 100% homology when aligned to the NCBI entry AF387739.1, which is the *APX* gene sequence (Figure 3.4.7). Since clone 8 (Figure 3.4.7, red boxes) differed at a few nucleotides, it was decided to subject clone 7 to further analysis.

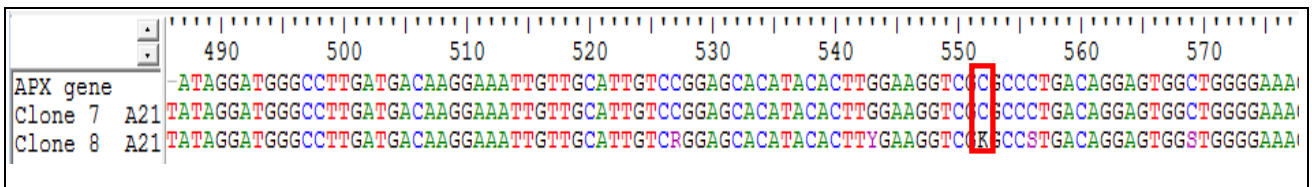


FIGURE 3.4.7: Sequence alignment of *APX* to clones 7 and 8.

For *GSTF6b*, a consensus sequence was found between clone 9 and 12 when aligned to NCBI entry of AJ441055.1 *GSTF6b* gene sequence (Figure 3.4.8) and clone 9 was used in the study.

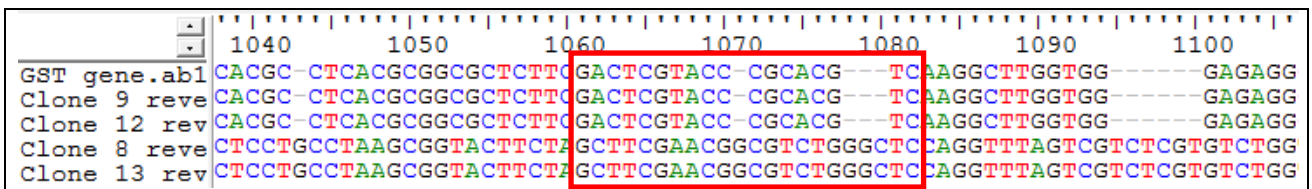


FIGURE 3.4.8: Clustal W alignment of the sequence of clones 8, 9, 12 and 13 to the reference sequence used to obtain a gene fragment from wheat.

3.4.5. Tissue culture and transformation via particle bombardment

In order to test the association of the genes with RWA resistance in wheat, a tissue culture procedure enabling transformation was required. For this, immature seeds (Figure 3.4.9B) were obtained from 11 to 16 days post anthesis (Figure 3.4.9A). Gamtoos-R (*Dn7*) and Gamtoos-S (*Dn0*) cultivars were used to isolate immature embryos (Figure 3.4.9C).

Embryos were pre-cultured on Induction A medium for 4 to 6 days in a dark growth room at 26°C. After 6 days of culturing in the dark, embryonic tissue was bombarded with pUBI plasmids containing the *APX* and *GSTF6b* respectively.

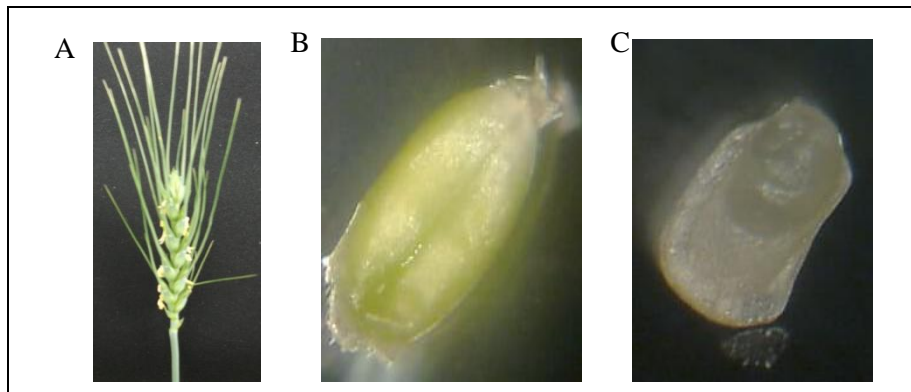


FIGURE 3.4.9: Immature embryos isolated from wheat inflorescence. (A) represents inflorescence of cultivar Gamtoos-R (*Dn7*) at the beginning of anthesis. (B) Entire immature seed before the embryo has been isolated. (C) Representing an isolated embryo that is still intact 11 to 14 days after anthesis.

Bombarded tissue (Figure 3.4.10A) underwent a series of subculturing on different media with various supplements (Table 3.7.1, Appendix A). After bombardment, embryogenic tissue was co-cultured on induction medium for a period of 14 days in a dark growth room. Fourteen days later embryogenic tissue was transferred to the regeneration medium (Table 3.7.1, Appendix A) in a 26°C light growth room and covered with protective screens to avoid direct light.

After 10 days on regeneration medium, embryonic tissue showing first leaf stage (Figure 3.4.10C-F) was subcultured on shoot initiation medium (Table 3.7.1, Appendix A) for a period of 10 days. This was followed by culturing the embryonic tissue on shoot elongation medium (Table 3.7.1, Appendix A) for a period of 14-20 days with direct light exposure in a 26°C growth room. The medium contained the same supplements as that of the shoot initiation medium except no geneticin was added to this medium (Table 3.7.1, Appendix A).

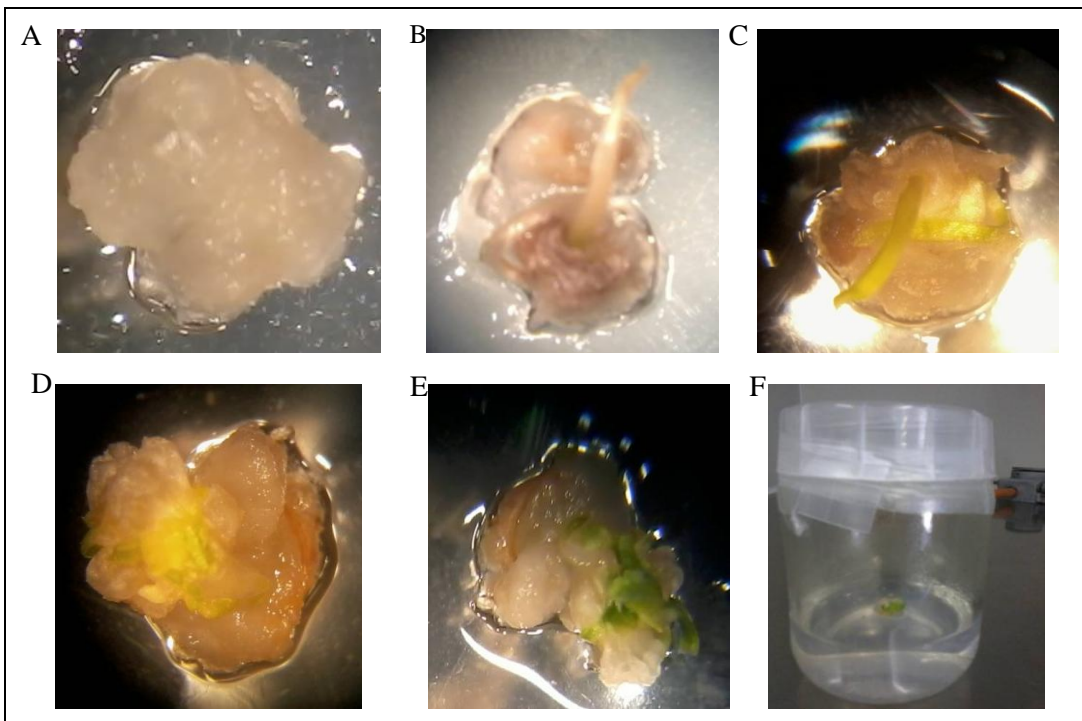


FIGURE 3.4.10: Different stages of tissue culturing: (A) Callus tissue on induction A medium for 14 days. (B-D) Embryonic tissue on regeneration medium for 10 days; (B) and (C) Embryonic tissue differentiating into plantlets, i.e. Gamtoos-S (*Dn0*) and Gamtoos-R (*Dn7*) (D) non-differentiating calli; (E) Plantlet on shoot elongation medium for 14 days in a 90 mm petri dish; (F) Plantlet on shoot elongation medium for 14–20 days in a tissue culture flask.

The next step entailed transfer of the surviving plantlets, with regenerating roots (Figure 3.4.11A-D) on shoot elongation media, to soil (Figure 3.4.12). The soil consisted of sand, palm peat/autoclaved potting soil and vermiculite. Plantlets were placed in a greenhouse with protective screens preventing direct sunlight exposure for a period of three days as shown in Figure 3.4.12.

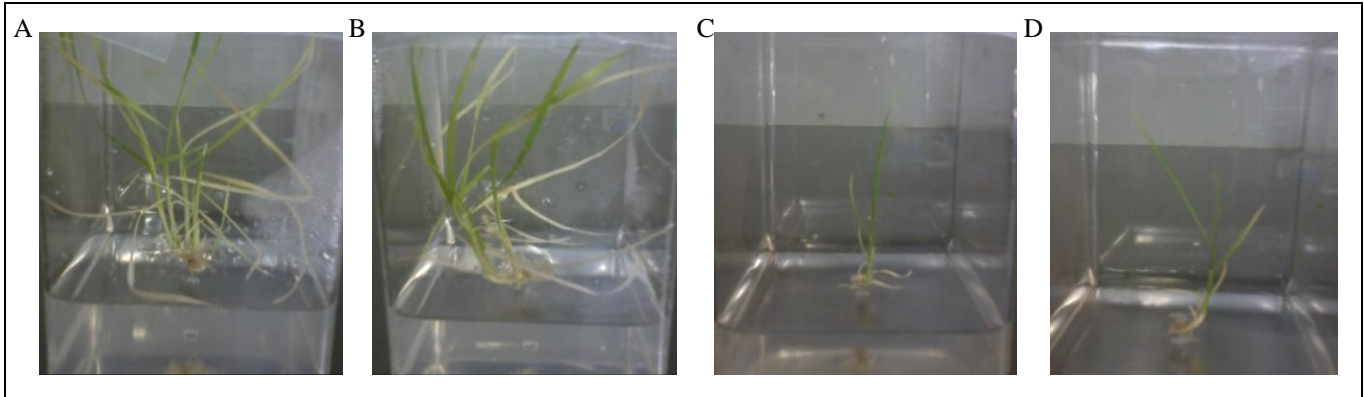


FIGURE 3.4.11: Different stages of plantlets on shoot elongation medium: (A and B) Plant transformed with construct pUBi-GST; (C) Plant transformed with construct pUBI-APX; and (D) Control (tissue culture derived plant).

With Zeatin-rich medium, two plantlets were obtained with a transformation efficiency of 3% (Table 3.4.6). However, no plantlets were obtained with the benzyladenine purine (BAP)-rich medium. Embryogenic tissue bombarded with the silencing construct pUBI-APX instead of pUBi-GST, produced a plantlet on the BAP-rich medium with a transformation efficiency of 2%. When comparing delayed selection against early selection, only bombarded tissue, which underwent the delayed selection process, generated plantlets. When comparing the hormones under these conditions, medium containing zeatin (3%) gave higher transformation efficiency than the BAP-containing medium (2%). Thus the delayed selection process together with the addition of zeatin is very likely to be more preferable for culturing bombarded tissues from Gamtoos-S and Gamtoos-R wheat cultivars

TABLE 3.4.6: Comparing mortality rate of isolated embryos — cultivars Gamtoos-S (*Dn0*) and Gamtoos-R (*Dn7*) — bombarded with constructs pUBI-*GSTF6b* and pUBI-*APX* when cultured on delayed selection

Cultivar	# Embryos isolated	Gene Construct	Regeneration Media ^a		Shoot Elongation Media ^a		Type of Selection	Hormone	% Efficiency
			-	+	-	+			
Gamtoos-S (<i>Dn0</i>)	27	pUBI-GST	22	5	4	1	Delayed	Zeatin	3%
Gamtoos-S (<i>Dn0</i>)	38	pUBI-GST	32	6	6	-	Delayed	BAP	0%
Gamtoos-S (<i>Dn0</i>)	22	pUBI-GST	19	3	3	0	Delayed	BAP	0%
Gamtoos-R (<i>Dn7</i>)	30	pUBI-GST	21	9	8	1	Delayed	Zeatin	3%
Gamtoos-R (<i>Dn7</i>)	44	pUBI-GST	44	-	-	-	Delayed	BAP	0%
Gamtoos-R (<i>Dn7</i>)	20	pUBI-GST	20	-	-	-	Delayed	BAP	0%
Total # plantlets obtained	181		158	23	21	2			
Gamtoos-S (<i>Dn0</i>)	50	pUBI-APX	38	12	12	0	Delayed	BAP	0%
Gamtoos-R (<i>Dn7</i>)	47	pUBI-APX	41	6	5	1	Delayed	BAP	2%
Total # plantlets obtained	97		79	18	17	1			

a. Where “-“ denotes no proliferation and “+” denotes proliferation on media

TABLE 3.4.7: A comparison between the mortality rate of isolated embryos — cultivars Gamtoos-S (*Dn0*) and Gamtoos-R (*Dn7*) — bombarded with construct pUBI-*GSTF6b* when cultured on early selection medium

Cultivar	Isolated Embryos	Gene Construct	Regeneration Media ^a		Shoot Elongation Media ^a		Type of Selection	Hormone	% Efficiency
			-	+	-	+			
Gamtoos-S (<i>Dn0</i>)	40	pUBI-GST	37	3	3	0	Early	BAP	0%
Gamtoos-R (<i>Dn7</i>)	53	pUBI-GST	36	17	17	0	Early	BAP	0%
Total # plantlets obtained	93		73	20	20	0			

a. Where “-“ denotes no proliferation and “+” denotes proliferation on media

In total 371 immature embryos were isolated and bombarded, 310 died on the regeneration medium and 58 died on the shoot elongation medium. From 371 bombarded embryogenic tissues, only three were able to withstand the entire selection process on geneticin. These putative transgenic plantlets are shown in figure 3.4.12.

