UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Using cDNA-AFLP and microarray analysis for rapid identification of *Diuraphis noxia* induced genes from near-isogenic *Triticum aestivum* lines

by

M. Thuto Matsioloko

Submitted in partial fulfilment of the requirements for the degree

Magister Scientiae

In the Faculty of Natural and Agricultural Sciences

Department of Genetics

University of Pretoria

Pretoria

June 2011

Under the supervision of

Prof. A.-M. Botha-Oberholster

and Prof. A. A. Myburg

DECLARATION

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

M. Thuto Matsioloko

June 2011

SUMMARY: Using cDNA-AFLP and microarray analysis for rapid identification of *Diuraphis noxia* induced genes from near-isogenic *Triticum aestivum* lines.

M. Thuto Matsioloko

Supervised by **Prof. A.-M. Botha-Oberholster and Prof A. A. Myburg**

Submitted in partial fulfillment of the requirements for the degree ***Magister Scientiae***

Department of Genetics

University of Pretoria

This is a study of transcriptional gene regulation in wheat (*Triticum aestivum*, L.) in response to Russian wheat aphid (RWA) (*Diuraphis noxia*, Kurdjumov) infestation. The Russian wheat aphid feeds on the phloem sap in the leaves of wheat plants, and causes the leaves of susceptible wheat plants to curl. This forms a protective barrier for the RWA from insecticides and natural enemies. Chlorosis also results from the RWA feeding. In cases of high infestation, death of susceptible plants can also occur. Eleven wheat genes that confer resistance to the Russian wheat aphid have been identified, but their mechanism at molecular level is still not clearly understood.

Wheat near-isogenic lines (NILs) were used in a genome-wide, transcriptome analysis using cDNA-AFLP technology. RWA-resistant cultivar ‘Tugela DN’ and RWA-susceptible cultivar ‘Tugela’ were infested with the RWA and leaves were collected from the infested plants at different (0-, 1-, 2-, 6-, 12-, 24-, 48- and 120-) hours post infestation. cDNA samples derived from these leaves was then analyzed by cDNA-AFLP which revealed 18 clusters of differential gene regulation between the two NILs. The results of this experiment show that differential regulation of transcripts occur even within the first hour of infestation. All types of regulation were observed within the clusters. Differentially expressed transcript derived

fragments (TDFs) that were randomly isolated from PAGE gels and sequenced (41 TDFs) included sequences in the functional groups similar to those observed in the microarray analysis. The functional categories are cell structure and maintenance [protein synthesis (14%), chaperone (2%), protein degradation (2%), transcription factor (5%)]; photosynthesis [sugar metabolism (5%), carbohydrate metabolism (2%), energy related (7%)]; defense-related [signaling (7%), defense-related (10%)] while the rest did not have any significant homology to any known or characterized proteins.

Previous suppressive subtractive hybridization experiments identified transcripts that are differentially expressed in wheat in response to RWA feeding. More transcripts were identified by PCR from cDNA pools derived from RWA-infested plants as having conserved motifs common in pathenogenesis related proteins. The isolated transcripts were used to generate a defense response-biased microarray chip that was used to investigate the regulation of these transcripts during infestation of RWA resistant wheat plants ('Tugela DN') in a time trial. Dual hybridization of *CyDye* labeled probes derived from the induced 'Tugela DN' plants to the microarray chips revealed differential regulation of the immobilized transcripts in wheat, at different time points post infestation with the RWA.

Statistical analysis of the *CyDye* intensities on the 380 spots mounted on the cDNA microarray slides showed 29 transcripts to be significantly regulated ($P \leq 0.05$) during the time of the experiment. These included ESTs that were grouped into four functional categories, namely cell structure and maintenance (9 ESTs); photosynthesis (8 ESTs); defense-related (4 ESTs) and those with no significant homology found or proteins with unknown function (8 ESTs). Patterns of regulation of these transcripts in all of the functional categories included all types of regulation e.g. mainly down-regulation, mainly up-regulation, and a combination of up-/down-regulation in response to RWA feeding.

In conclusion, data obtained utilizing cDNA microarray and cDNA-AFLP analyses in infested wheat suggest that the ability to maintain structures involved in photosynthesis by regulating the relevant transcripts through-out infestation is an important determinant in plant survival during RWA feeding. The timing of regulation is also important as some of the transcripts are also regulated in RWA susceptible ‘Tugela’ plants but not in a timely manner which leads to loss of energy and subsequent death of susceptible plants.

PREFACE

The Russian wheat aphid is a serious pest of wheat in almost all the countries that cultivate wheat except for Australia. It is said to have originated from the southern parts of Russia where it then spread to other countries like South Africa. The Russian wheat aphid has been a serious pest of wheat in South Africa since its introduction in the late 1970s. The Russian wheat aphid being an introduced pest has been challenging to successfully control. Conventional breeding programmes to produce wheat cultivars that are resistant or tolerant to the Russian wheat aphid have been only partially successful as new biotypes of the aphid develop that overcome the resistance of the plants and induce the same symptoms on resistant cultivars as in the susceptible lines. New Russian wheat aphid biotypes have been reported in the USA in 2003 and in 2006 the development of Biotype SA2 was reported in South Africa. More recently a third Russian wheat aphid biotype was reported, known as Biotype SA3.

A possible solution to control this crippling pest in wheat cultivation, is (i) to identify and introduce sources of resistance into susceptible wheat lines through normal breeding as well as biotechnological methods; and (ii) to study the underlying genetic mechanisms confering resistance to allow for the introduction of multiple sources of resistance which may lead to durable resistance against the Russian wheat aphid.

The dissertation comprises the following: **Chapter 1** provides a review of what is already known about the interactions of wheat with the RWA, the general interactions of plants and their insect pests and pathogens, the result of these interactions at molecular level and methods of improving how the wheat and other plants respond to attack. **Chapter 2** gives a description of how cDNA-AFLP and microarray technologies were employed in EST expression analysis in RWA induced ‘Tugela DN’ (RWA resistant) and ‘Tugela’ (RWA

susceptible) which is followed by a presentation of the data generated from the experiments conducted (**Chapter 3**) and a discussion on the significance of these results to the elucidation of the Russian wheat aphid resistance in wheat (**Chapter 4)**. Seven novel sequences that were obtained during this study were submitted to GenBank EST database (www.ncbi.nih.gov) with the following accession numbers: ES697585; ES697586; ES697587; ES697588; ES697589; ES697590; ES697591 (**Appendix A**). The microarray hybridizations conducted in this study contributed to the preparation of the manuscript presented in **Appendix B**.

This dissertation represents outcomes of a study conducted in the Department of Genetics, University of Pretoria, under the supervision of Professor A.-M. Botha-Oberholster and Professor A. A. Myburg from March 2002 to September 2010. The following manuscript and conference presentations (oral and posters) were generated based on the results produced during this study:

Matsioloko MT and Botha A-M (2003). cDNA-AFLP Profiling in wheat upon Russian wheat aphid feeding. Proceedings of the 10th International Wheat Genetics Symposium, Paestum, Italy, (1-6 September 2003). Volume 3 pp. 1275-1277.

Botha A-M, Lacock L, van Niekerk C, Matsioloko MT, du Preez FB, Myburg AA, Kunert K and Cullis CA (2003). Gene expression profiling during *Diuraphis noxia* infestation of *Triticum aestivum* cv. 'Tugela Dn' using microarrays. Proceedings of the 10th International Wheat Genetics Symposium, Paestum, Italy, (1-6 September 2003). Volume 1 pp. 334-338.

Botha-Oberholster A-M, Matsioloko MT, du Preez FB, van Eck L and Walters RSG (2004). Russian wheat aphid mediated elicitation of the wheat defense-transcriptome. International Plant Resistance to Insects (IPRI) – 16th Biennial Workshop, Baton Rouge, Louisiana, USA, March 21-24.

Matsioloko MT, Myburg AA and Botha A-M (2004). Expression profiling in wheat upon Russian wheat aphid infestation. 18th congress of the South African Genetics Society. Stellenbosch, 4-7 April 2004. Oral presentation.

Botha-Oberholster A-M, Matsioloko MT, Du Preez FB, van Eck L and Walters RSG (2004). Russian wheat aphid-mediated elicitation of the wheat defense-transcriptome. Plant Biology 2004 programme, pp 126. Lake Buena Vista, Florida, USA, July 24-28, 2004.

Botha A-M, Lacock L, van Niekerk C, Matsioloko MT, Du Preez FB, Loots S, Venter E, Kunert KJ and Cullis CA (2006). Is photosynthetic transcriptional regulation in *Triticum aestivum* L. Cv. 'Tugela DN' a contributing factor for tolerance to *Diuraphis noxia* (Homoptera: Aphididae)? Plant Cell Reports 25 (1), 41-54.

ACKNOWLEDGEMENTS

I would like to express my heartfelt appreciation and gratitude to the following people in no order of preference.

- Professor Anna-Maria Botha-Oberholster for her belief in me and her continued support and guidance through this research and for providing me with the platform in her research programme so as to realise my dream to grow as a scientist.
- Professor Alexander A. Myburg for his valuable contributions and advise.
- My friends in the Cereal Genomics Research Group, Adrene Laubscher, Karen Muller, Franco du Preez, Dirk Swanevelder, Rosie van Zyl, Robert Walters, Lynelle Lacock, Chantal van Niekerk, Edward Venter, for all the discussions and assistance.
- My husband David Ntsowe, for giving his love and necessary emotional support during the study.
- My parents, George and Dorothy Matsioloko, for their love and endless encouragement to pursue my academic interests.
- The Department of Genetics for a stimulating scientific environment with a good infrastructure.
- Forestry and Agricultural Biotechnology Institute (FABI) for their infrastructure.
- Pretoria University Personnel Development Fund (PUPPDF), National Research Foundation (NRF) and the Winter Cereal Trust (WCT) for their financial assistance to complete the study.
- The Small Grain Institute (SGI), Bethlehem (SA), for providing the germplasm required for this study.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ABA	abscisic acid
AFLP	amplified fragment length polymorphysms
ANOVA	analysis of variance
APS	ammonium persulphate
APX	ascorbate peroxidase
***Avr*-gene**	avirulence gene
bp	base pair
BSA	bovine serum albumin
BTH	benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester
CC-NBS-LRR	coiled-coil NBS-LRR
cAPX	cytosolic APX
CC	coiled coil
cDNA	complementary DNA
cDNA-AFLP	complementary DNA amplified fragment length polymorphysms
CDPK	calcium dependent protein kinase
ddH_2O	double distilled water
DDRT-PCR	differential display RT-PCR
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
ds	double stranded
EDTA	ethylenediaminetetraacetic acid
ESTs	expressed sequence tags
Et	ethylene
EtBr	ethidium bromide

GITC	guanidine isothiocyanite
H_2O_2	hydrogen peroxide
HR	hypersensitive response
hpi	hours post infestation
INA	2,6-dichloroionicotinic acid
IPTG	isopropylthio-β-D-galactoside
JA	jasmonic acid
LRR	leucine-rich-repeat
LZ-NBS-LRR	leucine-zipper NBS-LRR
MAPK	mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPK kinase kinase
MAS	marker assisted selection
NBS	nucleotide binding site
NIL	near-isogenic line
O^{2-}	superoxide
PAGE	polyacrylamide gel electrophoresis
PCD	programmed cell death
PCR	polymerase chain reaction
PI	plant introduction
PR-proteins	pathogenesis related proteins
***R*-gene**	resistance gene
RNA	ribonucleic acid
ROS	reactive oxygen species

RWA	Russian wheat aphid
SA	salicylic acid
SAR	systemic acquired resistance
SDS	sodium dodecyl sulfate
SSH	suppression subtractive hybridization
TDFs	transcript derived fragments
TE	tris-EDTA
TEMED	tetramethylethylenediamine
TIR-NBS-LRR	Toll, interleukine-1 receptor like NBS-LRR
TMV	tobacco mosaic virus
Tris	tris(hydroxymethyl)aminomethane
WCI	wheat chemically induced
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction and background

Much focus has been on the development of better plant disease control strategies, which has led to substantial advances in this area. The global food supply is, however, still threatened by pests and pathogens in great amounts (De Maagd et al., 1999). Grain crops like wheat, maize and rice are staple foods for many nations of the world, but diseases constantly threaten their production. Wheat was the first crop to be domesticated and has a high trade value compared to other cereals. Seventeen percent of all crop area is occupied by wheat plantation (Gill et al., 2004). Wheat is a very important crop and yet an annual loss of about 25% occurs due to biotic (pathogens and pests) and abiotic stresses (heat, frost, drought etc.). This is one of the reasons why significant advances in the development of disease control strategies are required – to protect the crops from stresses and increase crop yield (Gill et al., 2004).