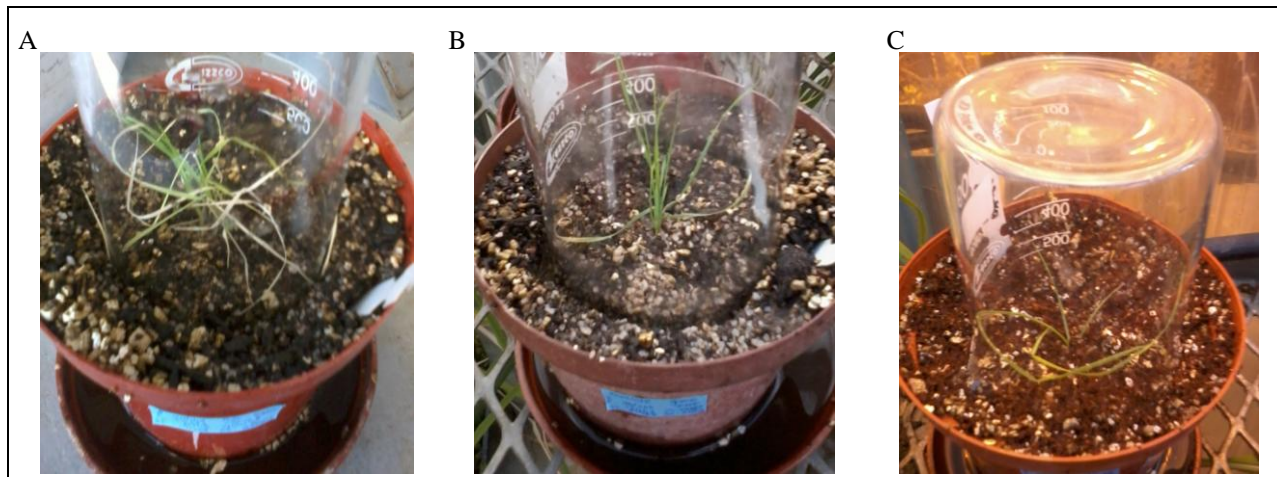


FIGURE 3.4.12: Hardening off: (A) transformed Gamtoos-S (*Dn0*) pUBi-GST; (B) transformed Gamtoos-R (*Dn7*) pUBi-GST; and (C) transformed Gamtoos-S (*Dn0*) pUBi-APX.

After four weeks hardening off, clear phenotypic height differences between the control and transformed plants were observed. The control had a height of 30 cm (Figure 3.4.13) and the putative *GSTF6b* plant was only 14 cm long. This difference in height was also observed between the control and the putative *APX* transgenic plant. The stunted growth was not permanent in the transgenic plants they reach the normal height as non-transgenic plants within eight weeks of hardening off.

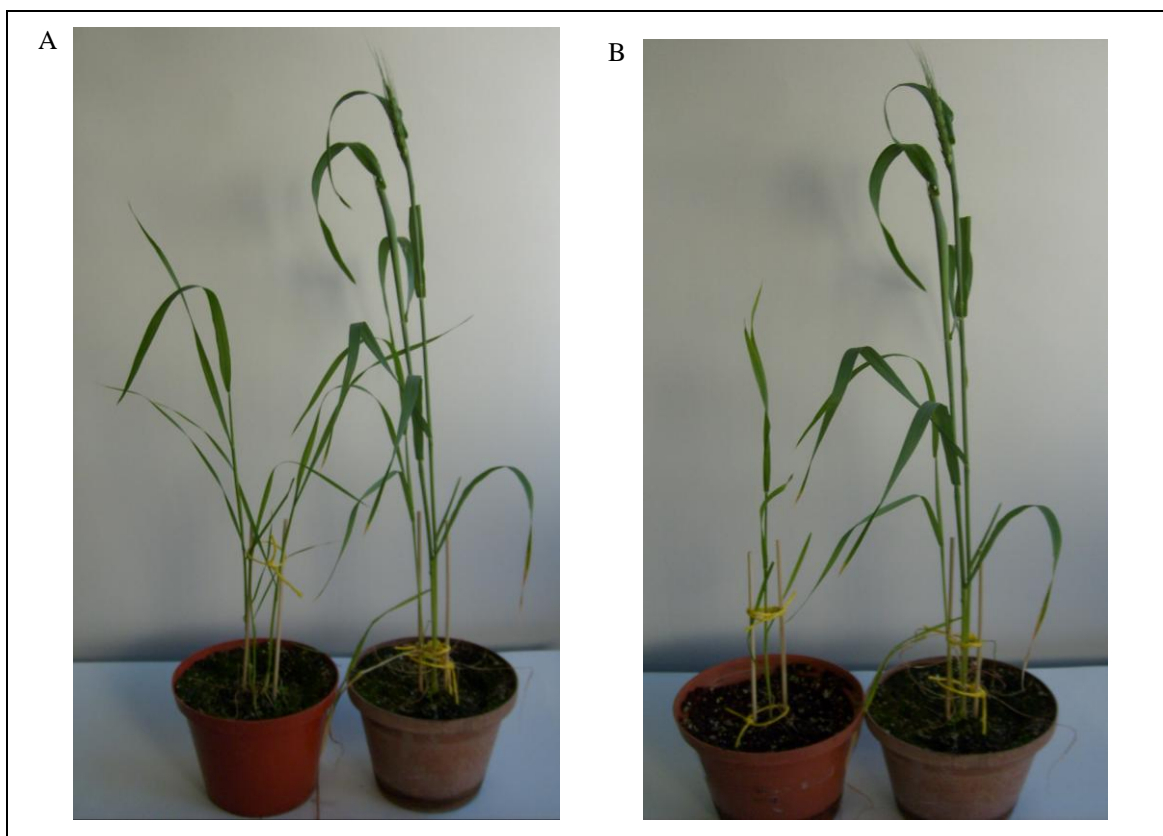


FIGURE 3.4.13: (A) Hardening off: Left: transformed Gamtoos-S (*Dn0*) pUBi-GST, Right: tissue culture derived control Gamtoos-R (*Dn7*); (B) Hardening off: Left: transformed Gamtoos-S (*Dn0*) pUBi-APX, Right: tissue culture derived control Gamtoos-R (*Dn7*).

3.4.6. Aphid infestation analysis

The phenotypical screening 48 hours after RWA infestation indicated significant difference between the controls and the transgenic plant (Figure 3.4.14). Chlorosis was observed in the Gamtoos S (*Dn0*)-APX line, no white and yellow longitudinal streaking of the leaves was observed in the controls.

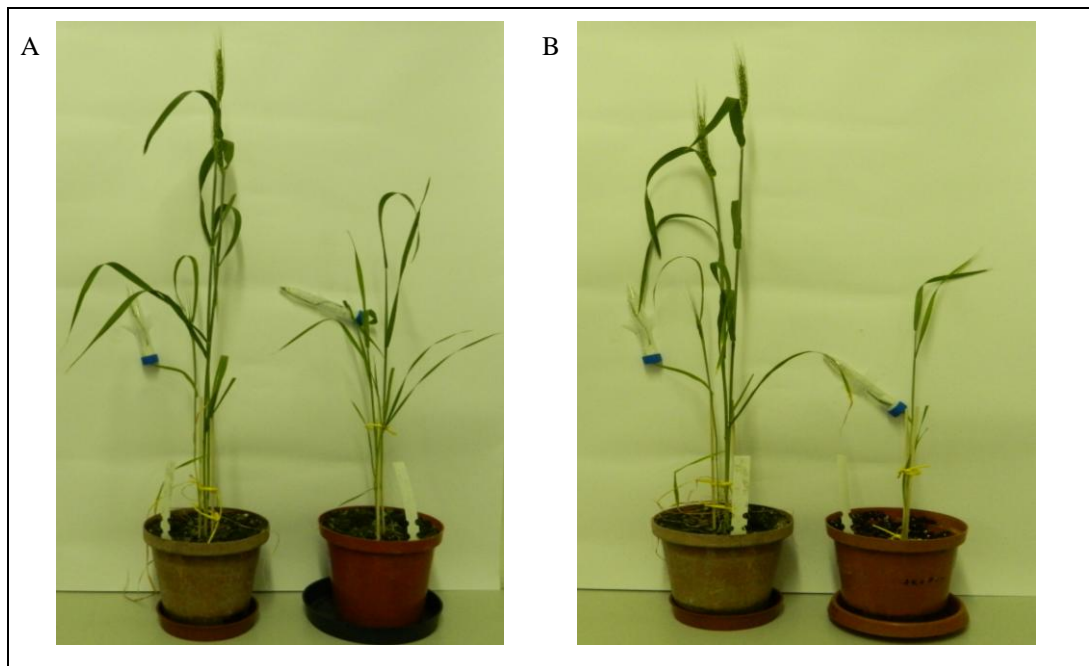


FIGURE 3.4.14: (A) Aphid infestation: Left: tissue culture derived control Gamtoos-R (*Dn7*), Right: transformed Gamtoos-S (*Dn0*) pUBi-GST; (B) Aphid infestation: Left: tissue culture-derived control Gamtoos-R (*Dn7*), Right: transformed Gamtoos-S (*Dn0*) pUBi-APX.

Typical visible symptoms of infestation were observed in the transformed Gamtoos-S (*Dn0*) pUBi-APX, i.e. chlorosis, while leaf rolling was observed Gamtoos-R (*Dn7*) control plant when compared to a leaf without infestation (Figure 3.4.15A). APX primary function in plants is to protect the chloroplast against hydrogen peroxide damage. The fact that chlorosis was observed in the Gamtoos-R (*Dn7*) control plant is an indicator of oxidative burst implying plant susceptibility in the absence of APX.

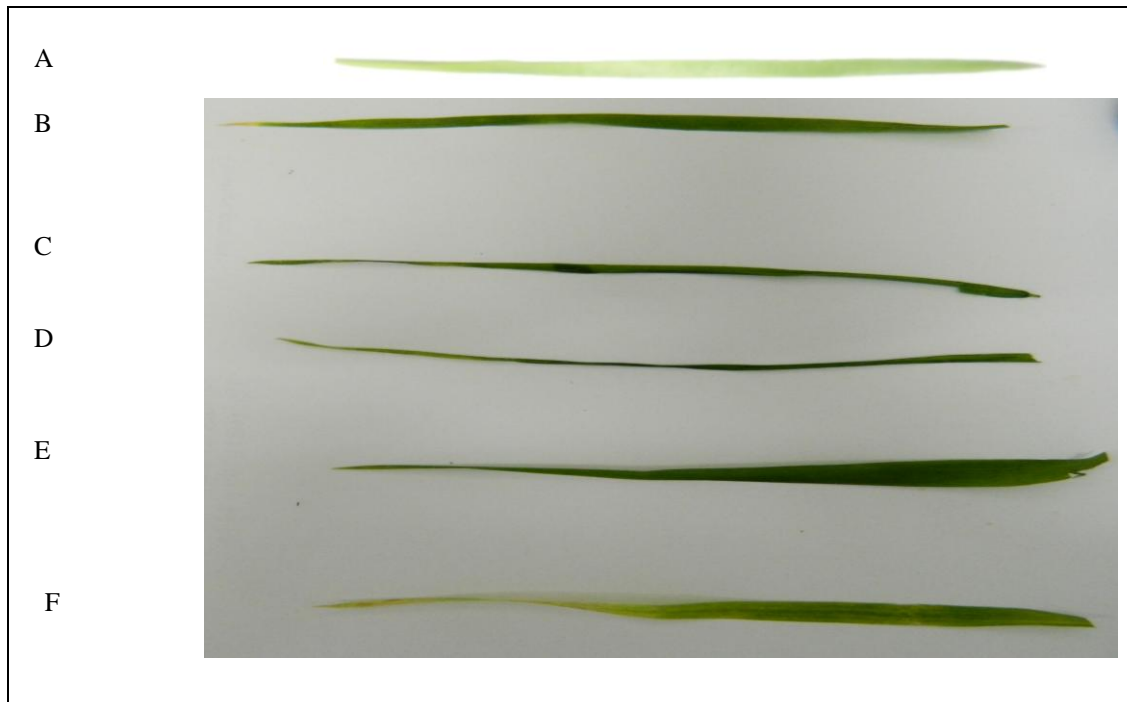


FIGURE 3.4.15: Symptoms before infestation:(A) Gamtoos-S (*Dn0*), Symptoms due to infestation: (B) Gamtoos-S (*Dn0*), (C) Gamtoos-R (*Dn7*), (D) tissue culture-derived control Gamtoos-R (*Dn7*), (E) transformed Gamtoos-S (*Dn0*) pUBi-*GSTF6b*, (F) transformed Gamtoos-S (*Dn0*) pUBi-*APX*.

3.4.7. Quantifying expression levels of transgenes

To validate if these putative transformants were indeed transgenic, the next step was to test for the presence of the transgene in the respective plants. qRT-PCR analyses were performed to test the expression levels of the transgenes with and without RWA infestation. Extracted RNA quality and concentrations were verified using a spectrophotometer (Table 3.4.8).