1.1.1 Origins of hexaploid wheat and its genome structure

Polyploidy played a major role in the evolution of the plant kingdom. This includes most flowering plants and crops like wheat, oat, cotton, coffee, sugarcane and potato. The present polyploids resulted from sexual chromosome hybridisation of autopolyploids or allopolyploids (Naranjo and Corredor, 2004). The major driving force behind cereal genome expansion was chromosome segment and transposable element duplication, the effects of which were exacerbated by polyploidisation in wheat. Grasses originated 70-55 million years ago. The common ancestor of wheat, maize and rice diverged 15-30 million years later. Compared to rice and maize, wheat has the largest genome with a size of 16 000 MB (Arumuganathan and Earle, 1991). Wheat (*Triticum aestivum* L.) is an allopolyploid with three genomes, the A-, B- and D-genome, each with seven chromosomes (2n = 6x = 42) adding up to a 42 chromosome hexaploid species (AABBDD). About three million years ago, the A, B, and D diploid wheat progenitor species diverged from a common ancestor. *Triticum uratu* (AA) and *Aegilops speltoides* (BB)

hybridized about 200 000 years ago to form *Triticum turgidum* (AABB) in the Middle East. Wheat is estimated to have only been domesticated 15 000 years ago marking the start of civilization. The hexaploid wheat (AABBDD) was then formed when the tetraploid wheat hybridised to a diploid species *Aegilops tauschii* (DD) about 8 000 years ago (Gill et al., 2004). The chromosomes of the genomes A, B and D are homoeologous, but are, however, prevented from pairing by a gene situated on the long arm of chromosome 5B, the *Ph1* (pairing homoeologous) gene, causing wheat to behave or function like a diploid organism (Gill et al., 1993; Griffiths et al., 2006). Kimber and Riley (1963) showed in their research that strict bivalent formation occurs in hexaploid wheat and is genetically controlled. They showed that when the A-, B- and D-genomes of wheat were combined in the presence of chromosome 5B, there was no homology, but, when chromosome 5B was removed, chromosome pairing occurred among genomes.

The structure of the wheat genome is very complex due to its origins. About 90% of the wheat genome constitutes repeated sequences, 70% of these are known to be transposable elements (TEs). The genome consists of some gene-rich regions (gene clusters) which are separated by long stretches of transposable elements as opposed to rice whose genes are fairly evenly distributed along the chromosomes. These stretches can either be gene-poor or gene-free. Low-copy and miniature inverted TEs are most often associated with active genes, while the high copy number TEs mainly insert in the intergenic regions (reviewed by Gill et al., 2004).

1.1.2 The Russian wheat aphid as a pest of wheat

Description

The Russian wheat aphid [RWA; *Diuraphis noxia* (Kurdjumov)] is a threat to the production of wheat in almost all countries where wheat is grown except for Australia (Webster et al., 1987;

Nkongolo et al., 1991; Rafi et al., 1996; Dong et al., 1997; Anderson et al., 2003). Its body is only about 2 mm in length, spindle shaped, with short antennae and a double tail above its cauda (Walters et al., 1980; Robinson, 1992; Figure. 1.1E).

Origins and distribution

D. noxia is thought to have originated in southern Russia and then spread from its native countries to South Africa in 1978, where its pest status was underestimated, leading to devastating effects on wheat production in this country (Walters et al., 1980; Du Toit, 1989; Basky, 2003). The RWA is estimated to cause 21-92% loss in crop yield following infestation (Du Toit, 1986; Basky, 2003). The RWA also spread to South America and Mexico where it was detected in 1980 (Smith et al., 1991; Basky, 2003; Anderson et al., 2003). More recently (in 2003), new RWA biotypes were reported in the USA (Hayley et al., 2004; Puterka et al., 2006) and South Africa (Boshoff and Du Toit, 2006; Tolmay et al., 2007). The development of these new RWA biotypes lead to the complete breakdown of previously known RWA resistant cultivars (e.g. all *Dn4*-carrying lines in the USA and *Dn1*-containing lines in South Africa) (Hayley et al., 2004; Puterka et al., 2006; Tolmay et al., 2007).

Feeding and symptoms

Diuraphis noxia is a phloem sucking aphid. It feeds by randomly probing host leaves with a stylet to establish the potential feeding sites. It is thought that the aphid injects a toxin-like substance into the host plant (Miles, 1999). This injection of toxin then results in the formation of longitudinal chlorotic streaks that are yellow, white or purple on the leaves of the plant. This chlorosis develops eventually into necrosis and plant death follows if the plants are highly susceptible. Aphid feeding also results in the curling of the leaves. In heavily infested plants, leaf curling traps the kernels and prevents them from protruding properly (Walters et al., 1980; Rafi

et al., 1997; Basky, 2003; Anderson et al., 2003; Figure 1.1C). The feeding also results in reduced photosynthetic capacity of the host due to the destruction of the chloroplasts (Fouché et al., 1984; Rafi et al., 1997; Botha et al., 2006). It has been observed that the aphids move upwards as the plant grows and continues to colonise new emerging leaves, especially at the base of the leaves, while they desert the previously infested leaves (Aalberg et al., 1989). The aphids also prefer to colonize the flag leaves during senescence and anthesis, leading to high yield losses. This loss is due to the fact that flag leaves produce most of the carbohydrates necessary for grain development (Gray et al., 1990).

Sources of resistance and biotypic variation

Resistance genes were identified in the relatives of wheat where the Russian wheat aphid originated. These were then crossed with hexaploid wheat through conventional breading to introduce the RWA resistance into hexaploid wheat. The wheat lines that resulted from the breeding programmes were then labelled as plant introduction (PI) lines, and were numbered accordingly. The PI lines therefore possess a specific *Diuraphis noxia* (*Dn*) resistance gene introgressed into their genome. The different *Dn* genes offer the wheat plants resistance to the Russian wheat aphids (Budak et al., 1999).

Biotypic variation between different RWA populations is measured by the degree of damage that is caused by the aphids on the plants. Puterka and colleagues (1992) tested virulence patterns of eight different RWA isolates from different countries on barley, triticale and wheat cultivars and found that they yielded seven unique patterns. The USA, Jordan and USSR 2 isolates were found to be moderately virulent on PI 149898 wheat, but they exhibited very low virulence on PI 372129 cultivar [donor of the *Dn4* resistance gene to most of the resistant cultivars in Canada (Haley et al., 2004)]. The USSR 1 isolate was highly virulent to PI 372129 and TAM W-101 while it was moderately virulent on PI 149898.

Figure 1.1. Images showing damage caused by the Russian wheat aphid infestation on wheat near-isogenic lines 'Tugela DN' (RWA resistant) and 'Tugela' (RWA susceptible). A- leaf rolling in 'Tugela'; B- 'Tugela DN' plants were able to withstand prolonged infestation by the RWA while 'Tugela' plants eventually died; C and D- heavy infestation on the flag leaves resulting in failure of the next leaf or head to successfully emerge; E- two adult forms of the Russian wheat aphid, apterous (wingless) and alate (winged); F- chlorotic streaking on 'Tugela' leaves. (Photographs courtesy of Leon van Eck).

A Turkish isolate was very poorly virulent to all three wheat cultivars while a Syrian isolate was moderately virulent in all cases (Puterka et al., 1992). Other isolates of Russian wheat aphid that have been reported include: (1) A Hungarian isolate virulent to wheat lines carrying *Dn1, Dn2 and Dn4* resistance genes (Basky, 2003), (2) A Chilean isolate virulent to wheat lines carrying the *Dn4* resistance gene, but not virulent to those carrying *Dn2*, *Dn5*, *Dn6*, and *Dny* (Smith et al., 2004).

Haley et al. (2004) confirmed the occurrence of a new aphid biotype (Biotype 2), different to the one reported by Smith and colleagues in 2004, in south-eastern Colorado that was virulent to *Dn1*, *Dn2*, *Dn3*, *Dn4*, *Dn5* and *Dn6* carrying genotypes but not to the accession 94M370 carrying the *Dn7* resistance gene.

The use of biological control is not very effective against the Russian wheat aphid although its use would help in protecting resistance genes incorporated into wheat cultivars from the development of a new aphid biotype (Basky, 2003). The presence of other pests also limits the predation on the Russian wheat aphid, as the biological control insects will also feed on the other pests. The leaf curling pose an additional problem as it protects the aphids from their natural enemies (Walters et al., 1980; Robinson, 1992; Basky, 2003). Another problem with the use of biological control agents is that they are also killed by the use of insecticides (Robinson, 1992). *Aphelinus asychis* and *A. albipodus* are examples of parasitoids with a potential for controlling the RWA populations because they are able to penetrate into the rolled wheat leaves and attack the RWA. Populations of these species have been established in some wheat producing areas, but their effectiveness in controlling the RWA has still to be observed over an extended period (reviewed by Lee et al., 2005). Biological control of the Russian wheat aphid is also considered to require a multilateral approach, whereby all the potential host plants for the RWA, all potential parasitoids, alternate host aphids and their host plants, should be considered as there is

no single species expected to offer complete control for the Russian wheat aphid (Tanigoshi et al., 1995).

A more environmental-friendly and economical approach is the development of resistant wheat cultivars (Dong and Quick, 1995; Saidi and Quick, 1996; Basky, 2003). Resistance to the Russian wheat aphid is searched for in wheat cultivars that originate from countries where the RWA originated from, i.e. the Middle East (Nkongolo et al., 1991; Du Toit, 1989). To date, several lines have been identified. The introgression of this resistance sources into susceptible wheat cultivars involves conventional breeding methods, which are time-consuming, but eventually gives rise to resistant cultivars that can be used in breeding programs, whereafter less chemical spraying is required (Webster et al., 1987; Du Toit, 1992; Anderson et al., 2003).

A potential solution lies with the development of Marker-assisted selection (MAS) tools that accelerate breeding efforts and thus, will help to reduce the amount of time spent in trying to identify resistant cultivars based on phenotype. Several molecular markers have been identified thus far that are used to identify resistant wheat lines and all known *Dn* genes have been mapped to specific chromosome regions (Liu et al., 2001; Myburg et al., 1998; Table 1.1). Genetic markers are found in areas flanking a gene of interest, upstream and downstream of a gene. The identified markers therefore indicate areas in the wheat genome where the resistance genes are located. These markers can be used as sites to amplify genes of interest from cDNA and clone these into susceptible wheat lines and evaluate their effect instead of relying on conventional breeding programmes.

1.2 Wheat as host to the Russian wheat aphid

1.2.1 Host plant resistance

Eleven genes that offer resistance against the Russian wheat aphid have been identified in wheat and its relatives. Table 1.1 provides a list of the identified *D. noxia* resistance genes (*Dn* genes) and information regarding their inheritance and locations in the wheat genome. The resistant

gene *Dn1* was identified in the wheat germplasm accession PI 137739 from Iran, while the *Dn2* gene was identified from the wheat accession PI 262660 from Russia (Du Toit, 1987, 1989; Ma et al., 1998). These two genes were identified in South Africa (Liu et al., 2001). A recessive gene *dn3* was identified in the *Aegilops tauschii* line SQ24 (Nkongolo et al., 1991).

Dn4 was identified in the Russian bread wheat accession PI 372129 and is located on chromosome 1DS (short arm of chromosome 1D) (Nkongolo et al., 1991). The *Dn5* gene was identified from the Bulgarian wheat accession PI 294994 (Du Toit, 1987; Marais and Du Toit, 1993). *Dn6* was identified from the Iranian wheat accession PI 243781 and the *Dn7* gene was derived from a rye accession and transferred to wheat via a IRS.IBL translocation (Liu et al., 2001; Marais et al., 1994; Anderson et al., 2003). The *Dn1*, *Dn2*, *Dn5* and *Dn6* genes are all located on chromosome 7DS (short arm), near the centromeric region and seem to form a linkage group (Liu et al., 2001). *Dn8* was identified in PI 294994 (located near the distal end of 7DS). *Dn9* was identified and located to a defense gene-rich region on wheat chromosome 1DL in PI 294994. The tenth gene, *Dnx* was identified in PI 220127 and was also shown to be located on wheat chromosome 7DS (Liu et al., 2001).

All of the South African wheat cultivars were found to be susceptible to the Russian wheat aphid and therefore it became necessary to use some of the exotic wheat lines to develop new cultivars for South Africa. Four *D. noxia* (*Dn*) resistant wheat lines have been developed and used in breeding programs in South Africa for the development of RWA resistant lines (Du Toit, 1989, Marais and Du Toit, 1993; Marais et al., 1994; Labuschagne and Maartens, 1998; Liu et al., 2001).

It has been shown that the mode of inheritance of the resistance genes in these lines, except for the PI 294994 accession (*Dn5*), was single gene dominance (Du Toit, 1989; Nkongolo et al., 1991). There has been controversy over the mode of inheritance of the *Dn5* gene (Anderson et al., 2003). Three modes of inheritance have been suggested by different authors. These are that

the mode of inheritance for the *Dn5* gene is (1) single dominance, (2) one recessive and one dominant genes are involved and (3) that two dominant genes are involved (Marais and Du Toit 1993; Dong and Quick, 1995; Saidi and Quick, 1996).