TABLE 3.4.8: Spectrophotometer measurements of isolated total RNA from uninfested and infested plant material

[]	ng/ μ l	260/280	260/230
Gamtoos-R (<i>Dn7</i>) Tissue culture	407.2	2.07	2.20
Gamtoos-R (<i>Dn7</i>)	58.3	1.65	1.19
Gamtoos-S (<i>Dn0</i>)	43.0	1.31	0.63
Gamtoos-S (<i>Dn0</i>) pUBi-APX	324.3	2.05	2.20
Gamtoos-S (<i>Dn0</i>) pUBi-GST	265.3	2.05	2.15
Gamtoos-R (<i>Dn7</i>) pUBi-GST	323.5	1.48	1.18
Gamtoos-R _{infested} (<i>Dn7</i>) Tissue culture	226.7	2.06	2.23
Gamtoos-R _{infested} (<i>Dn7</i>)	475.4	1.98	2.15
Gamtoos-S _{infested} (<i>Dn0</i>)	291.9	2.01	2.23
Gamtoos-S _{infested} (<i>Dn0</i>) pUBi-APX	350.8	1.99	2.14
Gamtoos-S _{infested} (<i>Dn0</i>) pUBi-GST	326.5	2.06	2.19

The housekeeping gene used for qPCR analysis was the 18S rRNA gene (Table 3.3.3). When comparing the relative *APX* expression measured in the control Gamtoos-S (*Dn0*) to the transformed Gamtoos-S (*Dn0*) pUBi-APX, the control had a relative *APX* expression level of 1.05 when compared to transformed Gamtoos-S (*Dn0*) pUBi-APX plant was only 0.52. Therefore, down-regulation of gene expression was found when compared to the control plant. The transcript level of the transgenic plant is approximately 50% less than that of the control, implying partial silencing in the transgenic Gamtoos-S (*Dn0*) pUBi-APX (Figure 3.4.16).

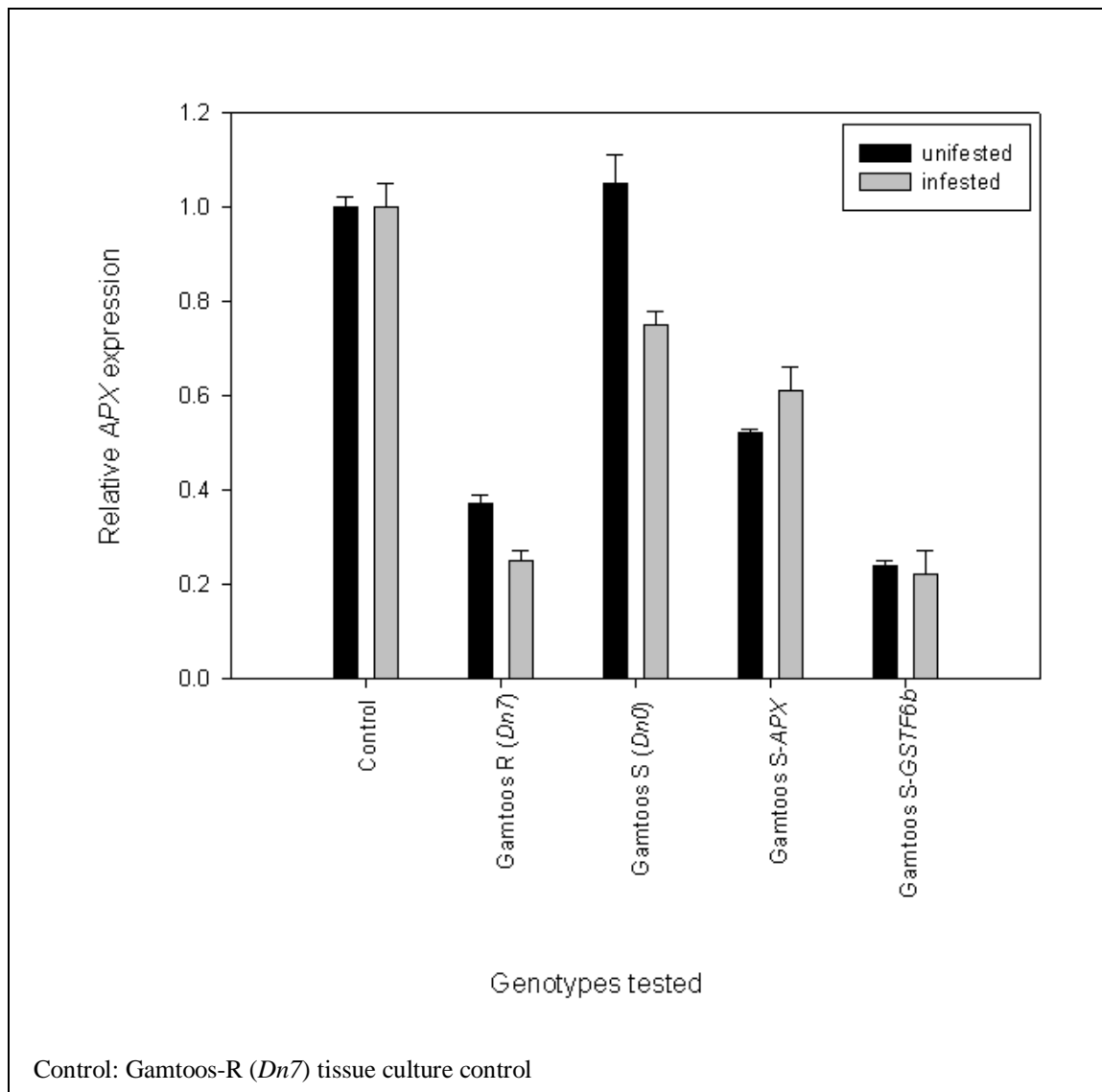


FIGURE 3.4.16: Relative *APX* expression in uninfested and infested transgenic Gamtoos-S (*Dn0*) pUBi-*APX* wheat significantly lower than the Gamtoos S (*Dn0*) control plant.

While after infestation when comparing the relative *APX* expression measured in the infested control Gamtoos-S (*Dn0*) to the infested transformed Gamtoos-S (*Dn0*) pUBi-*APX*, the control had a relative *APX* expression level of 0.78 while that of the transformed Gamtoos-S (*Dn0*) pUBi-*APX* plant was only 0.57. The transcript level of the transgenic *APX* plant were found not to be significantly different from the control material (Figure 3.4.16).

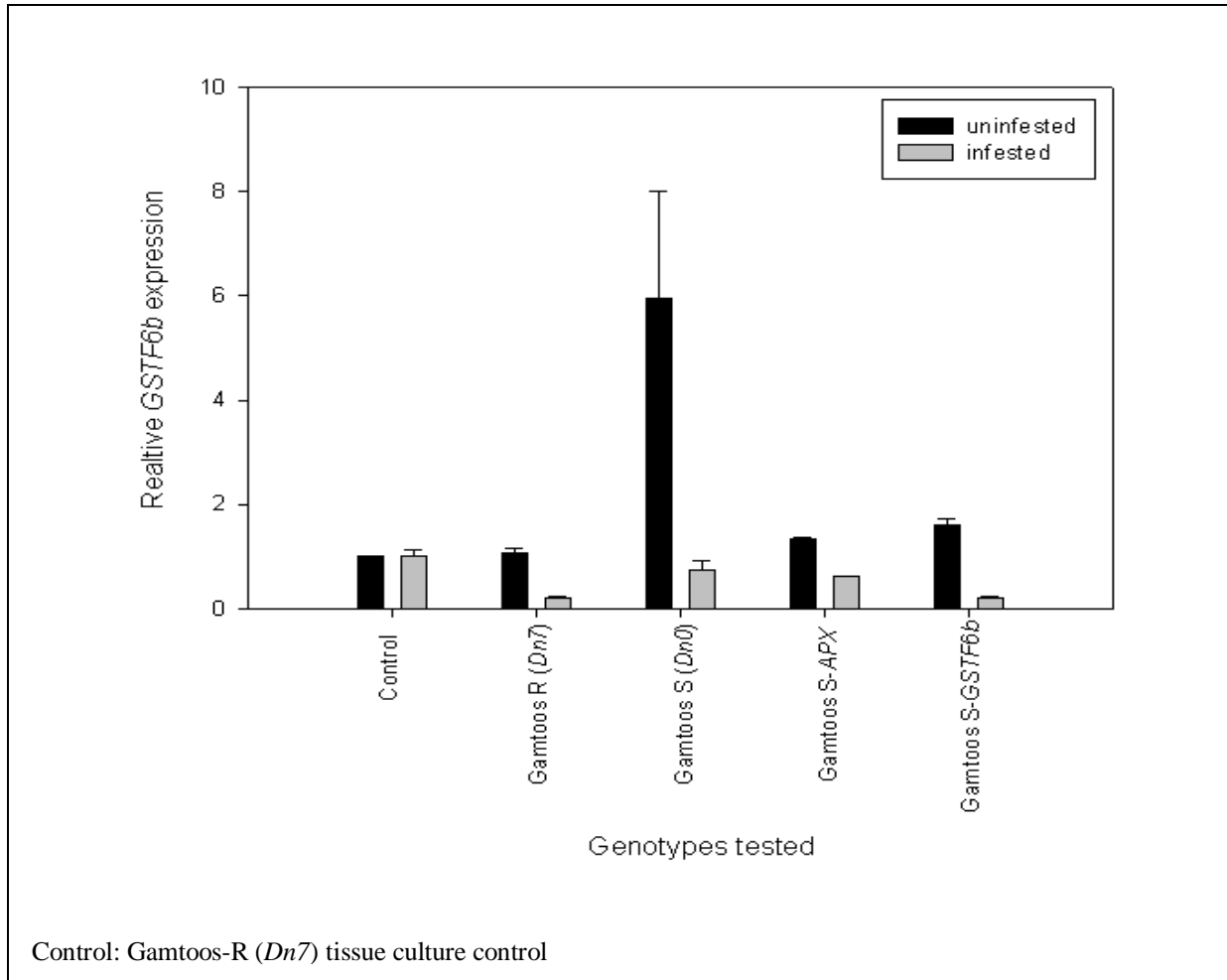


FIGURE 3.4.17: Relative *GSTF6b* expression in uninfested and infested transgenic Gamtoos-S (*Dn0*) pUBi-*GSTF6b* wheat compared to control Gamtoos S (*Dn0*) line.

When comparing the relative *GSTF6b* expression measured in the control Gamtoos-S (*Dn0*) to the transformed Gamtoos-S (*Dn0*) pUBi-*GSTF6b*, the control had a relative *GSTF6b* expression level of 5.96 while that of the transformed Gamtoos-S (*Dn0*) pUBi-*GSTF6b* plant was only 1.62, showing more than 50 % reduction in *GSTF6b* expression in the transgenic Gamtoos-S (*Dn0*) pUBi-*GSTF6b* (Figure 3.4.17).

When comparing the relative *GSTF6b* expression measured in the infested control Gamtoos-S (*Dn0*) to the infested transformed Gamtoos-S (*Dn0*) pUBi-*GSTF6b*, the control had a relative *GSTF6b* expression level of 0.75 (Figure 3.4.17).

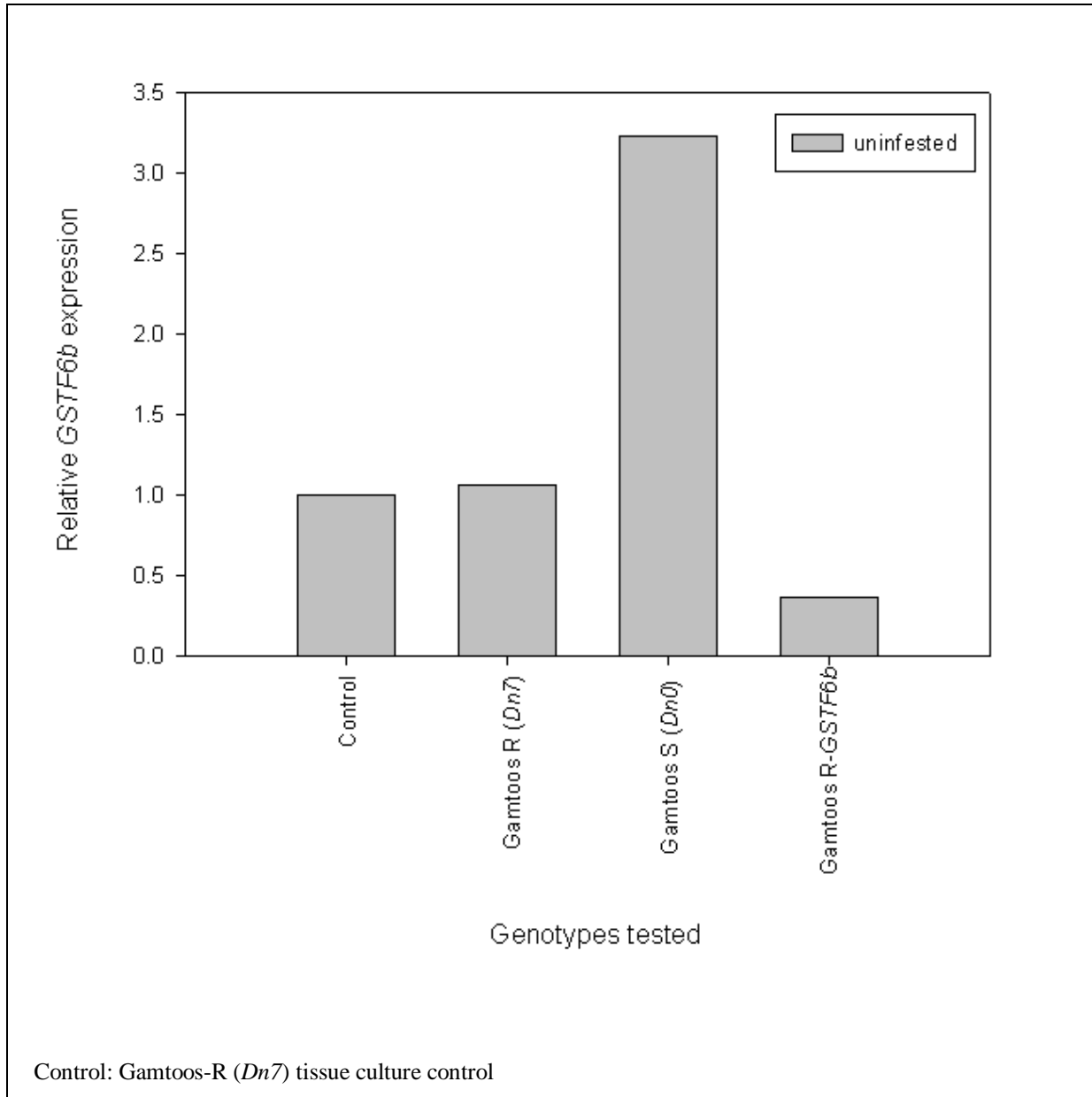


FIGURE 3.4.18: Relative *GSTF6b* expression in the transgenic Gamtoos-R (*Dn7*) pUBi-*GSTF6b* wheat compared to the Gamtoos R (*Dn7*) line.