The modes of resistance of the *Dn* genes are antibiosis, antixenosis and tolerance or a combined effect thereof (Basky, 2003). The introduction of these genes into susceptible cultivars reduces the formation of chlorotic streaks, lowers the production of nymphs and prevents the curling of the leaves (Du Toit, 1992; Formosoh et al., 1994).

When the fecundity of the aphids is lowered after feeding on the resistant plant, the mode of resistance is referred to as antibiosis. Antixenosis results in the resistant plant being less edible to the aphids and they therefore leave the plant to search for more edible plants. Tolerance is the mode of resistance whereby plants are able to tolerate the feeding of the aphids without showing severe symptoms of infestation (Smith, 1989; Webster et al., 1987; Du Toit, 1989, Formosoh et al., 1994; Kindler et al., 1995; Rafi et al., 1996; Basky, 2003). Although the actions of the genes have been documented, the gene sequences themselves still have to be identified on a molecular level. For this to happen, the genes have to be cloned and characterized.

1.3 Plant-insect and plant-pathogen interactions

Plants and insects existed together for many millions of years and have been interacting throughout. Over this period, beneficial and unfortunate deleterious interactions evolved between the two, but, in most cases it has been the plants that suffer damage by herbivorous insects, which might lead to the plant being killed, depending on the intensity of infestation (Stotz et al., 1999; Mello and Silva-Filho, 2002).

Table 1.1. *Dn* genes currently identified, the source from which they come and their chromosome positions in wheat.

Gene	Source	Position	Inheritance	Authors
Dn1	PI 137739 from Iran	7D	SD	Du Toit, 1989
Dn2	PI 262660 from Russia	7D	SD	Du Toit, 1989
Dn3	*Aegilops tauschii* line SQ24	Unknown	SR	Nkongolo et al., 1991
Dn4	PI 372129 from Russia	1DS	SD	Nkongolo et al., 1991
Dn5	PI 294994 from Bulgaria	7D	SD; 2DR; 2D	Marais and du Toit, 1993; Dong and Quick, 1995; Saidi and Quick, 1996
Dn6	PI 243781 from Iran	1DS	SD	Saidi and Quick, 1996
Dn7	Rye accession	1RS·1BL translocation	SD	Marais *et al*., 1994
Dn8	PI 294994	7DS	Unknown	Liu et al., 2001
Dn9	PI 294994	Unknown	Unknown	Liu et al., 2001
Dnx	Unknown	Unknown	Unknown	Liu et al., 2001
Dny	Unknown	Unknown	Unknown	Liu et al., 2005

S = single; D = dominant; R = recessive; 2DR = two genes, one dominant the other recessive; 2D = two dominant genes

Plants benefit from having associations with insects through pollination, while they provide shelter, oviposition sites and food to the insects (reviewed by Mello and Silva-Filho, 2002). As a result of the damage caused by herbivorous insects, plants have evolved their own defense mechanisms against these pests for millions of years and there is a sharing or similar defense mechanisms

across many plant families. At the same time insects also have evolved over millions of years to overcome the plant defenses in order to survive. Not all defense mechanisms are therefore able to protect the plants fully (De Maagd et al., 1999; Mello and Silva-Filho, 2002).

The damage caused by insects in the agricultural sector is very costly. Trying to combat these pests also has a negative impact on the economy and the environment (De Maagd et al., 1999). Plant diseases reduce crop yields dramatically and this effect is particularly acute in developing countries where the use of pesticide (chemical) control is beyond the means of farmers (McDowell and Woffenden, 2003). Estimates on crop yield losses due to RWA infestations amounts to $ 900 million from 1987 to 1993 in the USA alone (Webster and Amosson, 1994; reviewed by Botha et al., 2005). It is for these reasons that breeders started to look at host-plant resistance to generate crops that are able to withstand insect attack and, at the same time, are still able to give better crop yields. It is therefore important to study and get a better understanding of plant defenses against insect attack (Stotz et al., 1999).

Plants are able to activate defense responses via the hypersensitive response (HR) and this can, in turn, switch on a longterm systemic acquired resistance (SAR), providing resistance against a wide range of pathogens (McDowell and Woffenden, 2003; Stotz et al., 1999). Although this resistance is reasonably well understood when it comes to plant-pathogen interactions, much less is known about plant-insect interactions. The situation changed with the first isolation and identification of a resistance/recognition gene (*Mi* gene of tomato). Knowledge about plant-pathogen interactions is used as the basis to understand the interaction between plants and their insect pests, e.g. the *Mi* gene was shown to be effective against the root knot nematode and a potato aphid species *Macrosiphum euphorbiae* (Stotz et al., 1999; Martin, 1999).

Downstream defenses or secondary responses to RWA attack have been extensively studied by a number of authors (Botha et al., 2005). The response has been indicated to be a typical HR [very specific to reactive oxygen species (ROS), outburst] that occurs during pathogenesis and is

characterized by the activation of intercellular β-1,3-glucanases (Van der Westhuizen et al., 1998a) (implicated in fungal cell wall destruction), peroxidases (Van der Westhuizen et al., 1998b; Moloi and Van der Westhuizen, 2006) (functioning to strengthen plant cell walls by lignification and cross linking) and chitinases (Botha et al., 1998; Van der Westhuizen et al., 1998b).

Plants maintain their ability to recognize invading pathogens by expressing a wide spectrum of *R*-genes. In many of cases, *R*-genes have been shown to provide susceptible plants with complete resistance to one or more strains of some pathogens, thus *R*-genes have been used in many breeding programs (McDowell and Woffenden, 2003). The function of the *R*-genes is to recognize specific pathogen expressed avirulence products in a gene-for-gene interaction (Flor, 1942). There is, however, a problem with this approach in that the genes are quite often overcome by the co-evolving pathogens/ pests, which develop new biotypes to overcome the resistance by escaping early detection by the host. One example of this is the recent development of several Russian wheat aphid biotypes in the USA, which resulted in yield losses in already established RWA resistant wheat cultivars. It was found that all of the commercial lines carrying the *Dn4* gene are susceptible to these new biotypes (Haley et al., 2004; Botha et al., 2005). Another good example of biotype evolution is seen in the Hessian fly – wheat interaction where the Hessian fly larvae are believed to inject some proteins into wheat that are recognized as avirulent factors in a classical gene-for-gene interaction (reviewed by Sardesai et al., 2005). Studies on Hessian fly resistance in wheat have revealed about thirty-two genes conferring resistance to the Hessian fly (genes *H1-H32*; reviewed by Sardesai et al., 2005). Only a few of these have been individually introgressed into commercial cultivars (Williams et al., 2003). This only renders the wheat resistant against specific biotypes for about 8 – 10 years before a new, more virulent biotype develops (Sardesai et al., 2005).

Recently, studies have been undertaken to obtain better insight into the molecular action of *R*-genes and downstream signal transduction pathways and much effort has been put into mapping *R*-genes to enable resistance gene-pyramiding. This strategy promises to improve the durability of pest resistance in wheat cultivars (Sardesai et al., 2005).

1.3.1 The Hypersensitive response

Rapid development of cell death at and around the site of infection is a common feature in pathogenesis of plants (Lam, 2004). This process is termed the hypersensitive response. The HR is triggered by an interaction (either direct or indirect) of a product of the invading pathogen avirulence (*avr*) gene and a corresponding plant resistance (*R*) gene product. Compatible reactions result in the development of disease symptoms because the pathogen overcomes the defense response of the plant, while incompatible reactions result in the plant being able to halt pathogen growth, and therefore no disease symptoms develop (reviewed by Botha et al., 2005).

Programmed cell death (PCD) resulting from the onset of HR usually follows changes in ion fluxes (Ca^{2+} and H^{+} intake; Cl^{-} and K^{+} efflux), generation of ROS, superoxide (O^{2-}) and hydrogen peroxide (H_2O_2) accumulation, cell wall strengthening and activation of various defense genes (reviewed by Morel and Dangl, 1997; Figure 1.2) The ROS are usually directly protective in that they initiate the collapse of challenged cells thereby containing and killing the pathogen in the process, stopping it from spreading in the plant (Moloi and Van der Westhuizen, 2006; McDowell and Dangl, 2000).

ROS directly induce the expression of cellular protectant and defense genes. The expression of the NADPH oxidase gene has been shown to be induced by tissue damage and wounding as well as pathogen infection. The generation of ROS is directly linked to the activation of the NADPH oxidase enzyme (Figure 1.2). The NADPH oxidase complex mediates the formation of superoxide from oxygen at the plasma membrane or at the apoplast and the superoxide is in turn

transformed to hydrogen peroxide with the aid of the superoxide dismutase enzyme (reviewed by Del Río et al., 2002).

ROS are normally produced within cells as byproducts of metabolic reactions. They are able to cause unrestricted oxidation of various cellular components, and as a result, plants have developed mechanisms to detoxify the ROS. O_2^- are scavenged by the enzyme superoxide dismutase while H_2O_2 scavenging is catalysed by ascorbate peroxidase (APX) and catalase (reviewed by Mittler et al., 1999).

Stresses and interactions of plants with pathogens normally disrupt normal homeostasis of the plant cell and this results in an increased ROS production and hence an increased APX expression (Mittler et al., 1999). A study by Mittler and colleagues (1999) suggests that a cytosolic *APX* (cAPX) expression in tobacco is controlled by an HR signal transduction pathway. cAPX expression was activated only after changes in ion fluxes and protein phosphorylation following inoculation of tobacco plants with tobacco mosaic virus (TMV).

Programmed cell death

Plant cell death often results when plants interact with pathogens, irrespective of whether the interaction is compatible or not. Hypersensitive cell death (a localized cell collapse rapidly induced at the site of infection), is genetically programmed. PCD serves to limit the spread of disease to uninfected areas in the plant following HR induction (Morel and Dangl, 1997).

Characteristic morphological markers of PCD include systematic DNA degradation and formation of apoptotic-like bodies, which are similar to animal apoptosis (reviewed by Lam, 2004). The key difference between plant PCD and animal apoptosis is the absence of engulfment by neighboring cells in plants.

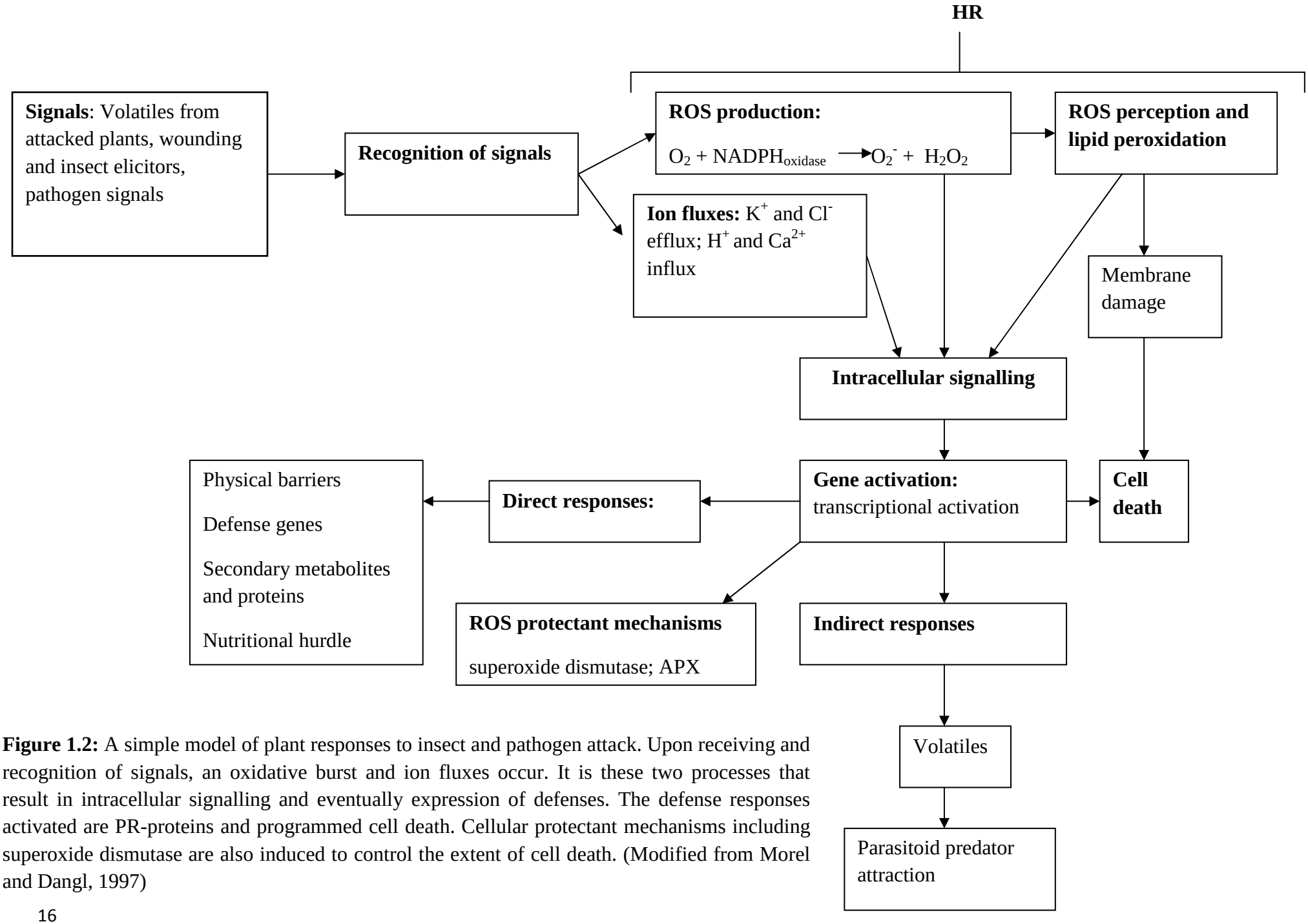


Figure 1.2: A simple model of plant responses to insect and pathogen attack. Upon receiving and recognition of signals, an oxidative burst and ion fluxes occur. It is these two processes that result in intracellular signalling and eventually expression of defenses. The defense responses activated are PR-proteins and programmed cell death. Cellular protectant mechanisms including superoxide dismutase are also induced to control the extent of cell death. (Modified from Morel and Dangl, 1997)

Recent advances in molecular techniques are revealing some interesting regulators of plant PCD which have similar and also some unique properties compared to their animal counterparts (Lam, 2004).