When comparing the relative *GSTF6b* expression measured in the control Gamtoos-R (*Dn7*) to the transformed Gamtoos-R (*Dn7*) pUBi-*GSTF6b*, the control had a relative *GSTF6b* expression level of 1.00 while that of the transformed Gamtoos-R (*Dn7*) pUBi-*GSTF6b* plant was only 0.36, showing a reduction of approximately 70%, implying partial silencing in the transgenic Gamtoos-R (*Dn7*) pUBi-*GSTF6b* (Figure 3.4.18).

3.5. Discussion

3.5.1. The isolation of genes from the wheat genome

World wide, bread wheat is grown on 17% of all the cultivatable land, making it one of the most important cereal crops in the world (Jones 2005; Xia *et al.* 2011), providing essential proteins, calories and vitamins to the human diet. With a genome size of 16 000Mb of which more than 80% are repetitive DNA fragments (Brenchley *et al.* 2012), the task of isolating genes from allohexaploid species is difficult.

Initially it was attempted to isolate the targeted genes (*APX* and *GSTF6b*) from wheat using designed primers from alignments with existing NCBI sequences (Table 3.4.1). Polymerase chain reactions (PCR) were attempted to isolate targeted genes from the wheat genome. Through trial and error the predicted fragment sizes (600 bp) were obtained but results obtained after sequencing and Blast N homology searches confirmed that the clones did not have correct identity, i.e. *APX* or *GSTF6b*.

Alternative route was therefore followed which has been previously successfully used to isolate *APX* and *GSTF6b* fragments from the wheat genome (Schultz, unpublished). Genes sizes of 350 bp and 250 bp for *APX* and *GSTF6b*, respectively, was confirmed in this study and the correctness of the sequence was further confirmed. The availability of the genes made the process of constructing a silencing construct possible.

3.5.2. Establishing a transformation process

In order to test the association of the genes with RWA resistance in wheat, a tissue culture process enabling transformation was required which was achieved in this study. The practical application of this technique is fundamental in understanding the molecular basis of plant/pathogen/pest interactions to improve agronomically important crops (Gurr & Rushton 2005). It also enables plant scientists to investigate genetically controlled traits of wheat, as in the case in the present study.

For the purpose of cereal transformation the most commonly used tissues are embryogenic suspension cells and embryogenic callus cultures (Jahne *et al.* 1995, Jones 2005). For this study, immature wheat embryos were excised from seeds and used as explant of choice for establishing an effective and

sufficient tissue culture procedure. Following this approach proved to be amenable for transformation (Figure 3.4.9), as previously also reported by Barlett *et al.* (2008).

The optimal stage for embryo isolation found in this study was between 11-16 days post anthesis. This confirms work done by Pastori *et al.* (2001) who also found 11-16 days after anthesis works the best. Working with immature embryos brings about several advantages and isolation of these embryos is easier when harvested from younger plants allowing efficient growth of plants (Jones 2005). The main limitation working with immature embryos is the availability of flowering plants which is seasonal dependent.

Immature embryos developed into embryogenic calli tissue (Figure 3.4.10C) with somatic embryos, which was distinguishably different from non-embryogenic callus tissue. Embryogenic callus was nodular and yellowish in color, and they contained definitive structures (Figure 3.4.9C), whilst non-embryogenic callus tissue was white in color, soft in texture and were enclosed by watery tissue.

3.5.2.1. Medium

The basic composition of tissue culture media for wheat transformations consists of basic MS (Murashige and Skoog, 1962) salts medium together with sugars, vitamins and hormones (Jones 2005). For callus induction, an auxin 2,4-D (2,4-dichlorophenoxyacetic acid), and either zeatin or benzyladenine purine (BAP) are used as cytokinins (Jones 2005). In this study, two types of media were tested, one using zeatin and another containing BAP.

The results showed a higher mortality rate of bombarded embryogenic tissue when cultured on BAP rich medium in comparison to zeatin (Table 3.4.6). Furthermore, two out of three transformed plants were obtained from the zeatin-rich medium and only one from the BAP (Figure 3.4.6). Thus, it is recommended for future work that zeatin might be used as the cytokinin of choice for wheat transformation. The results are not statistically significant, due to the small number of transformants obtained in this study. Therefore, a large number of isolated embryos should be implemented in future studies to verify this result.

3.5.2.2. Promotors

For stable wheat transformation, the cauliflower mosaic virus (CaMV) 35S promotor and the maize ubiquitin (Ubi 1) promotor are the most commonly used promotors (Jones 2005). Although these are the preferred promotors, they have low success rates during silencing experiments with wheat (Jones 2005). For this study, these two promotors were used in combination and resulted in the generation of three genetically modified plants.

3.5.2.3. Transformation efficiency and difficulties associated with wheat transformation via particle bombardment

The entire process of transformation, i.e. from tissue culture establishment to hardening off of transgenic plantlets, is very laborious and time consuming (Li *et al.* 1993). Even though many researchers attempted and practice transformation of bread wheat using immature embryos as explant, the reported average transformation efficiencies remained low between 1-5% (Jordan 2000; Abdul *et al.* 2010). This is mainly ascribed to bread wheat's large genome size and the fact that wheat is a monocot, and thus recalcitrant to *Agrobacterium* transformation (Abdul *et al.* 2010).

Even though the transgenic approach is technically challenging in wheat (Manmathan *et al.* 2013), this study, has succeeded in the establishment of a tissue culture and transformation process and produced three putative transgenic plants. These were successfully hardened off from which seeds were obtained and are presently growing the T₁ generation of Gamtoos S (*Dn0*)-pUbi-*GSTF6b* and Gamtoos R (*Dn7*)-pUbi-*GSTF6b* plants no seeds were obtained from the Gamtoos S (*Dn0*)-pUbi-*APX* line. In order to optimize this technique the amount of immature embryos isolated should be increased which could possibly lead to an increase in transgenic plants obtained in future studies.

3.5.2.4. Particle bombardment as a relevant technique for knockout studies

Particle bombardment has proven to work in this study and generated three transformed plants. Several disadvantages are still related to this technique which include a genotype-dependent transformation efficiency of 10% (Lacock *et al.* 2003, Xia *et al.* 2011). Studies conducted by Lacock and Botha (2001) and also Jordan (2000) have found that the largest limitation with particle bombardment is that higher efficiencies can only be obtained with certain genotypes (i.e. Florida and Fielder). Additionally, complex transgene integration patterns are found which leads to analysis problems (Jones 2005).

With this study this was confirmed, initially a few cultivars (such as Tugela, Tugela *Dn1*, Palmiet and Yumar) were introduced *in vitro* with limited cultivars surviving the process with Gamtoos having the highest *in vitro* regeneration ability (le Roux unpublished results). Therefore, spring wheat cultivars are thought to be suitable plant material to be implemented in future studies. The challenges affecting wheat transformation, especially for commercial purposes, include direct transformation of commercial cultivars, rapid regeneration protocols, marker-free regenerated plants and single gene insertion with no extra foreign DNA (Bhalla 2006). This study has contributed to the above-mentioned statement in the sense that the transgenic plants are marker-free, it is a rapid regeneration protocol and no extra foreign DNA was inserted into the plants as in the case of *Agrobacterium* transformation.

3.5.3. qPCR analysis of R_0 plants

qPCRs were performed on transgenic plants before and after infestation to confirm the degree of silencing of ascorbate peroxidase (*APX*) and glutathione S-transferase (*GSTF6b*). One of the most powerful mechanisms by which plants can prevent pest/pathogen attacks is the hypersensitive response (HR) (Pellegrineschi 2005). The HR is triggered by both biotic and abiotic threats (Danna *et al.* 2003) which leads to the activation of ascorbate peroxidase (*APX*) and glutathione S-transferase (*GSTF6b*) (Ishikawa & Shigeru 2008), which are antioxidants that play a major role in the removal of reactive oxygen species (ROS) (Danna *et al.* 2003). These ROS scavenging mechanisms protect cereals against pest/pathogen attacks (Pellegrineschi 2005) and prevent peroxide damage (Dalton *et al.* 1986).

In this study, qPCR analysis conducted on the Gamtoos-S-pUbi-*APX* transgenic plant before infestation indicated lower levels of *APX* activity than in the susceptible control plant. When comparing the resistant and susceptible controls, lower levels of *APX* were found in the resistant control than in the susceptible control before infestation. Aphid infestation after 48 hours caused an increase in *APX* activity in the transgenic susceptible Gamtoos-S-pUbi-*APX* while lower *APX* transcript levels were present in the Gamtoos-S-pUbi-*GSTF6b*. This was expected since the Gamtoos-S-pUbi-*GSTF6b* line does not contain the *APX* gene silencing construct. Higher *APX* levels were found in the Gamtoos-S-pUbi-*APX* in comparison to the susceptible control. This implies that gene silencing has failed because with silencing the transcript amount is supposed to decrease and not increase. This can be ascribed to antisense gene silencing which generates levels of silencing and not complete gene silencing. Furthermore, the resistant control plant contained higher levels of *APX* activity when compared to the susceptible control plant post infestation.

Lukasik *et al.* (2012), tested ascorbate peroxidase content in aphid infested *Triticale*, also found higher levels of *APX* content in resistant cultivars than the susceptible cultivars (Lukasik *et al.* 2012). Further, Mittler *et al.* (1999) suppressed the activity of *APX* in tobacco plants by introducing antisense *APX* gene fragments into the plant and found that plants were more sensitive to pathogen attack. Resistant plants, expressing more *APX* activity, are very likely better equipped to withstand pest attacks by enhancing the defensive related genes. Furthermore, susceptible plants have more *APX* activity before infestation than after infestation. Therefore, it correlates to our study because upon stress higher levels of *APX* must be present, while upon little stress less *APX* must be found in cells. The results implies that a decrease in *APX* activity may be related to susceptibility of wheat to Russian wheat aphid feeding.

3.5.3.1. qPCR analysis of Gamtoos-S-pUbi-*GSTF6b* before and after infestation

qPCR analysis of the Gamtoos-S-pUbi-*GSTF6b* transgenic plant before infestation contained lower amounts of *GSTF6b* transcripts in comparison to Gamtoos-S control plant. The resistant control plants had also a lower amount of *GSTF6b* transcripts than the Gamtoos-S-*GSTF6b* plant and the Gamtoos-S-*APX* plant. After aphid infestation, higher amounts of *GSTF6b* transcripts were found in the resistant control than in the susceptible control plant and less *GSTF6b* transcripts was found in the susceptible Gamtoos-S cultivar than in the control Gamtoos S (*Dn0*) plant. The interesting result of this qPCR analysis showed higher levels of *GSTF6b* in the Gamtoos-S-pUbi-*APX* plant than Gamtoos-S-pUbi-*GSTF6b* transgenic plant after infestation. The opposite was suppose to be observed due to the fact that the Gamtoos-S-pUbi-*APX* does not contain the *GSTF6b* gene construct. Furthermore, considerable decrease levels of *GSTF6b* in the Gamtoos-S-pUbi-*GSTF6b* plant after infestation was observed. Implying partial silencing of the Gamtoos-S-pUbi-*GSTF6b* plant although no visible symptoms of infestation was observed after infestation.

3.5.3.2. qPCR analysis of Gamtoos-R-pUbi-*GSTF6b* before infestation

qPCR analysis of the Gamtoos-R-pUbi-*GSTF6b* transgenic plant before infestation showed significant lower levels of *GSTF6b* in the transgenic plant when compared to the resistant control plants. While higher levels of *GSTF6b* were found in the susceptible control plant. Out of the three transgenic plants the Gamtoos-R-pUbi-*GSTF6b* were the most highly silenced plant with silencing percentage of 70%. Implying partial silencing of the Gamtoos-R-pUbi-*GSTF6b* plant. No phenotypic symptom could be ascribed to the silencing of *GSTF6b* in Gamtoos-R. According to literature, GST's primary role is to prevent or minimize oxidative stress (Frova 2003). Therefore, in the absence of the gene a

disease phenotype would be expected but this was not the case with this study but on a gene expression level decrease gene transcripts were found.

3.5.4. Aphid-cereal interaction

Plant productivity is largely affected by environmental stress which is associated with oxidative damage as a result of increased production of ROS at the cellular level (Le Martret *et al.* 2011). RWA feeding causes a variety of chemical and defensive response to be triggered in the host plant. Susceptible cultivars are more prone to phloem feeding insects infestation causing chloroplast-breakdown giving rise to chlorosis (Smith *et al.* 1991; Botha *et al.* 2005), which is one of the most frequently found symptoms upon infestation.