The cytological process of PCD involves chromatin aggregation and DNA cleavage before the disruption of the vacuole which only occurs during the late stages of cell death, blebbing of the vacuole and plasma membranes, and late destruction of organelles. Finally, the plasma membrane collapses and separates from the cell wall, ending with the leakage of the dead cell's content into the apoplast (Lam, 2004). One example of the cytological events is that of the interaction between cowpea and the biotrophic fungus *Uromyces vignae* during an incompatible interaction. Chen and Heath (1991) observed that the nucleus starts migrating to the site of penetration and there is cytoplasmic streaming at 15 hours following inoculation. Halting of the cytoplasmic streaming and the Brownian motion of organelles then follows. The nucleus then condenses, granules accumulate at the edge of the cytoplasm and the protoplast shrinks. Eventually the cytoplasm collapses and the infected cell dies (reviewed by Morel and Dangl, 1997).

Recent molecular studies have identified regulators and signalling molecules of PCD (Lam, 2004). Two steps are necessary for the induction of HR-PCD; 1) recognition of pathogen or stress signal and 2) transduction of the perceived signals to the effectors of PCD (Morel and Dangl, 1997). Some of the components of the defense response are potentially toxic for the plant cell and they could participate directly in PCD. ROS for example have elevated reactivity towards membrane lipids, proteins and the nucleic acids, which can cause loss of cell integrity and viability (Lam, 2004; Morel and Dangl, 1997; Figure 1.2).

Uncontrolled cell death would lead to highly deleterious effects at the tissue level. The plant has thus evolved some protectant mechanisms (Figure 1.2) and anti-cell death pathways. Plant-specific PCD regulators have been identified. The *A. thaliana LSD1* gene and barley *MLO* gene (reviewed by Lam, 2004) are both conserved in monocots and dicots. LSD1 is a repressor of cell death-progression during plant defense, while MLO mediates downstream signalling of calcium channels to suppress HR PCD (Lam, 2004; Morel and Dangl, 1997).

HR PCD induction involves several signals generated in the plasma membrane which then diverge into genetically and biochemically separable pathways that eventually induce expression of defense genes, ROS protectant mechanisms and ultimately cell death (Figure 1.2).

Signalling

The most important factor that ensures an effective defense against pathogens and pests is having efficient signal transduction events (Sessa and Martin, 2000). The signal event includes specific receptors on the plasma membrane or in the cytosol of the cells and proteins responsible for transferring the signal to the nucleus for induction of gene expression. Signaling molecules like salicylic acid (SA), jasmonic acid (JA), systemin and ethylene (Et) are also known to play a role in this signaling event and they have been shown to activate pathogenesis related (PR) proteins like β-1,3-glucanases and chitinases, protease inhibitors and phytoalexins (Keen, 1990; Dangl, 1998; Lamb et al., 1989).

- Receptor proteins

R-genes are classified into five groups based on the structure of their protein products. These proteins are receptors involved in binding specific elicitors (avr proteins). They are: 1) the largest class that encodes the coiled coil (CC) or Toll, interleukine-1 receptor like (TIR) domain fused to a central nucleotide binding site (NBS) domain and a carboxy terminal region containing Leucine rich repeats (LRR), CC-NBS-LRR or TIR-NBS-LRR; 2) receptor-like protein kinases with an extracellular LRR motif; 3) intracellular NBS-LRR proteins with a region similar to TIR proteins; 4) extracellular LRR proteins that are membrane bound and 5) a probable membrane bound protein with a probable intracellular coiled-coil domain (e.g. an *Arabidopsis RPW8* gene, Rathjen and Moffetty, 2003; McDowell and Woffenden, 2003; Stotz et al., 1999; Dangl and Jones, 2001).

- Recognition specificity

Much is still to be discovered on how the R proteins recognise avr proteins and transfer this information in the plant cell to induce/initiate defense. Most plant pathogens live in the extracellular environment and it was for this reason that R proteins were expected to encode extracellular receptor-like proteins. This is true for only a few proteins (like Xa21 and Cf proteins; Martin, 1999) while the majority of *R*-genes encode for intracellular proteins. A suggestion that recognition might actually be occurring inside the cell was brought about by a discovery in bacteria whereby a type III phytopathogenic secretion system allowed injection of the pathogen products directly into plant cells and this was confirmed by the transient expression of many avr proteins inside plant cells (Martin, 1999).

It has been proposed that the LRRs are involved in the recognition of avr proteins since they have been shown to be involved in other protein-protein interactions (Kobe and Deisenhofer, 1995). This was also supported by the fact that the LRRs vary greatly among members in family clusters and, in a few cases, this variation has been directly correlated to new recognition specificities. Furthermore, the rice blast *R*-gene *Pi-ta* was shown to interact with the AVR-Pi-ta protein in a yeast two-hybrid system (reviewed by Martin, 1999). This is, however, still not clear evidence of the general direct involvement of the LRRs in recognition. Domain swap experiments show the involvement of LRRs in pathogen recognition (reviewed by Martin, 1999). There has been evidence of R-avr protein interaction in the *Pto-avr* system, where *Pto* encodes a serine-threonine kinase that lacks a receptor-like domain, but confers resistance to *Pseudomonas syringae* strains and mutations that lead to the disruption of this interaction lead to loss of recognition in the plant cell. This mutation is said to occur at a threonine at position 204 which is conserved in a large number of protein kinases (reviewed by Martin, 1999).

- Localization in the plant cell

Very little is known about the localization of R proteins in the plant cell, although it would be expected that the majority be localised to the cell membrane to intercept incoming pathogen proteins. In one case, an LZ-NBS-LRR protein, RPM1, was found to be enriched in the plasma membrane fraction (Boyes et al., 1998). This supports its direct role in recognition. RPM1 mediates recognition of a type III effector protein from *Pseudomonas syringae* AvrB (Asfield et al., 2004).

Additional evidence of R protein localization is given by the Pto kinase with an amino terminal sequence MGSKYSK. This is similar to a myristoylation motif consensus sequence. Myristoylation plays an important role in mammals in the localization of various kinases and phosphatases to cellular membranes. There was a report that the myristoylation motif of the Fen kinase (related to Pto) was required for its function but this was later disputed by a site directed mutagenesis study that showed that mutations in the critical glycine residue in this motif did not affect the Pto kinase function (reviewed by Martin, 1999).

- Role in signal transduction

NBS-like R-proteins have been noted to be very similar in structure to the mammalian CED-4 and APAF-1 proteins (reviewed by Martin, 1999). These two proteins activate proteases involved in apoptosis. CED-4 and APAF-1 proteins form heterodimers with their respective proteases (CED-3 and caspase-9) through interactions at homologous domains in their amino terminal portions. It has therefore been proposed that by similarity, the NBS-LRR proteins form heterodimers through their LZ/TIR motifs with downstream proteins, and that the NBS would serve as an activation signal of the downstream signalling events. In support of this, studies of the *R*-gene *RPS5* showed that mutations in the third LRR suppressed resistance conferred by multiple *R*-genes (Warren et al., 1998; Martin, 1999), and that overexpression of the wild-type *RPS5* gene in mutants did not completely restore resistance by these genes. These results led to a proposal that the mutation in the LRR increased binding to a pathway component shared by several *R*-genes and interfered with essential signaling (Martin, 1999).

Receptor-like protein kinases

Disease resistance signaling in plants has some common elements to the signaling leading to innate immunity in animals, for example, the NPR1 protein shares homology with the components of the innate-immunity pathways in *Drosophilla malanogaster* (reviewed by Menke et al., 2004). Protein kinases, including those linked to mitogen-activated protein kinase (MAPK) cascades, have been shown to be involved in signaling pathways in both plants and animals (Sessa and Martin, 2000). MAPK transduces extracellular signals into a wide range of intracellular responses. This generally involves three functionally linked protein kinases: a MAPK kinase kinase (MAPKKK), a MAPK kinase (MAPKK) and a MAPK. MAPKKK activates MAPKK upon recognition of external stimuli. Activation occurs via phosphorylation of serine and serine/threonine residues within the SXXXS/T motif (X = any amino acid). MAPKK then phosphorylates the threonine and tyrosine residues in the TXY motif of MAPK, thereby activating it. Then MAPK in turn phosphorylates specific effector proteins leading to the activation of cellular responses (reviewed by Menke et al., 2004).

Plant homologs for all three components of this cascade have been identified and reported by various authors (reviewed by Menke et al., 2004). A number of these MAPKs have been found to play a role in plant defense response. Menke et al. (2004) reported that *MPK6* plays a role in resistance gene-mediated and basal resistance in *Arabidopsis thaliana*, by showing that plants with silenced *MPK6* had enhanced disease susceptibility to virulent and avirulent stains of *Pseudomonas syringae*. A membrane bound calcium-dependent protein kinase (CDPK) was identified by Romies et al. (2000) in *Cf9* transgenic tobacco cells. The accumulation of superoxide

and hydrogen peroxide require a Ca^{2+} influx and protein kinase activity (Romies et al., 2000).

Other MAPKs identified in plants shown to be involved with signal transduction during defense include: wound-induced protein kinase (WIPK) from tobacco, OsBIMK1 in rice and SIMK and MMK3 in *Arabidopsis* (Seo et al., 1995; Song and Goodman, 2002).

Signaling molecules

- Salicylic acid

SA plays an essential role in the establishment of SAR and the development of HR induced by pathogens and their elicitors (Xie et al., 1998). One example of the role of SA in defense signaling was shown by Ryals and colleaques (1995) where tobacco transformants over-expressing the *NahG* gene of *Pseudomonas putida*, which codes for salisylate hydrolase (enzyme responsible for converting SA to catecol), failed to accumulate SA following TMV infection and also failed to induce SAR. The *Arabidopsis* mutants, *sid1*, *sid2* (salicyclic acid induction-deficient 1 and 2) and *pad4* (phytoalexin deficient 4), are defective in SA accumulation upon attack by *P. syringae* pv. *tomato* and *Peronospora parasitica* resulting in increased susceptibility to these pathogens (Pieterse et al., 2001), providing further evidence that SA is important in basal resistance against pathogens.

- Jasmonic acid and Ethylene

Evidence that JA signaling is involved in plant defense and basal resistance against several pathogens came about when JA-response mutants of *Arabidopsis*, *coi1* and

jar1, showed increased susceptibility to *Alternaria brassicicola*, *Botrytis cinerea*, *Erwinia carotovora* and *P. syringae* pv. *tomato* (reviewed by Pieterse et al., 2001). Another study by McConn et al. (1997) on the *fad3*-2, *fad7*-2, and *fad8* mutants, deficient in the jasmonate precursor, linolic acid, showed exaggerated susceptibility (high mortality) to attack by larvae of *Bradysia impatiens* (Diptera: Sciridae) as compared to the wild-type plants that showed very little effect. When methyl jasmonate was applied exogenously on the mutants, they were protected and their mortality rate reduced to approximately 12%. This is evidence that JA plays an important role in resistance against insect herbivory (McConn et al., 1997).

The evidence for the involvement of Et in defense has been contradictory. Some studies have shown that Et-dependant signaling is required for increased resistance to some pathogens (reviewed by Pieterse et al., 2001) while, in other cases, Et was shown to be involved in disease symptom development (Hoffman et al., 1999). In their study, Hoffman and his colleagues used soybean mutants with reduced sensitivity to ethylene to show that the mutants (*Etr1* and *Etr2*) developed similar or less severe disease symptoms when challenged with virulent *Pseudomonas syringae* pv *glycinea* and *Phytophthora sojae*, when compared to the wild-type parents. When some of the mutants were challenged with *Septoria glycines* and *Rhizoctonia solani*, they developed similar, or more severe symptoms, in comparison to the wild-type plants. This suggests that reduced Et sensitivity in plants can be both beneficial and deleterious against different pathogens.

- Systemin

Plants respond to wounding by herbivory insects by expressing proteinase inhibitors to interfere with the digestive processes of the insects. In tomato leaves, systemin, a

polypeptide that is 18-amino acids long, activates the synthesis of proteinase inhibitor I and II proteins in response to injury by chewing insects (McGurl et al., 1994). This polypeptide is cleaved by proteolysis from a protein called prosystemin before or during the injury. Studies on polypeptides that may function in the activation of plant defense genes were initiated by the finding that systemin was capable of inducing expression of protease inhibitors in tomato leaves even without wounding (McGurl et al., 1994). Expression of systemin is normally found throughout the tomato plant (except in the roots) and is wound inducible in the leaves. When systemin was overexpressed in tomato plants, proteinase inhibitor I and II accumulated in the leaves, which is in contrast to wild-type plants that only show production of these proteins in response to chemical inducers or wounding. This study therefore implicates systemin in wound signaling (McGurl et al., 1994).

Pathogenesis related proteins

Plants lack chitin and β-1,3-glucan is not a major component in the plant cell wall. Evidence has however shown that the expression of chitinases and β-1,3-glucanases is upregulated by pathogen invasion and insect attack (Van der Westhuizen and Pretorius, 1996; Van der Westhuizen et al., 1998a, b; Van der Westhuizen et al., 2002; Zhou and Thornburg, 1999). Chitin and β-1,3-glucan are, however, components of fungal cell walls which means that these enzymes are directed against fungal pathogens and therefore the expression of chitinases and β-1,3-glucanases limits growth of their targets (Zhou and Thornburg, 1999). A study by Botha et al. (1998) showed that chitinase activity increased following infestation of wheat plants with the RWA which suggests that chitinase may be involved in the defense against the aphid.

Protease inhibitors

Many studies have focused on the potential use of protease inhibitors against insect pests (Lecardonnel et al., 1998; Stotz et al., 1999). Plant proteases mediate the degradation of storage proteins for the assimilation of nitrogen into biosynthesis pathways during germination. These proteins however, have been implicated in developmental processes such as programmed cell death during the formation of tracheary elements and interaction between plants and other organisms for example pathogen infection and digestion of plant proteins by herbivores (Michaud et al., 1995). Plant protease inhibitors contribute to defense against insects by targeting the digestive proteases in the guts of insects resulting in reduced fitness of the insects and mortality from starvation (Stotz et al., 1999).

Plant proteases are classified into four classes according to their catalytic mechanisms: 1) serine proteases, those with an active serine or histidine in the active centre; 2) cysteine proteases, with a cysteine in the active centre; 3) aspartic proteases, with an acidic amino acid in the active centre and 4) metalloproteases which possess an essential metal involved in the catalytic reaction (Thie and Houseman, 1990). Insects employ different proteases to hydrolyse ingested proteins. Among these are catheptic cysteine and aspartate proteases in Hemiptera, pepsin-like enzymes in some Diptera, and trypsin-like enzymes in Lepidoptera (Thie and Houseman, 1990). It is due to the fact that protease inhibitors specifically inhibit insect proteases that much effort is directed to the genetic manipulation of protease inhibitor genes to enhance insect resistance (Stotz et al., 1999). It is therefore important that the products of these genes are studied for each target insect's the digestive protease system. The gene

products should also be screened to find the most effective inhibitors before plants are transformed with the candidate genes (Girard et al., 1998).

Jongsman (2004) reported two new types of genes to fight sucking insects. The one type is protease inhibitors and the other mono- and sesquiterpene synthase genes. A chymotrypsin inhibitor (Chy8) was found to be five times more effective against the pea and peach aphid (*Myzus persicae*) than the parent trypsin inhibitor, MTI-2 (Jongsman, 2004). Another protease inhibitor tested was a dual inhibitor from sea anemone, equistatin. This protease inhibitor was found to be very effective at inhibiting both the cysteine and aspartic gut proteases of many insects, including western flower thrips (*Frankliniella occidentalis*).

Shortcomings to this approach are that some insects are able to overcome this line of defense by inducing different proteolytic enzymes insensitive to the corresponding plant PIs, increasing the proteolytic activity, or by degrading the plant protease inhibitors with their proteases (Girard et al., 1998; Stotz et al., 1999). Another problem is that the insects can, or may, rapidly evolve a tolerance to the transgenic protease inhibitor, even when it is from sources they have never encountered before.

1.3.2 The systemic acquired resistance (SAR)

Invasion by one pathogen can result in increased defense against another. This is brought about by a signal produced by the first pathogen at the site of infection which then systematically spreads throughout the plant. The spreading signal results in expression of a broad-spectrum, long-lasting immunity in the infected and uninfected areas (Heil, 1999). This resistance is termed systemic acquired resistance. SAR is activated by a variety of compounds like JA, SA, Et and systemin. Induced defenses

during SAR do not depend directly on the type of inducing pathogen. The systemic reaction leads to production of PR proteins like chitinases, β-1,3-glucanases, which protect the cells against further infection (reviewed by Heil, 1999).

Genes whose induction is tightly linked to the onset of SAR have been termed SAR marker genes. A protein can therefore be classified as an SAR protein if its activity directly affects the systemic acquired resistance of plants (reviewed by Ryals et al., 1996). Many of these proteins belong to the class of PR proteins. In tobacco, the SAR marker proteins are: acidic forms of PR1 (PR-1a, PR-1b and PR-1c); the basic isoform of PR-1; β-1,3-glucanase (PR-2a, PR-2b and PR-2c); an extracellular β-1,3-glucanase (PR-1Q'); Class II chitinase (PR-3a and PR-3b); acidic and basic forms of class III chitinase; hevein-like protein (PR-4a and PR-4b); thaumatin-like protein (PR-5a and PR-5b) and a basic protein family (SAR-8.2). In *Arabidopsis*, the SAR markers are PR-1, PR-2 and PR-5. In wheat, chemically induced (*WCI*) genes encoding a cysteine proteinase, lipoxygenase, and three other genes were identified as markers for chemically induced SAR (reviewed by Ryals et al., 1996).

Lignification is regarded to be an important part of plant resistance to pathogens. Lignification has been shown to occur in plants shortly after attack by a pathogen. Lignification strengthens plant cell walls mechanically making them more resistant to degradation by pathogen secreted enzymes. Lignified cell walls are also expected to act as barriers to nutrient flow, causing the pathogen to starve. All these changes to the structure of the cell wall allow the plant to stop or slow down the invasion by a pathogen and allow the plant more time to switch-on further defensive mechanisms (reviewed by Sticher et al., 1997).

Salicylic acid is a very important signalling compound in the induction of SAR. SA and other chemicals can be applied to plants exogenously to induce SAR. For a chemical to be considered a SAR inducer, it must meet the following criteria: (1) the chemical or its metabolites must not exhibit direct anti-microbial activity; (2) the induced SAR must show resistance against the same spectrum of pathogens as the one activated biologically; (3) it should lead to the expression of the same marker genes as in SAR activated by a pathogen (Kessman et al., 1994). The chemicals 2,6-dichloroisonicotinic acid (INA) and benzo(1,2,3)thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) were shown to activate SAR with a broad spectrum disease resistance (reviewed by Ryals et al., 1996).

Cao and colleagues reported in 1994 that an *Arabidopsis* mutant, *npr1* (nonexpressor of *PR* genes), which lacks the expression of SA-, INA-, and pathogen-induced chimeric reporter genes is unable to express other *PR* genes as well. Wild-type plants and the *npr1* mutants were pre-treated with SA, INA or an avirulent pathogen and then later challenged with *Pseudomonas syringae*. They discovered that in the mutants, the lesion was less confined and *PR* gene induction was disrupted whereas the wild-type plants were protected by the pre-treatment in all three cases.

Further evidence that SA is important for SAR induction is provided by the study reported on transgenic tobacco expressing the bacterial gene *nahG*. These transgenic plants were unable to accumulate SA in their leaves and, as a result, could not induce SAR and were therefore not protected against the tobacco mosaic virus. The nahG protein (salicylate hydroxylase) is a catalyst for the reaction to change SA to catecol which is inactive (Gaffney at al., 1993). Delaney et al. (1994) reported that *nahG*

plants are susceptible to many pathogens and are unable to express some *R*-gene defense pathways.

1.3.3 Tritrophic interactions

Induction of plant responses can affect herbivores either directly or indirectly. Herbivore development can be negatively affected or the performance of natural enemies greatly enhanced (Havill and Raffa, 2000). In a study using clonal poplar trees (*Populus nigra*), the gypsy moth, *Lymantria dispar* (L.) and the gregarious parasitoid, *Glyptapanteles flavicoxis* (Marsh), Harvil and Raffa (2000) observed that the parasitoid was three times more attracted to leaf odours from herbivore damaged leaves than those from undamaged leaves. The parasitoids were also more attracted to moth larvae fed on leaves than when they were fed on an artificial diet. Another interesting observation from this study was that induction of a systemic response resulted in a reduced developmental success of both the herbivore and the parasitoid. This study indicates the importance of induced responses on the performance of parasitoids and the effect thereof on the use of parasitoids as biological control agents.

1.4 Genomics and gene discovery

Invasion of plants by a pathogen results in the activation of a hypersensitive response and a signal for acquired resistance is systematically spread throughout the plant. This renders the plant resistant to a wide range of pathogens. The response has been extensively studied and great advances have been made to date with respect to elucidating pathways involved in the interaction of plants and disease causing pathogens. This holds at least for some model plant species like *Arabidopsis thaliana*,

Nicotiana tabacum, *Oryza sativa* etc. With evidence presented to date, it seems that the resistance of wheat to the Russian wheat aphid follows the path of HR induction which is followed by PCD and SAR.

In the "omics" era, scientists are looking more at integrative biology approaches to solve challenges they face in terms of improving living standards by providing food security, health and nutrition. The omics include genomics (the quantitative study of genes, regulatory and noncoding sequences), transcriptomics (RNA and gene expression), proteomics (protein expression), metabolomics (metabolites and metabolic networks) and glycobolomics (glycobiology-focused proteomics). The integration of different "omics" is important if we are to fully understand what drives the plant's ability to withstand, tolerate or defend itself against biotic and abiotic stresses. This would make the production of 'super-plants' for the future possible.

Gill et al., (2004) proposed in a workshop that the hexaploid wheat genome be sequenced for use as a model for all the wheat genomes, including the progenitors of common wheat. This will particularly be advantageous over using rice as model plant for cereals since its genome is very small. For example the genome structures of wheat and rice are very different as a result of how hexaploid wheat evolved. This makes positional cloning of wheat genes with agricultural importance using microlinearity based on rice, an impossible task. Having a fully sequenced wheat genome will make the task of identifying markers for important agricultural traits less complex.

1.4.1 Technologies for gene discovery

Screening for differentially expressed genes is one of the most direct approaches to elucidate the molecular basis of a biological pathway (Lievens et al., 2001). For a

typical eukaryotic cell, a mass of approximately 100 000 mRNAs contains approximately 15 000 to 30 000 unique mRNAs. Numbers of these unique mRNAs range from 1 to several thousands. Abundant transcripts make approximately 50% of the transcript population (a few hundreds). The abundant transcripts represent 1% of the distinct mRNA species in a cell. Rare mRNAs fall within the other 50% of the transcript population (Wan et al., 1996). Trying to isolate a gene responsible for a specialized function, thus becomes a very daunting task because of the fact that it is expressed at low levels, while the rest of the transcriptome is composed of highly abundant mRNAs (Lievens et al., 2001).

With the availability of PCR, it became possible to amplify rare transcripts by increasing the probability of isolation. Differential Display of mRNA by Reverse Transcriptase PCR (DDRT-PCR) was one of the first differentiating methods to take advantage of this (Liang and Pardee, 1992). DDRT-PCR amplifies subsets of mRNA which has been reverse transcribed with anchored oligo dT primers. The oligo dT primer consist of 11 or 12 T's plus two additional 3' bases which provide specificity (Liang et al., 1993). These are then used in conjunction with a 5' arbitrary decamer oligodeoxynucleotide for the subsequent PCR amplification. The resulting cDNA fragments are then separated on denaturing PAGE gels and visualized by autoradiographically (Liang et al., 1993).

Advantages of this procedure are that the method is fast, based on simple and well-established techniques, has increased sensitivity, can be used to compare many samples at a time, and only a small amount of starting material is required. Moreover, it results in an increase in the total number of differential (rare) transcripts leading to the increase in the probability of detecting some of the low abundant ones (Lievens et

al., 2001). There are however drawbacks/ limitations to differential display. These are that the frequency of cloning false positives is high, the frequency of redundancy is also very high leading to a reduced screening efficiency (Lievens et al., 2001). Because of the amount of false positives and the redundancy obtained, DDRT-PCR seemed less attractive than when it was originally presented. Furthermore the downstream verification processes not only become labour intensive, but also require significant amounts of RNA (Lievens et al., 2001).