Other alterations also includes white and yellow longitudinal streaking of the leaves (Botha *et al.* 2005; Franzen *et al.* 2007), necrosis and impairment of new growth (Lukasik *et al.* 2012), in comparison to resistant cultivars which are more reluctant to infestation. Plants have developed many defensive mechanisms which include cell wall modifications, hypersensitive induced cell death and plant volatiles (Lukasik *et al.* 2012).

3.5.4.1. Phenotype of R₀ transformants

Initially phenotypically smaller transgenic plants were obtained after four weeks of hardening off when compared the control plants. Stunted growth was ascribed to silencing but after eight weeks of hardening off the transgenic plants were exactly the same height as the control plants. Normal development and flowering were found in the transgenic plants as in the case of non-tissue culture wheat plants. Two of the three transgenic plants were able to produce seeds i.e. Gamtoos-S-pUbi-*APX* (no seeds) Gamtoos-S-pUbi-*GSTF6b* (34 seeds) and the Gamtoos-R-pUbi-*GSTF6b* (1 seed).

With RWA feeding, phenotypic symptoms associated with infestation was observed in infested controls and transformed plants. Typical symptoms of infestation i.e. chlorosis were displayed especially in the Gamtoos-S-pUbi-*APX*- transgenic plant. In literature, *APX* is responsible for protecting the chloroplast and other cells from peroxide damage. Therefore, in the absence of *APX* it is expected to observe a susceptible phenotype. The observed phenotypic symptom confirms gene involvement in protecting the photosynthetic machinery of the plant (Murtaza *et al.* 2011). In a recent study by Zaaerman *et al.* (2009) chlorosis, as a consequence of infestation, was also found in Gamtoos

susceptible wheat plants upon RWA feeding. Maccdo *et al.* (2003) further found significant reduction of chlorophyll in susceptible plants upon *D. noxia* infestation under continuous light.

In conclusion, in this study, it was shown that susceptible plant phenotypes were observed in the absence of the *APX* gene and not the *GSTF6b*. *APX* and *GSTF6b* are candidate genes associated with the hypersensitive response activated by *Dn1* mediated resistance and not necessarily by *Dn7* mediated resistance. This could possibly be why no phenotypic disease symptom can be associated the *GSTF6b* gene. Therefore, in future studies these two genes should be silenced in a *Dn1* background. Due to time constraints the T₁ generation was not tested to determine transgene integration but will be analysed further in future studies.

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3.7. Appendix A

3.7.1. Embryo isolation: 4 -6 days prior to bombardment

Embryos were isolated using autoclaved forceps and scalpels from immature seeds as explants. Embryos were placed on Induction A medium (refer to Tables 3.7.1 and 3.7.2 for composition of the media) and sealed with parafilm tape for 4 to 6 days and incubated in a dark growth room at 26°C.

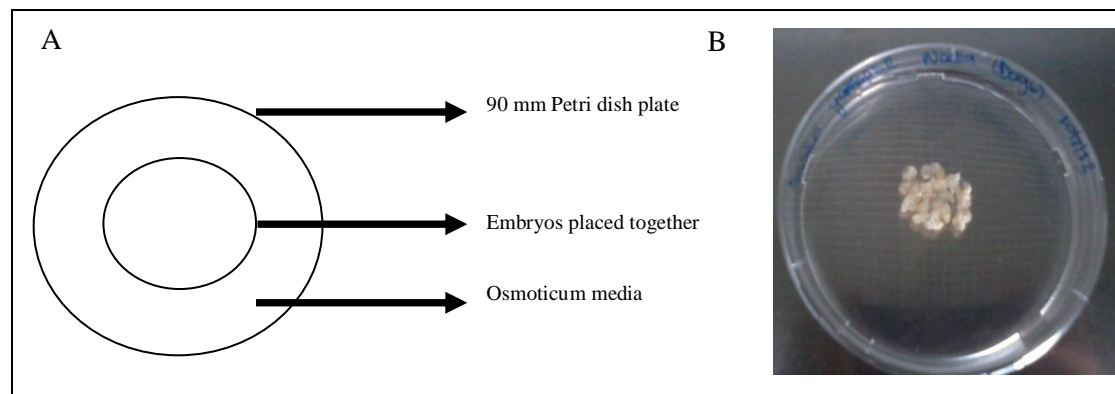


FIGURE 3.7.1: a) Schematic representation of callus tissue on osmoticum medium placed in the centre. b) A actual picture of callus tissue on medium 4 hours prior to bombardment.

3.7.2. Pre-bombardment

Four hours prior to bombardment 4 to 6 days old embryogenic tissue were placed on osmoticum medium (Table 3.7.1 and 3.7.2), embryogenic tissue were arranged in a 25 cm diameter petri dish as shown in figure 3.7.1. Embryogenic tissue were then stored in a dark growth room at 26°C for four hours.

TABLE 3.7.1: Different tissue culturing media for delayed selection

Components	Induction A	Osmoticum	Regeneration	Shoot initiation	Shoot Elongation
MS	2.2 g	2.2 g	1.25 g	1.075 g	1.075 g
Sucrose	20 g	20 g	10 g	7.5 g	7.5 g
Casein	50 mg	50 mg	-	-	-
L-Glutamine	250 mg	250 mg	-	-	-
2.4 D	5 mg/ml	5 mg/ml	-	-	-
Gelrite	1.1 g	1.1 g	1.1 g	1.25 g	1.25 g
Picloram	5 mg/ul	5 mg/ul	-	-	-
D-Manitol	-	9.1 g	-	-	-
D-Sorbitol	-	9.1 g	-	-	-
Geneticin	-	-	1.25 mg/ml	1.25 mg/ml	-
Zeatin/ BAP	-	-	2.5 mg/ml	-	-
Time period	4-6 days	16-20 hours	10 days	8-10 days	14-20 days

a. Where “-“ denotes when chemicals has not been added

b. Solution were made up to 500 ml

TABLE 3.7.2: Different tissue culturing media for early selection

Components	Induction A	Osmoticum	Regeneration	Shoot initiation	Shoot Elongation
MS	2.15 g	2.2 g	2.15 g	1.075 g	1.075 g
Sucrose	10 g	20 g	10 g	7.5 g	7.5 g
Myo-inositol	50 mg	-	50 mg	50 mg	50 mg
2.4 D	10 mg/ml	5 mg/ml	-	-	-
Gelrite	1.25 g	1.25 g	1.1 g	1.25 g	1.25 g
Picloram	-	5 mg/ml	-	-	-
D-Manitol	-	9.1 g	-	-	-
D-Sorbitol	-	9.1 g	-	-	-
Geneticin	-		1.25 mg/ml	1.25 mg/ml	-
Zeatin/ BAP	-		2.5 mg/ml	-	-
Time period	4-6 days	16-20 hours	10 days	8-10 days	14-20 days

a. Where “-“ denotes when chemicals has not been added b. Solution were made up to 500 ml

3.7.3. DNA precipitation

The process of DNA precipitation started with sterilising 10 mg tungsten (GradeM- 10, Bio-Rad # 165-2266) with 300 µl 100% absolute ethanol. Followed by vortexing and centrifuging the tungsten/ethanol mixture in a microfuge for ~ 30 s. The top layer of the mixture was then removed containing the ethanol, followed by resuspending the tungsten pellets in 300 µl dsH₂O. The next step was then to vortex and centrifuge the resuspended mixture, repeating this step three times.

After the washing step, the sterilised tungsten was resuspended in 100 µl dsH₂O, the sterilized tungsten was divided into two equal volumes of 50 µl per 1.5 ml eppendorf tube. The next step was then to add all the components together resulting in the precipitation mix as indicated in table 3.7.3 below.

TABLE 3.7.3: Components of the precipitation mix used for bombardment

[]	Components	Volume added
10 mg/50 µl in dH ₂ O	Tungsten	50 µl
1 ug/µl	DNA (pUbi 510-APX / pubi 510-GSTF6b)	5 µl
1 ug/µl	pEmuKN	5 µl
2.5 M	CaCl ₂	50 µl
0.1 M in dH ₂ O	Spermidine	20 µl

The precipitation mix contains the vector with the gene interest (1 ng/µl) and the selectable marker *nptII*). Stock solutions of both spermidine and CaCl₂ were prepared before hand and stored at -20°C and 4°C respectively. Spermidine (0.1 M) and CaCl₂ (2.5 M) was added simultaneously to the mixture while vortexing, followed by incubating on ice for 5 min before usage.

3.7.4. Bombardment

The 4 to 6 day old embryogenic tissue on osmoticum medium were placed inside the bombardment chamber on a grid at a distance of 16.5 cm. From the mixture prepared in 3.7.3, 100 µl of the supernatant from the settled precipitation mix was removed and discarded. The remaining solution was mixed rapidly several times, ensuring thorough dispersion of the particles.

Dispersed tungsten DNA preparation (5 μ l) was placed in the centre of the support screen in a 13 mm macrocarrier which were screwed onto the chamber. A gas pulse was released when a pressure of 84-85 kPa was reached resulting in the discharge of the particles into the target tissue. The shattered callus tissue was placed back onto the same osmoticum medium used for bombardment for 16- 20 h at 26°C in a dark growth room.

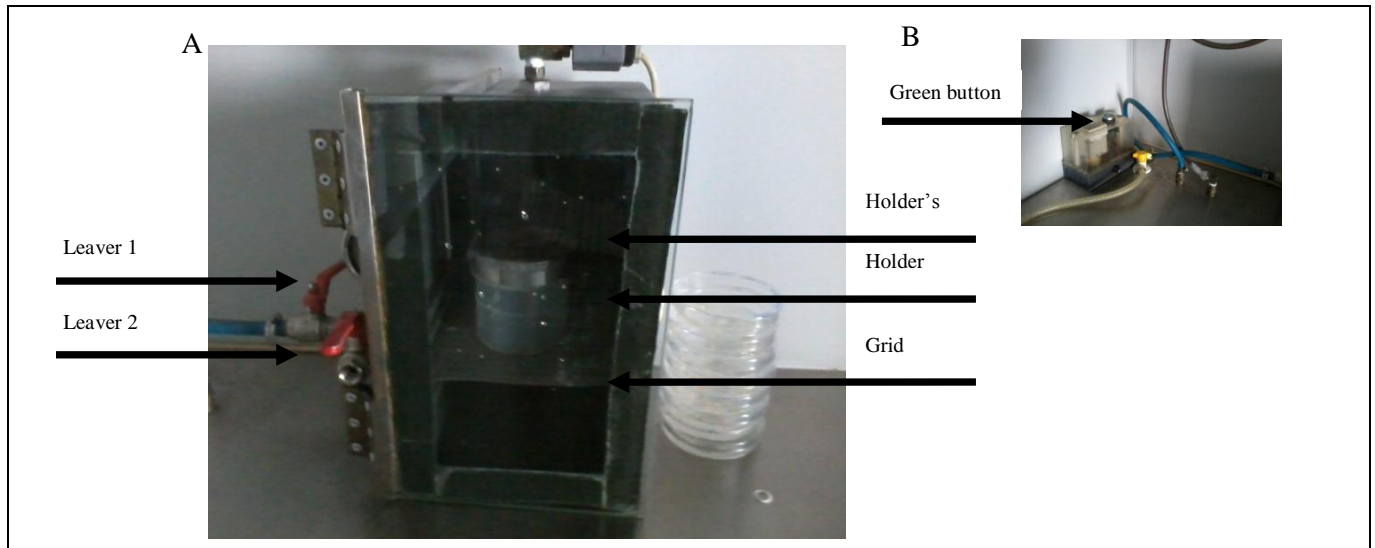


FIGURE 3.7.2: a) Showing all the different components of the particle bombardment gun. b) The green button used to accelerate the particle (DNA) in the explant (callus tissue).

3.7.5. Post-bombardment

After being 20 h on osmoticum medium, the embryogenic tissue were transferred to Induction A medium sealed with parafilm tape and placed in a dark growth room at 26°C for a period of 14 days. After being cultured in the dark for 14 days, the callus tissue were transferred to regeneration medium (Table 3.7.1 and 3.7.2) for selection. Embryogenic tissue were left on the medium for 8-10 days at 20°C in a light growth room covered with protective screens to avoid direct light.

After 10 days on regeneration medium, embryogenic tissue showing first leaf stage were subcultured to shoot initiation medium on a 100 mm petri dish. Plantlets were cultured for 14 days on this medium with direct light (16 hours light/ 8 hours dark) with no protection in a 26°C growth room. The next step was then to transfer the surviving plantlets to shoot elongation medium using tubes, instead of 100 mm petri dishes, for a period of 14-20 days. The medium contained the same supplements as the shoot initiation medium except no geneticin was added to the medium.

Chapter 4

***In situ* hybridization of Repulsion phase marker *Dn9*, *APX* and *GSTF6b* in hexaploid wheat**

4.1. Abstract

Genome analysis of bread wheat is complicated by its large genome of ~17 Gbp with highly repetitive regions. This creates great challenges for gene discovery and general wheat improvements. The Russian wheat aphid (*Diuraphis noxia*, Kurdjumov RWA) is a serious pest of wheat and barley (*Hordeum vulgare* L.) and feeding induces a variety of developmental, morphological, physiological and biochemical responses i.e. host defence. Host defence occurs at the infested site, referred to as the hypersensitive response (HR). Activation of the hypersensitive response releases essential anti-oxidant enzymes such as ascorbate peroxidase (APX) and glutathione S-transferase (GST). The objective of this part of the study was to establish the distribution and abundance of the targeted genes (i.e. *APX*, *GSTF6b* and *Dn9* marker) in the hexaploid genome using fluorescent *in situ* hybridization (FISH). Wheat material of both spring and winter varieties, which were either susceptible or resistant near isogenic lines (NILs), were utilized. And genomic DNA was fluorescently labelled with Texas red to detect targeted genes using Texas red. Two types of methods were used to obtain metaphase chromosomes i.e. chromosome squashes and chromosome suspensions, and chromosome squashes using roots of 3 cm generated the best results. Fluorescence could only be observed with chromosome suspensions but no fluorescence could be detected with the chromosome spreads. This technique therefore requires further optimization.