Many methods using PCR were introduced following the introduction of differential display. Most of these relied on generating an image profile on a gel of the expression patterns of different mRNA samples. These methods avoided the use of arbitrary primers and relied on the presence of restriction enzyme sites on the cDNAs (Lievens et al., 2001). cDNA amplified fragment length polymorphism (cDNA-AFLP) is one such method (Bachem et al., 1996) as it utilized restriction enzyme sites to generate a subset of fragments differing in size which are then amplified with primers specific to previously ligated primers. Eventually one or more nucleotides are added to the 3' ends of the primers to further reduce the cDNA subset that will be displayed. The cDNA-AFLP analysis is suitable for genome-wide expression analysis (Breyne et al., 2003). This method is advantageous over the differential display method in that, it is very efficient, no prior sequence information is required and the results are highly reproducible. Also, it enables the identification of novel genes and amplification is highly specific. The cDNA-AFLP method is an efficient tool for quantitative transcript profiling and a valid alternative to microarrays. The sensitivity and specificity of this method allows for detection of poorly (rarely) expressed genes. Here, expression profiles can be accurately analyzed quantitatively based on banding intensities (Breyne et al., 2003; Yang et al., 2003).

cDNA-AFLP has been successfully used in the past to study differential expression and identify genes involved in developmental processes such as potato tuber development (Bachem et al., 1996), *Arabidopsis thaliana* seed germination (De Diego et al., 2006), seminal root elongation during water deficit (Yang et al., 2003), fruit ripening (Jones et al., 2000), as well as abiotic stresses like salt tolerance (Chen et al., 2003). More interestingly, the cDNA-AFLP technology has been a very useful tool in the quest to study the molecular basis of interactions between plants and disease causing pathogens, including the interaction with environment (Borrás-Hidalgo et al., 2005; Chen et al., 2003; Nyamsuren et al., 2003).

Different approaches have been employed to identify wheat genes that show alteration in their expression patterns during infestation with the RWA. Microarray technology has been widely used to profile transcripts in disease resistance in other plant species. Although thousands of transcripts can be analyzed simultaneously by microarray application, this technique requires prior knowledge and identification of the analyzed transcripts (reviewed by Thompson and Goggin, 2006). Transcripts can be first be identified through methods like suppressive subtractive hybridization and different PCR techniques like DDRT-PCR and cDNA-AFLP (Liang and Pardee, 1992), then used in microarray assays.

The identified cDNA fragments are then amplified and spotted at high densities onto a microarray glass slide. The cDNAs are able to bind to the glass slides because of the presence of some special surface chemistry on the glass slide. Following the immobilization of the transcripts onto the glass slides, the target organism is treated or exposed to certain conditions to induce transcriptional responses. Two different total RNA or mRNA samples are then isolated from the organism and used in the synthesis

of two cDNA probes that have been labeled with the green *Cy3*- and red *Cy5*-flourescent dyes, independently. These probes are then hybridized to the cDNA microarray slides. Probes that have not hybridized to the spots on the slides are then washed off and the slides subsequently scanned using lasers that excite the fluorescent dyes on the hybridized probes. The ratio of the *Cy3*:*Cy5*-induced fluorescence computed for each of the spots on the array corresponds to the relative amount of that particular transcript in the cDNA preparation (Naidoo et al., 2005).

cDNA microarrays have been successfully employed to profile expression of rice (*Oryza sativa*) transcripts during cold, drought and high salinity stress and also following abscisic acid (ABA) application (Rabbanni et al., 2003). Several studies have employed this technology to profile the expression in wheat following infestation with the RWA (Smith et al., 2010) as well as in the development of wheat caryopsis (Laudencia-Chinguango et al., 2007). The caryposis is the fruit of grasses in which the pericarp is fused to the seed coat at maturity and is also commonly referred to as the grain in cereals (Laudencia-Chinguango et al., 2007).

1.5 Study objectives

The objective of this study is to elucidate how early defense response in wheat is regulated during Russian wheat aphid infestation. To do this, we used high throughput gene expression technologies to study responses in near-isogenic wheat lines, to identify and characterise transcripts involved in the defense against RWA.

1.5.1. Scientific question

Which genes are differentially expressed in tolerant and susceptible near-isogenic wheat lines grown in South Africa during early response to attack by *Diuraphis noxia*? Are the expression patterns temporal and/or spatial?

In order to answer the question above, the following technical objectives were pursued:

A. To identify differentially expressed transcripts by cDNA-AFLP and microarray in RWA infested ‘Tugela DN’ and ‘Tugela’ plants at different time points post infestation.

B. To characterise the differentially expressed transcripts identified in response to the RWA attack by cloning, sequencing and BLAST searches

C. To generate expression clusters of differentially expressed genes in near-isogenic wheat lines using cDNA-AFLP generated data.

CHAPTER 2

MATERIALS AND METHODS

2.1 General

2.1.1 Plant material and RWA infestation

'Tugela DN' (SA1684/ *5 Tugela, *Dn1*, RWA resistant) and 'Tugela' (RWA susceptible) seeds were planted in the green house in a 1:1 mix of peat and sand and grown at 22 ± 2 °C. The plants were left to grow until the third to fourth leaf stage, while watering regularly (once daily). Wheat plants were infested with the RWAs. Approximately ten aphids were applied on each plant with a soft paint brush (Botha et al., 1998). Second, third and fourth leaves were then collected from infested and uninfested plants at 0, 1, 2, 6, 12, 24, 48 and 120 hours post infestation (hpi). The leaves were rinsed with water and wiped to remove the aphids and prevent contamination with aphid nucleic acid molecules as described by Zaayman et al. (2009).

2.1.2 Aphid population

RWA (*Diuraphis noxia*, Kurdjumov) population SAM1 (Van Zyl, 2007) was maintained in net cages on the RWA resistant wheat line, 'Tugela DN' to manage the aphid population size. Prior to infestation the RWAs were starved for six hours to force immediate probing.

2.1.3 mRNA preparation

Total RNA extraction:

All glassware, plastic ware and mortars and pistils were treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC) solution overnight and autoclaved for 30 minutes at 121 °C. The mortars and pistils were then baked at 200 °C for at least 4 hours before use (Sambrook et al., 1989). Distilled water was treated with 0.1% (v/v) DEPC overnight and autoclaved to deactivate the

DEPC. All buffers and solutions were then prepared with RNase free water and autoclaved for 30 minutes.

A modified total RNA extraction method of Chomczynski and Sacchi (1987) was used. One gram of leaf material was collected. After wiping the plant leaf material with tissue paper to remove all RWAs, the leaves were frozen in liquid nitrogen, and then ground to powder ensuring that it remained frozen at all times. One ml of GITC buffer [4 M GuanidineIsoThioCyanate, 100 mM Tris-HCl (pH 8), 25 mM Sodium Citrate (pH 8), 100 mM β-mercaptoethanol and 0.5% (w/v) N-Lauroyl Sarcosine] per 100-200 mg of leave tissue was then added to the powdered leaf material. After proper mixing of ground tissue with the extraction buffer, the mixture was incubated for 10 minutes at room temperature and centrifuged for 20 minutes at 10 000 x *g*. The supernatant was then transferred to a new tube where after 0.05 ml 2 M Sodium Acetate (pH 4) and 0.50 ml H_2O-buffered Phenol/ Chloroform (1:1) per 1 ml of 4 M GITC buffer were added. Vigorous shaking by vortex for 15 seconds ensured proper mixing of the mixture. The sample was then incubated at room temperature for 10 minutes and then centrifuged at 10 000 x *g* for 10 minutes. The supernatant was carefully transferred to a new tube for RNA precipitation by the addition of 1 volume isopropanol to 1 volume supernatant. Complete mixing by a gentle inversion followed. The nucleic acids were then precipitated by incubation at -20 °C for 1 hr and collected by centrifugation at 13 000 x *g* for 30 minutes. The pellet was washed three times with 500 µl RNase free 75% (v/v) ethanol and centrifuged at 10 000 x *g* for 15 minutes. The pellet was finally air-dried for ten minutes and resuspended in 100 µl RNase free 0.1% (v/v) DEPC-treated water.

An aliquot of the total RNA sample was analysed on 1% (w/v) TAE (0.04 M Tris-acetate, 0.001 M EDTA electrophoresis buffer) agarose gel containing ethidium bromide (agarose/EtBr) at 90 V for 20 minutes to verify the integrity of the extracted RNA. The samples were visualized under UV light following separation. The concentrations of the extracts were determined

spectrophotometrically using the NanoDrop® ND-1000 spectrophotometer (V3.0.1). All samples were then stored at -80 °C in the freezer until further use.

Poly (A^+) mRNA purification:

Before isolating mRNA from total RNA, contaminating DNA was removed by the addition of RNase free DNase enzyme (10 U/ 20 µl reaction) in the presence of a 5 X reverse transcriptase buffer containing Mg^{2+} ions and incubation at 37 °C for 30 minutes. Digested DNA was removed from total RNA samples using the RNeasy Plant Mini kit (Qiagen, USA) according to manufacturer's instructions. Cleaned RNA samples were eluted from the RNeasy columns in 50 µl RNase free water. Total RNA integrity then was verified by agarose gel electrophoresis as described above. Poly(A^+) mRNA was purified from total RNA using the Oligotex mRNA purification Kit by Qiagen (USA) following the manufacture's instruction and eluted in 25 µl RNase free water. A small sample was quantified using the NanoDrop® ND-1000 spectrophotometer (V3.0.1).

2.2 Complimentary DNA (cDNA)-amplified fragment length polymorphysms (AFLPs)

2.2.1 Double stranded cDNA synthesis

Double stranded (ds) cDNA was synthesized from each of the mRNA (500 ng) samples using a cDNA synthesis system (Roche Molecular Biochemicals, Germany). Following the second strand synthesis reaction, the cDNA was purified by a MinElute reaction cleanup kit (Qiagen, USA). The cDNA was analyzed by 1% (w/v) agarose/EtBr gel and the concentration of the cDNA was determined spectrophotometericaly using the NanoDrop® ND-1000 spectrophotometer (V3.0.1).

2.2.2 cDNA-AFLP analysis

The cDNA-AFLP procedure was performed using the LI-COR Expression Analysis kit (LI-COR Biosciences, USA) following the supplier's instructions for the template preparation and the selective amplification. *TaqI* +2 and *MseI* +2 primers (represented by T-NN and M-NN respectively; where T or M are *TaqI* or *MseI* primers and N represents either of the four bases, T, C, A or G) were supplied in the kit for the selective amplification. Ten primer combinations (M-AC/T-GA; M-AC/T-GT; M-AC/T-TC; M-AC/T-TG; M-AC/T-CT; M-AC/T-CA; M-AC/T-AG; M-AC/T-AC) were used in the selective amplification reactions. All PCR reactions were done on a Perkin-Elmer GeneAmp PCR System 9700 DNA thermal cycler (Applied Biosystems, USA). Selective amplification products were then separated on 8% (w/v) LongRanger polyacrylamide gels [7.0 M Urea, 0.8 X TBE (0.072 M Tris borate and 0.0016 M EDTA, pH 8), 8% (w/v) acrylamide/bisacrylamide, 0.075% (w/v) ammonium persulphate (APS) and 0.075% (v/v) N,N,N',N'-Tetramethylethylenediamine (TEMED)] at 1500 V for 4 hours using the LI-COR Global edition IR^2 Automated DNA analyzers (Model 4200 LI-COR Biosciences, USA), which generated and captured the cDNA-AFLP images.

2.2.3 Image analysis and TDF quantification

Image analysis was done with the AFLP Quantar*Pro* software (KeyGene products B. V., Wageningen, The Netherlands). Band sizes and intensities on the images were determined using this software. The instructions provided in the AFLP-Quantar*Pro* user manual were followed to find lanes, bands, and size the bands. All TDFs visualized were quantified in AFLP-Quantar*Pro* and band intensities were exported to Microsoft Excel spreadsheets for further analysis.

2.2.4 Fragment recovery, cloning and sequencing

Following analysis of the cDNA-AFLP images, TDFs with differential expression between Tugela and 'Tugela DN' and across the time trial were identified and targeted for recovery. The selective amplification products were re-run on 8% LongRanger acrylamide gels for a shorter time (2 hours). The gels were then scanned on the Odyssey Infrared Imager (LiCor) to generate an image on which the gels could be aligned and the target fragments excised. These were then placed in separate tubes containing 20 µl of low TE-buffer [10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA], freeze-thawed four times by freezing them in liquid nitrogen and placing at room temperature to thaw and mixing by pipetting up and down. The recovered fragments (3 µl) were then reamplified by PCR to enrich abundance of the excised fragment. TaqI + 0 and MseI + 0 primers (see Table 2.1 for sequences) were employed in this step. The amplification program performed included 30 cycles of a denaturing step at 94 °C for 30 seconds, primer annealing at 56 °C for 30 seconds and elongation at 72 °C for a minute. A hold at 72 °C for 2 minutes was included at the end of the 30 cycles and was followed by a hold at 4 °C. Reamplification of the TDFs was verified on a 3% (w/v) agarose gel before cloning.