4.2. Introduction

The nuclear genome is organised into subunits referred to as chromosomes, which are found in all living organisms including humans, animals and plants (Dolezel *et al.* 2012). Although they are organised in the same manner, the complexity of genomes differs between organisms. This is largely ascribed to repetitive and duplicated DNA sequences found mostly in plants and animals (Dolezel *et al.* 2012).

Bread wheat (*Triticum aestivum* L.) is one of the major agricultural crops which have a very large and complex nuclear genome of almost ~17 Gbp (Bennet & Smith 1991). It is an allopolyploid species which contains three closely related genomes denoted A, B and D (Gill *et al.* 2004; Brechley *et al.* 2012) with two or more structurally similar chromosome sets (Dolezel *et al.* 2012). The genome of wheat is found to be a hundred fold larger than that of *Arabidopsis*, with a single wheat chromosome larger than the entire rice genome (Goff *et al.* 2002). Even though wheat is a allohexaploid possessing many unique features, the complex genome structure makes the search for gene discovery difficult (Dolezel *et al.* 2012).

The complexity of the wheat genome hampers the construction of physical maps being generated (Schwarzacher 2003; Dolezel *et al.* 2012). To date, different methods have been employed to determine the physical location of genes or DNA sequences but the most frequently used method is fluorescent *in situ* hybridization (FISH) (Dolezel *et al.* 2012). FISH is a standard method used for visualizing spatial distribution of targeted genes using probes (Lecuyer *et al.* 2008). This is a powerful technique for pinpointing the physical location of genes or DNA sequences on a chromosome, making it easier to group specific linkage groups to specific chromosomes (Schwarzacher 2003). This technique holds several advantages which include the ability to reconstruct three dimensional images using high resolution microscopy; it is also able to overlap multiple signals (Lecuyer *et al.* 2008). FISH employs fluorochrome conjugated probes or antibodies (Lecuyer *et al.* 2008). The most commonly used cytological targeted tissue for FISH mapping is the mitotic metaphase chromosomes. Metaphase chromosomes are obtained from roots, which contain actively dividing cells, and are readily available for most plant species (Jiang & Gill 2006).

Reactive oxygen species (ROS) production is elevated by both biotic and abiotic threats that cause cell damage. This is combated by ROS scavenging mechanisms *APX* and *GSTF6b* which is activated to ensure plant survival and productivity preventing peroxide damage (Belkhadir *et al.* 2004).

Therefore, the objective of this study was to determine the distribution, abundance and location of these genes in the wheat genome using fluorescent *in situ* hybridization (FISH).

4.3. Research Methodology

4.3.1. Plant material

Material used in this study included wheat seedlings from both winter and spring wheat varieties, i.e. Gamtoos-R, Gamtoos-S, Betta, Betta *Dn*, Betta *Dn2*, Betta *Dn9*, Tugela and Tugela *Dn1*, which was all utilized for fluorescent *in situ* hybridization.

4.3.2. DNA extraction

A DNazol (Molecular Research Centre Cincinnati, USA) protocol was used to isolate genomic DNA from wheat leaves. The DNA concentration of each sample was quantified using a ND-1000 Nanodrop spectrophotometer (NanoDrop Technologies) and resolved on a 1% agarose gel. Samples were then diluted to 25 ng/μl for PCR amplification.

4.3.3. Amplification

Three primer pairs (Table 4.3.1) were used in this study. Amplification reactions were conducted in a total volume of 20 μl containing DNA as template (25 ng/μl) together with the following components: 0.2 mM dNTPs (Fermentas, Cat no. FERo181), 10x buffer (Roche, Cat no. JMR-455) with a working stock of 1X, 2.5 mM MgCl₂ (Roche, Cat no. JMR-456), 10 pM primers and 5 U SuperTherm *Taq* Polymerase (Crimson *Taq* DNA polymerase from Bio Labs, Cat no. JMR-801). The Gene Amp 9700 (Applied Biosystem) thermo cycler was used for incubation, with a hot start amplification at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, various annealing temperatures (Table 4.3.1) for 30 s, and 72°C for 2 min, with a final DNA extension at 72°C for 7 min before cooling to 4°C. Amplification products were resolved by electrophoresis on 1.5% agarose gels (SeaKem® LE Agarose) at 80V (Bio-Rad Power Pac 1000) for 75 min in 1X Tris acetate (TAE) buffer (40 mM Tris acetate and 1mM ethylenediaminetetraacetic acid (EDTA) pH 8. Before samples were loaded onto a gel, the samples, DNA ladder and GR green stain (<http://thermoscientific.com/fermentas>) were denatured at 98°C for 3 min in the thermocycler. The Multi Genius Bio Imaging system (SynGene) and UV light were employed for visualisation.

TABLE 4.3.1: Primers used to amplify fragments to label probes

Primer #	Name	Sequence (5'-3')	T _m	Primer purpose
10/7	APX-L	CCACGTTTACCTGCTGAACA	55.4°C	PCR
10/8	APX-R	CGCAGCATCTGTAGGCAATA	55.1°C	PCR
10/17	GSTF6-L	CCTCAACCACTTCCCCTACA	56.3°C	PCR
10/18	GSTF6-R	ATTTGCCTTATTCGCGACAC	53.8°C	PCR
11/98	Amo <i>Dn9</i> -L	GCTGAGATAACCATAGATAAT	46.7°C	PCR
11/99	Amo <i>Dn9</i> -R	AGAGAGGCTAAGAGTGAGC	53.3°C	PCR

4.3.4. Probe Preparation

PCR products (*APX* and *GSTF6b*) was labeled with a 3' EndTag DNA Labeling system kit (Vector laboratories), according to the manufacturer's instructions. The reaction contained DNA as template (60 ng) together with the following components: 2 µl 10X TdT reaction buffer, 2 µl SH-GTP, 2 µl TdT in a total volume of 20 µl. The reaction was incubated at 37°C for 30 min, followed by 65°C for 30 min. Incubation was conducted in a Gene Amp 9700 (Applied Biosystem) thermocycler.

4.3.5. Chromosome squashes

4.3.5.1. Sample collection for chromosome squashes

4.3.5.1.1. Root sampling and Fixation

Seeds of each respective cultivar were germinated on moist filter paper in a petri dish and incubated at 25°C for a period of 3 days. Roots, which were 3 cm in length, were cut and stored in cold distilled water for a period of 29 hrs which was followed by fixation in methanol: proponic acid (3:1) between 1 to 13 days.

4.3.5.1.2. Embryo sampling and Fixation

Immature embryos were isolated from seeds of respective wheat cultivars, which were cultured on MS medium (2.2 g Murashige and Skoog, 20 g sucrose, 50 mg casein, 250 mg L-glutamine, 5 mg/ml 2.4D, 1.1 g gelrite and 5 mg/ml picloram) for a period of 10 days. After 10 days, embryos were collected and treated with ice water for 24 h at 4°C. After fixation, roots were stained with Feulgen solution (1 g fuchsin, 3 g potassium meta bisulfite, 30 ml 1N HCl in a total volume of 500 ml) for 24 hrs at 4°C which was followed by replacing the Feulgen solution with a 2.5% pecticlear (Serevac) solution (0.5 g pecticlear in 20 ml NaOAc buffer) for a period of 30 min at 37°C. This was followed

by replacing the pecticlear solution with water for 30 min. The next step was to squash the stained roots after 30 min incubation.

4.3.5.2. Squashes

Darkly stained pink roots were cut using a clean scalpel on a clean microscope slide. Acetocarmine (1 g carmine in 45% acetic acid) were added to root tips which were then squashed (Fig 4.31.) till the roots were in a liquid form. The liquid chromosome spread was then transferred to a 1.5 ml Eppendorf tube.

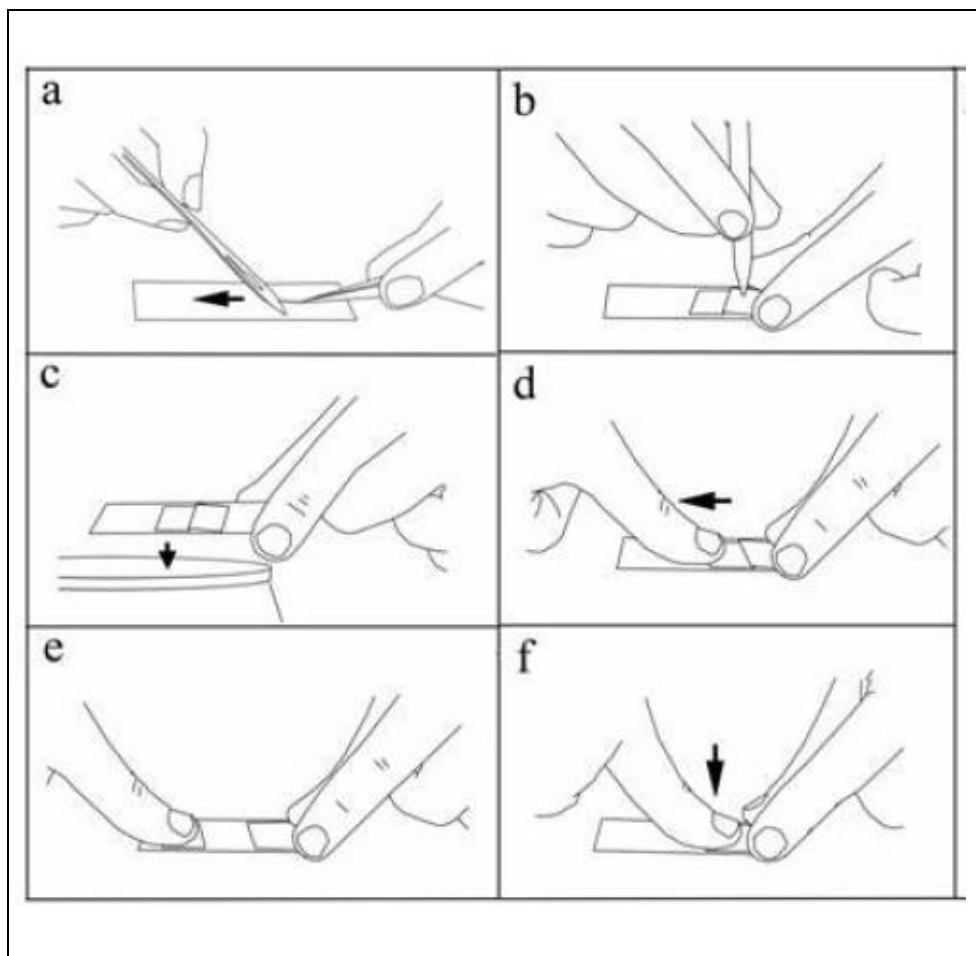


FIGURE 4.3.2: Diagrammatic representation in preparation of metaphase chromosomes from root tips (Mirzaghaderi 2010).

4.3.5.3. Hybridization

FISH was performed using three types of fluorochrome, i.e. blue, green and red. The gene of interest was labelled red while the telometric probe was labelled green and the chromosomes blue. Total

genomic DNA was labelled with all three probes (Table 4.3.1). The probe mixture contained formamide 40% (v/v), saline-sodium citrate (SSC) (Table 4.6.3), 50% (v/v) dextran sulfate and calf thymus. The mixture was denatured at 80°C for 45 s followed by an overnight incubation at 37°C.

4.3.5.4. Slide preparation

The hybridization mixture underwent a series of stringent washes (Appendix B, Table 4.6.3) at 42°C. A cover slip was placed on the liquid root tip and excess fluid was carefully removed using filter paper. Slides were viewed using a light microscope (Zeiss Axioskoop & Nikon Eclipse E600), followed by viewing fluorescence signalling using a confocal microscope (Zeiss Axioskoop). Images were analysed with the software NIS elements F30 (<http://www.nis-elements.cz/>).

4.3.6. Sample collection for chromosome suspension

4.3.6.1. Root sampling

Two methods of root generation were employed, in the first method approximately 30 seedlings were germinated on moist filter paper in 90 mm petri dishes. These were incubated at 25°C for a period of three days till roots were 3 cm in length. The second method involved planting seeds in autoclaved soil for a period of 3 days till roots were 3 cm in length.

4.3.6.2. Cells in metaphase

Once the optimal root length of 3 cm was obtained, roots underwent a series of incubation steps. Starting with freshly cut roots which were incubated in deionized water at 25°C for 3 hrs, followed by incubating roots in 2 mM hydroxyurea solution (121.6 mg hydroxyurea in 800 ml Hoagland's nutrient solution (Appendix B, Table 4.6.1) for 18 hrs at 25°C. After 18 hrs incubation, roots were transferred to Hoagland's solution without hydroxyurea (Appendix B, Table 4.6.1.) for period of 5 hrs and 30 min at 25°C in the dark. The next step was then to incubate the roots in 2.5 uM amiprofos-methyl (Appendix B, Table 4.6.2.) for 2 hrs in the dark at 25°C. The reaction was terminated by transferring roots to ice cold (1-2°C) deionized water and incubating for 16 hrs to stop the reaction.

4.3.6.3. Fixation

Roots were cut into 1 cm length pieces and transferred to 2% formaldehyde fixative (10 mM Tris, 10 mM Na₂EDTA, 100 mM NaCl, 0.1% Triton X-100 (v/v), pH 7.5 and 13.5 ml formaldehyde with a

total volume of 250 ml) for 20 min at 4°C. This was followed by washing the roots with Tris buffer (10 mM Tris, 10 mM Na₂EDTA and 100 mM NaCl with a total volume of 500 ml) for 5 min at 4°C. This step was repeated three times.