The re-amplified fragments were then cleaned by a Qiagen MinElute Reaction Cleanup Kit (USA) and ligated to pTZ57R vector provided in InsT/Aclone PCR Product Cloning kit

(Fermentas Life Sciences, USA). Ligation was performed overnight using half reaction volumes as was recommended in the cloning kit. JM109 high efficiency competent cells (>10^8 cfu/µg; Promega, UK) were transformed with the ligation mix following the manufacturer's instructions (Promega, UK). Blue-white colour screening for recombinants was done on LB plates (10 g bacto-tryptone, 5 g bacto-yeast extract, 10g NaCl, 15 g agar and water to 1 000 ml) containing 100 µg/ml ampicillin, 0.5 mM isopropylthio-β-D-galactoside (IPTG) and 40 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), where white colonies contained the insert. White colonies were picked and send to InqabaBiotech (South Africa) for sequencing. Sequencing reactions were done using the BigDye version 3.1 dye terminator cycle sequencing kit from Applied Biosystems. The cycling products were then analysed on Spectrumedix SCE2410 genetic analysis system with 24 capillaries (SpectruMedix LLC in Pennsylvania, USA). Sequence outputs were analysed using Chromas program (Version 1.45, Australia) where the sequences were edited based on the chromatogram peaks. Edited text sequences were assigned putative identities by nucleotide-nucleotide BLAST (BLASTn) and translating BLAST (BLASTx) analysis (Altschul et al., 1990). The threshold for significant homology was $1e^{-10}$.

Table 2.1 List of primers used for reamplification of excised fragments.

Primer name	**Sequence (5'-3')**	**T_m (°C)**
MseI +0	GATGAGTCCTGAGTAA	46.0
TaqI +0	TGTAGACTGCGTACCGA	52.0

2.3 cDNA microarray analysis

2.3.1 Fluorescent probe preparation

Cy3- and *Cy5*-fluorescently labeled cDNA probes were synthesized using the Cyscribe Post-Labelling Kit (Amersham Biosciences, Little Chalfont, UK). The cDNA probes were synthesized from the purified mRNA samples. The mRNA samples were thawed and

concentrated by drying of the samples in a SpeedyVac centrifuge and then resuspending in 5 µl RNase-free water and the entire sample used for probe synthesis. Depending on the concentration of the mRNA that was available 100 ng to 500 ng mRNA was used for the synthesis of the cDNA probes. For each of the mRNA samples of the different time points post infestation (day 0, -2, -5 and -8 p.i.), both *Cy3*- and *Cy5*-labeled cDNA probes were synthesized following the protocol supplied with the kit. The synthesized probes were protected from light. Following the probe synthesis, unincorporated dye molecules and nucleotides were removed using the Minelute cleanup kit (Qiagen Inc., USA) as per manufacturer's instructions. The concentration of the synthesized cDNA probe was determined using the NanoDrop® ND-1000 spectrophotometer (V3.0.1) spectrophotometer.

2.3.2 Microarray hybridization

Microarray slides were prepared by Botha and colleaques in 2006 as reported in appendix B. the target DNA printed onto the slide included 256 wheat ESTs, 50 flax and banana genomic clones and control genes. These microarray slides were used in the hybridization experiments.

For prehybridization, 35 µl of prehybridization solution [3.5x SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) 0.2% (w/v) sodium dodecyl sulfate (SDS), 1% (w/v) bovine serum albumin (BSA)] was added to the microarray slides and the slides were placed into a humidified hybridization cassette and incubated at 60 °C for 20 minutes in a waterbath. The slides were then washed in double distilled water (ddH_2O) for 1 minute and air dried using nitrogen gas. Dual colour hybridizations were performed whereby the two labeled probes were combined into one tube for all the different probe combinations. Table 2.2 lists the probe combinations that were performed.

Table 2.2 Probe combinations for dual dye microarray hybridizations.

Microarray slide number	Probe Combinations	
	Cy3 probe	*Cy5* probe
01 [13445]	Control 0-d.p.i. (C_0)	Induced 2-d.p.i. (I_2)
02 [13446]	Induced 2-d.p.i. (I_2)	Control 0-d.p.i. (C_0)
03 [13447]	Control 0-d.p.i. (C_0)	Induced 5-d.p.i. (I_5)
04 [13448]	Induced 5-d.p.i. (I_5)	Control 0-d.p.i. (C_0)
05 [13451]	Induced 2-d.p.i. (I_2)	Induced 5-d.p.i. (I_5)
06 [13452]	Induced 5-d.p.i. (I_5)	Induced 2-d.p.i. (I_2)
07 [13453]	Induced 2-d.p.i. (I_2)	Induced 8-d.p.i. (I_8)
08 [13454]	Induced 8-d.p.i. (I_8)	Induced 2-d.p.i. (I_2)

The handling of all the probes were performed at very low light conditions to minimize exposure of the probes to light. For each probe combination, equal quantities of each probe (30 pmol each) were combined into a single 0.5 ml tube and dried in a SpeedyVac centrifuge for 30 minutes at 40 °C. The mixture of probes was then resuspended in 35 µl of hybridization solution [50% (v/v) formamide, 25% (v/v) hybridization buffer, 25% (v/v) deionised water]. The probes were then denatured at 98 °C for 2 minutes and cooled down on ice for 30 sec. The entire hybridization mixture (~ 35 µl) was pipetted onto the part of the microarray slide where no clones were immobilized and then carefully covered with a coverslip ensuring no air bubbles get trapped beneath the coverslip, and that all the target clones on the slide are covered with the hybridization mix. The slides were then placed into a hybridization cassette and subsequently incubated at 42 °C for 12 – 18 hrs in a waterbath (creating a humid hybridization chamber).

Following hybridization, the slides were washed as follows: once in 1x SSC, 0.2% (w/v) SDS solution for 4 minutes at 37 °C; twice in 0.1x SSC, 0.2% (w/v) SDS solution at 37 °C for 4 minutes; twice in 0.1x SSC for 1 minute at room temperature. The washes were followed by rinsing the slides in deionized water for 2 sec. The slides were then dried using nitrogen gas. Two slides were hybridized per probe combination.

2.3.3 Microarray scanning and data analysis

Microarray slides were scanned using an Axon GenePix 4000 Microarray scanner and GenePix acquisition software (Axon Instruments, Inc., USA) according to the manufacturer's instructions. The level of the photomultiplier gains were adjusted in order to normalize between *Cy3*- and *Cy5*- fluorescent dye emission intensities ('global normalization'). Following the scanning and capturing of data, raw data was imported into Microsoft Excel for further analysis. Background intensities that were automatically calculated by the GenePix programme (http://www.moleculardevices.com) were subtracted from all fluorescent dye intensities obtained for the microarray spots before using them in any calculations. Transcripts of interest were then identified by computational analysis using ANOVA (Dudoit et al., 2001), the mixed model approach (Wolfinger et al., 2001; Chu et al., 2002) as well as SAS/STAT software version 8.0 (SAS Intitute Inc. 1999).

CHAPTER 3

RESULTS

3.1 Optimization of RNA isolation from wheat leaf tissue

Different RNA extraction methods were performed in order to determine which one would yield best quality total RNA in higher quantities for our study. The different extraction protocols were all based on modifications to the total RNA isolation method by Chomczynski and Sacchi (1987) that uses guanidinium thiocyanate and phenol-chloroform to isolate RNA. This method however cannot distinguish the RNA from the DNA and yields total RNA that is contaminated with genomic DNA (Figure 3.1). The different methods are listed in Table 3.1.

All the tested methods yielded fairly high amounts of total RNA. The high DNA contamination in the first three methods listed in the Table 3.1 skewed the initial concentration of the total RNA extracted. The contaminating DNA molecules were removed by RNAse free DNase treatment (Promega) and the concentrations re-determined. Total RNA extracted using the Trizol and the TriPure reagents was of good quality, with little or no DNA contamination and the RNA was visually assessed and seemingly not degraded. The total RNA extraction method 3 yielded the highest amount of total RNA (Figure 3.1) and was therefore selected as the method of choice for total RNA extraction for further use in the study.

Intact RNA samples are indicated by a smear ranging from 100 to about 10 000 base pairs (bp) with the bulk of it lying between 2 000 and 4 000 bp (Figure 3.2). The presence of two intense bands within the smear at position 3 000 and 2 000 bp which represents the most abundant rRNA molecules (25S and 18S, respectively) is an indication of good quality total RNA (Figure 3.2). Impurities in the total RNA interfere with the binding of the poly(A^+) mRNA to the oligo dT Qiagen oligotex mRNA purification kit, resulting in the reduced amount of mRNA purified. It was therefore important to ensure that the total RNA samples are pure. The ratio of the absorbencies at 260:280 nm wavelengths as determined spectrophotometrically indicate the purity the total RNA samples. Ratios indicative of pure samples are between the range 1.8-2.1 for the A260:A280.

Table 3.1 Different total RNA extraction methods tested for best total RNA yields.

Method	Extraction buffer	Phenol	Chlorofom	PVPP	Starting material	Total RNA quality
1	4 M GITC 25 mM sodium citrate 0.5% N-lauroylsarcosine 100 mM 2-Mecarptoethanol sodium acetate	Tris-EDTA (pH 8) equilibrated	Chloroform: acetic acid (49:1)	No	1 g wheat leaves ground in liquid nitrogen to a powder	DNA contamination, High salt/ carbohydrate contamination, 100-200 µg total RNA, slight degradation
2.	5M GITC 25 mM sodium citrate 0.5% N-lauroylsarcosine 100 mM 2-Mecarptoethanol sodium acetate	H_2O-buffered (1 ml DEPC-H_2O for every 5 g of phenol)	100% chloroform	Yes (1-2% (w/v) extraction buffer)	1 g wheat leaves ground in liquid nitrogen to a powder	DNA contamination, moderate salt/ carbohydrate contamination, 150-200 µg total RNA, slight degradation
3	4 M GITC 25 mM sodium citrate 0.5% N-lauroylsarcosine 100 mM 2-Mecarptoethanol sodium acetate	H_2O-buffered (1 ml DEPC-H_2O for every 5 g of phenol)	100% chloroform	No	1 g wheat leaves ground in liquid nitrogen to a powder	DNA contamination, moderate salt/ carbohydrate contamination, 150-250 µg total RNA, intact
4.	Trizol reagent (Invitrogen)	Included in the extraction buffer	100% chloroform	No	1 g wheat leaves ground in liquid nitrogen to a powder	Little/ no contaminating DNA, Less salt/ carbohydrate contamination, 100-150 µg total RNA/ g leaf material, intact
5.	TriPure reagent (Roche)	Included in the extraction buffer	100% chloroform	No	1 g wheat leaves ground in liquid nitrogen to a powder	Little or no contaminating DNA, Less salt/ carbohydrate contamination, 100-150 µg total RNA/ g leaf material on average, intact.