4.3.6.4. Chromosome suspension

Roots were transferred to 1 ml LB01 lysis buffer (Appendix B, Table 4.6.4) and, homogenized with an Eppendorf pestle, followed by centrifuging at 15 000 rpms for 13 s. The roots were then filtered using a 50 µm nylon mesh into a 1.5 Eppendorf tube which was followed by hybridization as described under 4.3.5.3 and 4.3.5.4.

4.4. Results

4.4.1. Optimization of chromosome spreads using the squashing technique

The analysis of Feulgen stained chromosome spreads varied in resolution. Figure 4.4.1A-D shows how the quality of spreads differs between different fixation periods.

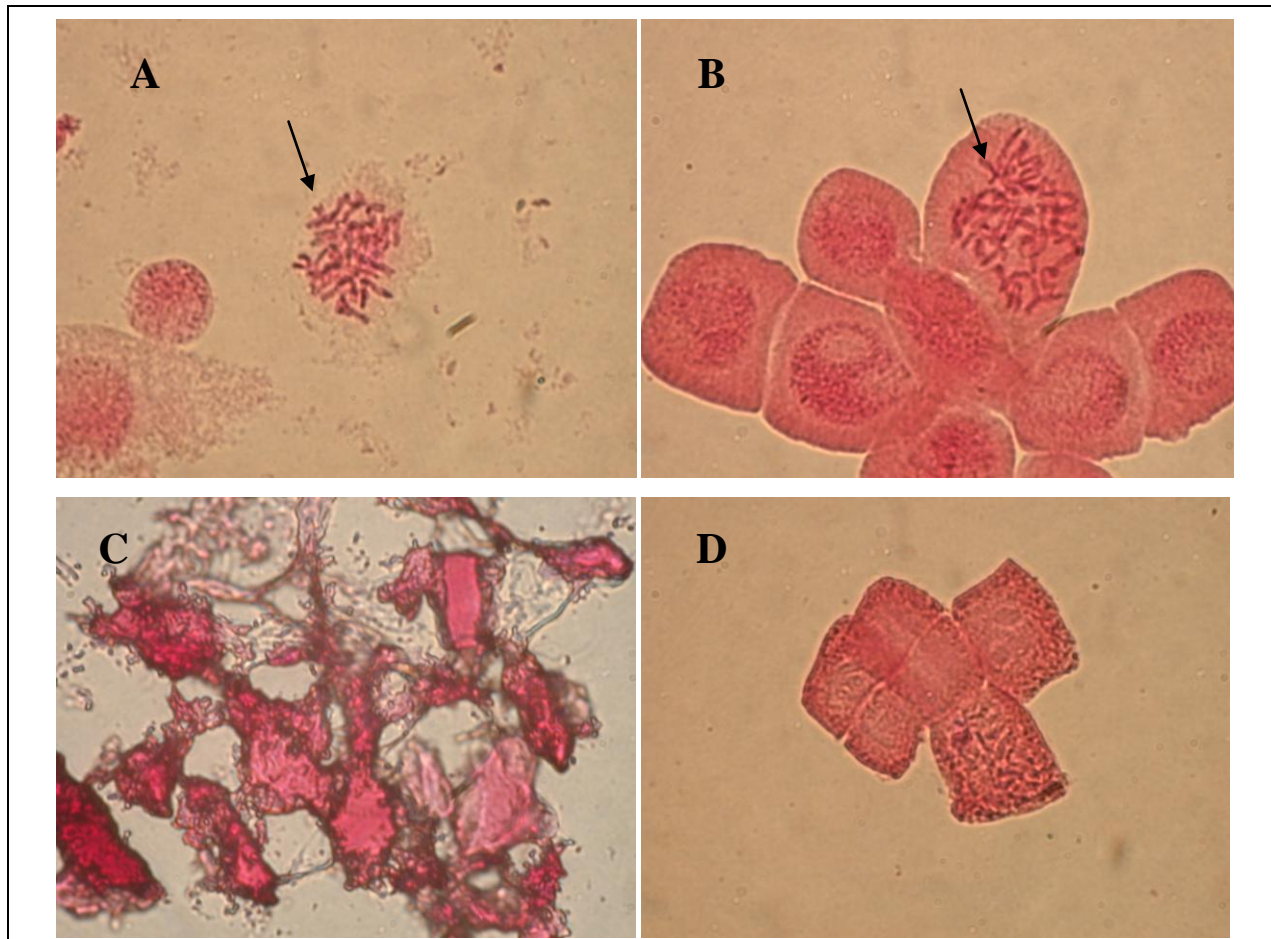


FIGURE 4.4.3: Metaphase root tips of Tugela *Dn* wheat cultivar cells: A-B: Chromosome spreads of root tips stored for 2 wks in 3 methanol: 1 proponic acid, C: Chromosome spreads of root tips stored in pecticlear solution for ~2 months, D: Chromosome spreads of root tips stored for 1 day in 3 methanol: 1 proponic acid visualized with the Zeiss Axioskoop microscope.

Root tips that were stored in the fixative for 2 wks arrested the cells in the metaphase. However, roots stored in the fixative for one day only, no metaphase chromosomes were detected. Root caps stored in pecticlear solution for ~2 months showed severe degradation of the cells.

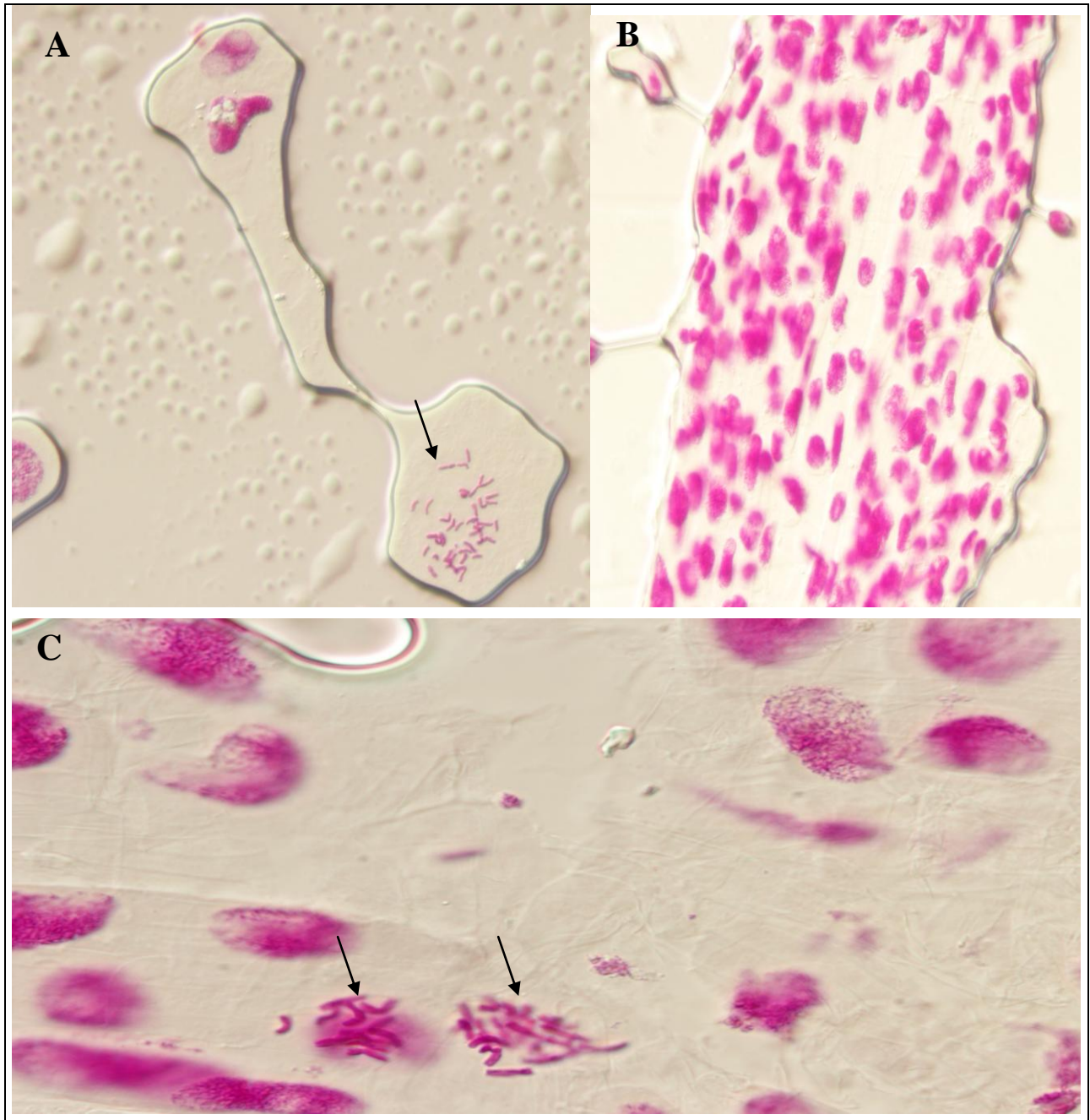


FIGURE 4.4.2: Metaphase root tips of Gamtoos R wheat cultivar cells fixed in 3 methanol:1propionic-acid and visualised using with Nikon Eclipse E600 microscope. A and C contains root caps that were in the fixative for a period of six days. And C are root caps that were in the fixative for 3 days.

Figure 4.4.2 shows root caps fixed for a period of 1 to 13 days. Six days of fixation gave the best quality chromosome spreads (Figure 4.4.2 A & C), while cells were still condensed with less of six days fixation(Figure 4.4.2B).

4.4.2. Analysis of chromosome spreads using suspension

Chromosome spreads obtained from suspensions gave the poorest quality spreads and the lowest resolution on the microscope. No structural chromosomes were obtained from these chromosome spreads (Figure 4.4.3A-D).

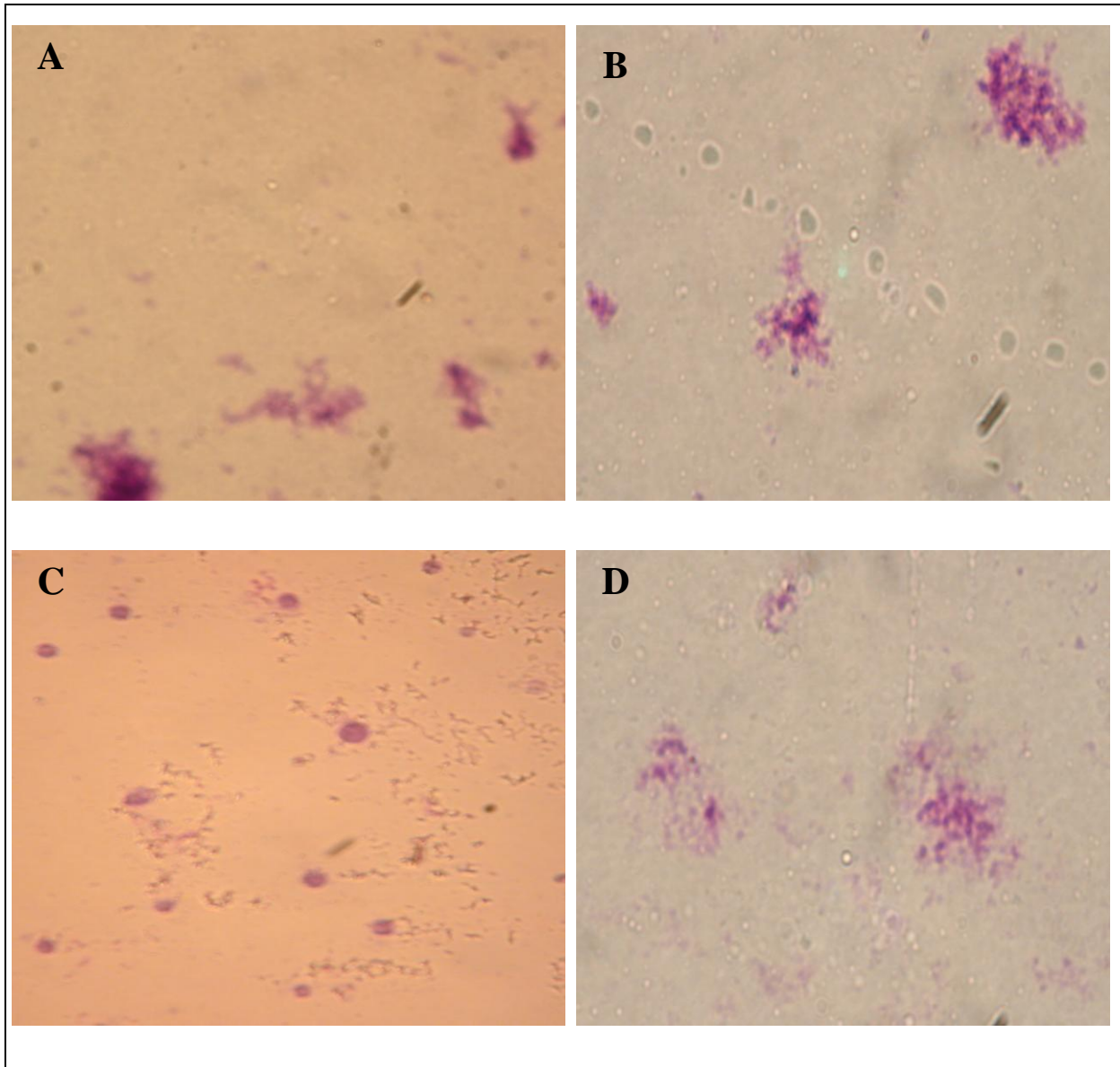


FIGURE 4.4.3: Metaphase root tips of Tugela *Dn1* wheat cultivar cells. A-D: Chromosome spreads of root tips in chromosome suspension using the Zeiss Axioskoop microscope.

4.4.3. FISH analysis using chromosome suspension

FISH analysis was carried out to identify the chromosome location of a *Dn9* repulsion phase marker (red) (Fisher 2011), telomeric probes (green) as well as chromosomes (blue). Results obtained showed that the chromosomes were present (Figure 4.4.4A).

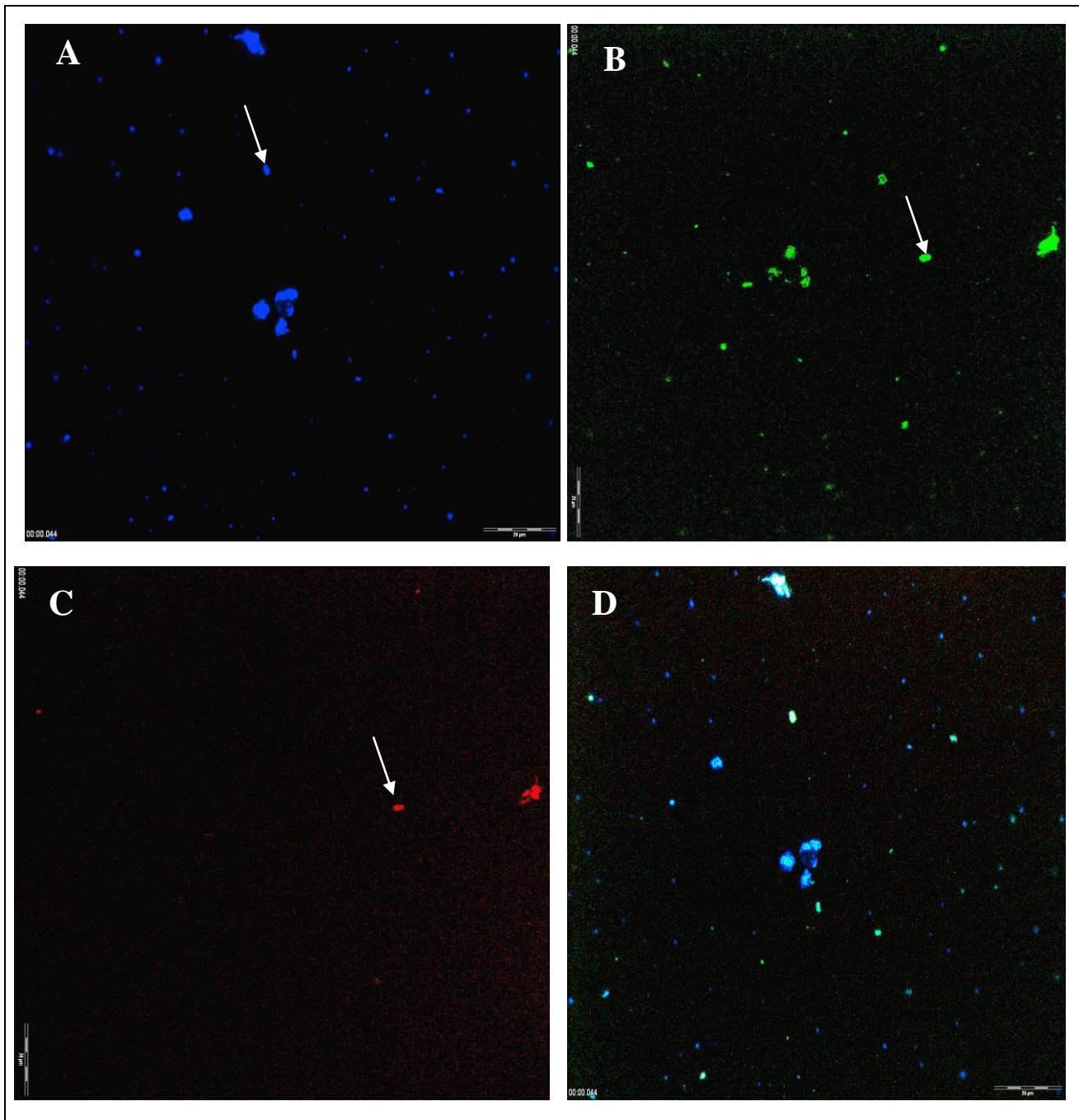


FIGURE 4.4.4: Fluorescence *in situ* hybridization to *Triticum aestivum* chromosomes. A: Chromosome presence, B: represents chromosome ends, C: Fluorescence signal of the gene marker of interest, D: Combination of all three fluorochromes.

Among the fluorophores applied, the blue fluorophore was the most abundant showing the amount of chromosome present. Less green signal was observed, which was expected, since the green fluorophore only detects the ends of the chromosomes. Differential fluorescence was found with the *Dn9* repulsion phase marker (red), which was significantly less than the blue and green signals. This is significant because it is a gene specific marker. Chromosomes contain long “worm like” structures and these structures were found in this study with the chromosome squashes, but no structural chromosomes could be detected with the applied FISH analysis. Only round like structures were detected (Figure 4.4.4A-D) (Dolezel *et al.* 2012).

4.5. Discussion

4.5.1. Optimal cultivars for cell cycle synchronization and accumulation of metaphase cells

Metaphase chromosomes from both spring and winter wheat cultivars were obtained in this study. It was further easier to obtain chromosomes from the resistant wheat varieties than from the susceptible counterparts which was unexpected since seeds were germinated under the same conditions. From the eight cultivars, only two (i.e. Gamtoos R and Tugela *Dn1* root caps) gave metaphase spreads. In the literature, spring wheat cultivars (Chinese spring wheat and Bob white) are commonly used for metaphase spreads which generally results in metaphase synchronization (Arzani *et al.* 2000).

4.5.2. Chromosome squashes vs chromosome suspensions

In this study, it was difficult to synchronize metaphase chromosomes from cell suspensions than from chromosome squashes. Using chromosome squashes, it was easy to obtain chromosome spreads while with chromosome suspensions no distinguishable chromosome structures were found. The most commonly used technique for preparation of chromosome samples is the use of the squashing technique using root tips as the starting material (Dolezel *et al.* 2012). They found that it is difficult to release chromosomes from the rigid wheat cell walls and that a low degree of metaphase synchrony is commonly found when chromosome suspensions are used (Dolezel *et al.* 2012). This makes it difficult to explore the wheat genome.

4.5.3. Limitations of microscopes used

Two types of light microscopes were used in this study to obtain chromosome spreads, the Zeiss Axioskope, and Nikon Eclipse E600. The Zeiss Axioskope gave the poorest resolution (Figure 4.4.2) while the Nikon Eclipse gave the highest resolution. While a confocal microscope was used to detect fluorescent signals.

4.5.4. FISH analysis

Initially, three fluorescently labelled probes were applied in separate hybridization reactions from which only one fluorescent signal could be detected i.e. the *Dn9* marker. It is possible that the other two probes (i.e. *APX* and *GSTF6b*) contained small amounts of repetitive DNA which might have prevented detection of these probes. This observation is supported by the work of Wang *et al.* (2006),

on maize and where difficulties with fluorescent signal detection were encountered when the probes had too little repetitive sequences.

Several factors made the FISH detection more challenging than the metaphase synchronization. First, the quality of chromosome spreads derived from chromosome squashes and chromosome suspensions. Work done by Mirzaghaderi *et al.* (2010) has, previously emphasized the importance of high quality chromosome spreads due to the fact that optimal hybridization is dependent on this. Secondly, the use of unique probes with small repetitive sequences is extremely important. And lastly the resolution of the microscopes employed also contributes to the outcome of the study.

In this study, the abundance, distribution and location of the targeted genes on wheat chromosomes were unable to determine. The root caps were found to be suitable to use as cytological tissue instead of immature embryos. The squashing technique yielded the best results to synchronized metaphase chromosomes. Optimization is required for the FISH technique however; the biggest limitation of this study was the resolution of the available microscopes.

4.6. References

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4.7. Appendix B

Table 4.6.1: Fluorescent <i>in situ</i> reagents 1		
Hoagland's Nutrient	Hoagland's Stock 10X	Hoagland's
100 ml Hoagland's Stock (10X)	4.7 g Ca(NO ₃) ₂ ·4H ₂ O 40 mM	Sol A:
5 ml Sol C	2.6 g MgSO ₄ ·7H ₂ O 20 mM	280 mg H ₃ BO ₃ 45 mM
In 1000 ml demonized water	3.3 g KNO ₃ 65 mM	340 mg MnSO ₄ ·H ₂ O 20 mM
	0.6 g NH ₄ H ₂ PO ₄ 10 mM	10 mg CuSO ₄ ·5H ₂ O 0.4 mM
	5 ml Sol A	22 mg ZnSO ₄ ·7H ₂ O 0.8 mM
	0.5 ml Sol B	10 mg (NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O 0.8 mM in 100 ml deionized water
	In 500 ml deionized water	
		Sol B:
		0.5 ml H ₂ SO ₄ 0.05 mM in 100 ml deionized water
		Sol C:
		3.36 g Na ₂ EDTA 18 mM
		2.79 g FeSO ₄ 20mM
		In 500 ml deionized water

Table 4.6.2: Fluorescent <i>in situ</i> reagents 2	
Amiprophos methyl treatment Stock 2.5 µM	Amiprophos methyl Stock 20 mM
101.3 µl APM Stock (20 mM)	60.86 mg APM
In 800 ml deionized water	In 10 ml ice cold acetone

Table 4.6.3: Fluorescent <i>in situ</i> reagents 3			
0.1X SSC washing buffer	2X SSC washing buffer	4X SSC washing buffer	20X SSC stock
5 ml 20X SSC	100 ml 20X SSC	200 ml 20X SSC	175.3 g NaCl 3M
1 ml Tween 20 0.1% v/v	In 900 ml deionized water	2 ml Tween 20 0.2% v/v	88.2 g $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ 300 mM
406 mg $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 2mM		In 1000 ml deionized water	In 1000 ml deionized water
In 1000 ml deionized water			Adjust to pH 7
			Autoclave

Table 4.6.4: Fluorescent <i>in situ</i> reagents 4		
LBO1 lysis buffer	Tris buffer	Formaldehyde fixative
0.363 g Tris 15 mM	0.606 g Tris 10 mM	0.242 g Tris 10 mM
0.149 g Na_2EDTA 2mM	1.861 g Na_2EDTA 10mM	0.931 g Na_2EDTA 10mM
0.0348 g spermine-4HCL 0.5 mM	2.922 g NaCl 100 mM	1.461 g NaCl 100 mM
1.193 g KCl 80 mM	In 500 ml deionized water	250 μl Triton X-100 0.1% v/v
0.234 g NaCl 20 mM	Adjust pH to 7.5	In 200 ml deionized water
200 μl Triton X-100 0.1% v/v		Adjust pH 7.5
In 200 ml deionized water		Add 13.5 ml formaldehyde
Adjust pH 9		
Add 220 μl mercaptoethanol		

Chapter 5

Summary

**Gene silencing in bread wheat (*Triticum aestivum* L.)
following a biolistics approach**

Gene silencing in bread wheat (*Triticum aestivum* L.) following a biolistics approach

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The long term goal of plant breeders is to breed for crop plants with increased and durable resistance. In order to achieve this both a tissue culture and effective transformation procedure are needed. This procedure can then be further utilized for resistance breeding by enhancing the expression of agronomically important genes ascorbate peroxidase (APX) and glutathione S-transferase (GST). In this study we succeeded in the establishment of effective tissue culture and transformation procedure for wheat (*Triticum aestivum* L.) at Stellenbosch University using the spring wheat cultivar Gamtoos S (*Dn0*) and its near isogenic line Gamtoos R, which contains the rye-derived *Dn7* resistant gene. The Gamtoos R cultivar is found to be the most resistant wheat variety against the Russian wheat aphid (RWA) in South Africa and allows increased yield under aphid feeding pressure. The interaction between the aphid and the host activates a cascade of defensive signalling pathways to assist the host plant to overcome the stress response. In this study, we were particularly interested in the hypersensitive response which minimizes aphid damage at the infested site. Stress response causes the activation of the HR resulting in the up regulation of ROS scavenging enzymes i.e. *APX* and *GST*. Therefore *APX* and *GSTF6b* silencing were investigated to further confirm their function in the defensive pathway of wheat and also where these two genes are located in the wheat genome. Their function was determined by decreasing the transcript amount of both genes using a reverse genetic approach i.e. gene silencing via particle bombardment. The transformation process from tissue culture establishment to hardening off of transgenic plantlets, took up 8 months to be completed and ultimately resulted in a small number of putative transgenic plants (Gamtoos S (*Dn0*)-pUbi-*APX*, Gamtoos S (*Dn0*)-pUbi-*GSTF6b* and Gamtoos R (*Dn7*)-pUbi-*GSTF6b* plants) which were partially silenced. Expression analysis was attempted on the three obtained transgenic plants before and after RWA infestation revealed that silenced plants were visually more chlorotic and had lower target gene expression due to silencing. These plants were successfully hardened off and generated a T₁ generation which are currently being further analysed. Further, an attempt was made to localize the abundance and distribution of the two genes in the wheat genome by performing fluorescent *in situ* hybridization. Two methods were tested for synchronizing metaphase chromosomes (i.e. chromosome squashes and chromosome suspensions) and chromosome squashes yielded the best results. However, fluorescence could not be observed with the chromosome squashing technique while fluorescence was

detected with the chromosome suspension. FISH was unsuccessful and the technique still needs to be optimised before this aim can be achieved.