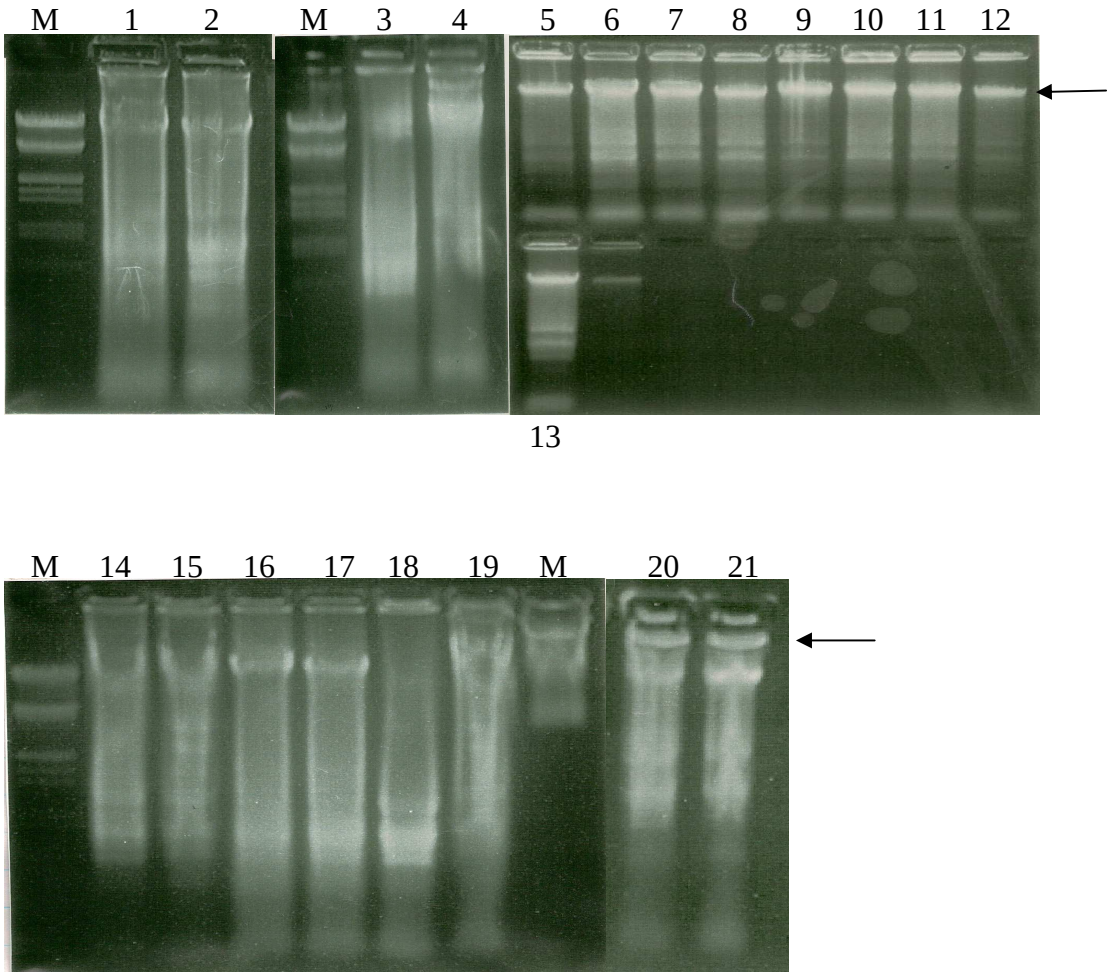


Figure 3.1 TAE agarose gel (1%) analysis (90 V, 20 minutes) of total RNA extracted from 'Tugela DN' and 'Tugela' leaves at different time points post infestation with the RWA. Lanes marked M represent molecular weight marker λIII. The total RNA extracts from 'Tugela' leaves are represented as follows: lanes 1 and 2 (0-hpi), lanes 5, 7, 9 11, 14, 16, & 18, represent leaf material collected at 1-, 2-, 6-, 12-, 24-, 48- (2 days), 120- (5 days) and 192-hpi (8 days), respectively. Total RNA extracts from 'Tugela DN' leaves are represented in lanes 3 and 4 (0-hpi), in lanes 6, 8, 10, 12, 13, 15 and 17 are from leaves collected at 1-, 2-, 6-, 12-, 24-, 48- (2 days), 120-hpi (5 days), respectively and lanes 19, 20, and 21 represent total RNA extracted from 'Tugela DN' leaves collected at 192-hpi (8 days). The arrows in the figure indicate the presence of genomic DNA molecules in all the samples (size ~20 kbp).

The quantities and the ratios A260:A280 and A260:A230 of the initial total RNA extracts, the clean total RNA samples and mRNA samples are given in Table 3.2. All the total RNA samples had salt contamination indicated by a low A260:A280 ratio of approximately 1.6 in all the extracts.

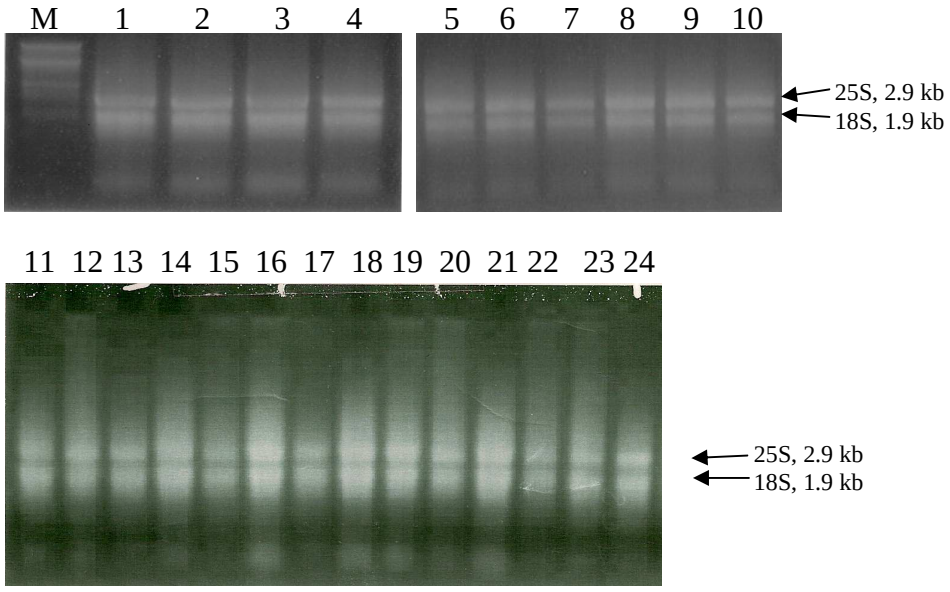


Figure 3.2 TAE agarose gel (1%) analysis of Total RNA following DNase treatment and purification through the RNeasy cleanup columns (Qiagen Inc., USA). Lane M is molecular weight marker λIII and the other lanes represent the cleaned total RNA samples from 'Tugela' and 'Tugela DN' and leaves collected at different time points post infestation with the RWA. The arrows in the figure indicate the presence of 25S and 18S rRNA bands that are 2.9 and 1.9 kb in size, respectively.

The total RNA clean-up with the Qiagen RNeasy cleanup columns (Qiagen Inc., USA) was successful in removing these salt contaminants. This is indicated by the improvement in the A260:A280 ratio ranging from 1.8-1.9 (Table 3.2). This falls within the desired range indicative of pure RNA samples.

Table 3.2 Concentrations (ng/µl) and the A260:A280 ratios of extracts obtained following total RNA extraction, total RNA clean up and mRNA purification, as determined spectrophotometrically. 'Tugena DN' extracts in the table are indicated as dn1, while 'Tugela' extracts are labeled as tu. The number of hours post infestation of the samples are provided after the sample names.

Total RNA extract (hpi)	Concentration (ng/µl)	A 260/280	Total RNA cleanup	Concentration (ng/µl)	A 260/280	Poly (A^+) mRNA	Concentration (ng/µl)	A 260/280
dn1-0	1752.46	1.61	dn1-0	1464	1.92	dn1-0	73.74	1.98
dn1-1	1607.03	1.61	dn1-1	1037	1.89	dn1-1	64.16	1.88
dn1-2	1756.45	1.68	dn1-2	1536	1.92	dn1-2	124.48	1.88
dn1-6	1612.47	1.61	dn1-6	1312	1.92	dn1-6	54.86	1.88
dn1-12	1743.44	1.63	dn1-12	1535	1.94	dn1-12	56.38	1.59
dn1-24	1665.42	1.64	dn1-24	1050	1.94	dn1-24	84.71	1.89
dn1-48	1599.02	1.62	dn1-48	1384	1.76	dn1-48	50.06	1.89
dn1-120	1764.42	1.62	dn1-120	1324	1.81	dn1-120	70.76	1.8
dn1-192	1291.05	1.69	dn1-192	989	1.84	dn1-192	49.45	1.85
tu-0	1775.16	1.65	tu-0	1521	1.97	tu-0	78.71	1.87
tu-1	1520.31	1.64	tu-1	1380	1.96	tu-1	103.36	1.96
tu-2	1506.03	1.62	tu-2	1291	1.81	tu-2	70.02	1.87
tu-6	2850.91	1.69	tu-6	1977	1.91	tu-6	103.86	2.06
tu-12	1727.06	1.64	tu-12	1678	1.88	tu-12	110.42	1.82
tu-24	1944.67	1.61	tu-24	1613	1.88	tu-24	62.86	1.86
tu-48	1856.34	1.63	tu-48	1343	1.68	tu-48	55.55	1.83
tu-120	1841.04	1.68	tu-120	1298	1.81	tu-120	67.12	1.81
tu-192	1237.45	1.64	tu-192	980	1.82	tu-192	37.05	1.8

Recovery of the total RNA following total RNA purification ranged from 60 to 80%, including that there was contamination with genomic DNA in the initial samples, which skewed the initial spectrophotometric readings (Table 3.2). The total RNA samples were eluted in 50 µl RNase free water and overall, the total quantities of the total RNA before mRNA purification ranged from 49 µg to 100 µg. For both the 'Tugela DN' and 'Tugela' leaf material, the least amount of RNA was obtained from leaf material collected eight days post infestation (192 hpi) with the Russian wheat aphid. The efficiency of mRNA purification from purified total RNA ranged from 3% to 5% of the total RNA samples (Table 3.2).

3.2 Optimization of cDNA-AFLP analysis

Double stranded cDNA synthesis and preparation of template for the cDNA-APLP procedure:

Five hundred nanogram of each mRNA sample was used for the synthesis of ds-cDNA. Analysis of the cDNA synthesized by 1% (w/v) agarose gel indicated cDNA with size ranges from 100 bp to above 1 500 bp (Figure 3.3). The results were indicative of a good cDNA pool.

Synthesized cDNA samples were purified of all unbound dNTPs and very short fragments before use in the cDNA-AFLP experiments. Analysis of the preamplification products on 1% (w/v) agarose gels revealed TDF pools ranging from 100-500 bp with the bulk of it around the 250 bp mark (Figure 3.4).

Selective amplification

Selective primer combinations were screened using the 'Tugela DN' derived cDNA samples of leaves collected at 0, 48 and 120 hours post infestation with the RWA. Figure 3.5 represents 8 primer combinations. The profiles revealed the presence of several bands throughout the time course at similar intensities. These are representative of constitutively expressed genes (red arrows, Figure 3.5).

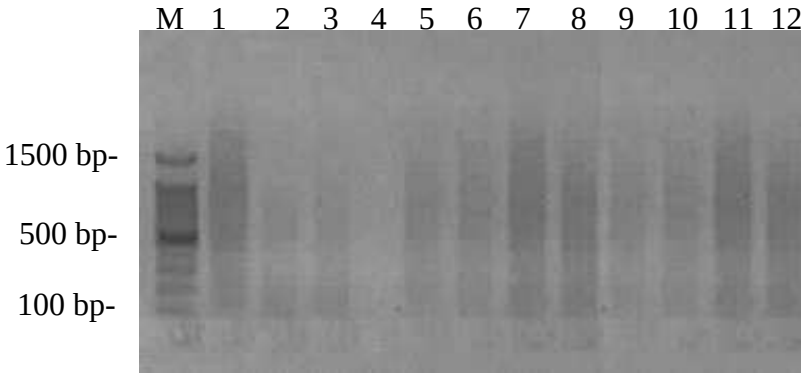


Figure 3.3 TAE agarose gel (1% w/v) analysis of ds-cDNA derived from leaf material collected from 'Tugela DN' and Tugela plants at 0-, 1-, 2-, 6-, 12-, and 24-hpi. The lane marked M represents a 100 bp ladder molecular weight marker. The sizes are indicated on the left of the image. Lanes 1, 3, 5, 7, 9 and 11 represent 'Tugela DN' derived cDNA samples while the 'Tugela'derived cDNA samples are represented in lanes 2, 4, 6, 8, 10 and 12.

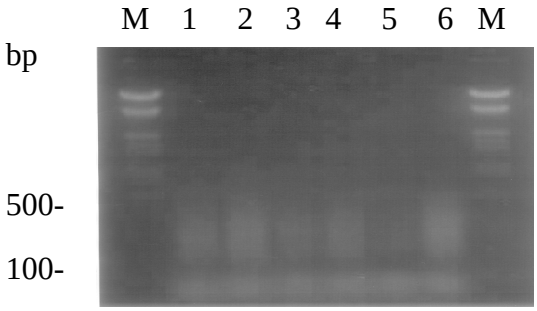


Figure 3.4 EtBr/Agarose gel (1.2% w/v) analysis of preamplification PCR products. Lane M = the molecular weight marker λIII, Lanes 1-6 preamplification product of 'Tugela DN' cDNA at 0, 1, 2, 6, 12, and 24 hpi, respectively.

Results also revealed some TDFs that were up-regulated at 48 hpi and down-regulated by 120 hpi and *vice versa* (black arrows, Figure 3.5).

Primer combinations were screened and selected according to the number of TDFs that resulted in clear and repeatable TDFs with differential regulation (Figure 3.5). The numbers of TDFs were counted for the primer combinations indicated in Figure 3.5, and they are listed in Table 3.3

Table 3.3 Expression profiles generated for selective amplification screening.

Primer pair	M-AC/ T-GA	M-AC/ T-GT	M-AC/ T-TC	M-AC/ T-TG	M-AC/ T-CT	M-AC/ T-CA	M-AC/ T-AG	M-AC/ T-AC
# TDFs	93	104	103	130	129	130	121	132

Primer combination M-AC/T-AC had the largest number of clear repeatable differentially expressed TDFs, whereas primer combinations M-AC/T-GA and M-AC/T-TC had the least number of clear TDFs. Transcript derived fragments from 'Tugela' and 'Tugela DN' obtained at eight different time points post infestation of the wheat leaves with the RWA were selectively amplified to generate expression profiles with 10 selected primer combinations (images not shown) for large scale screening purposes.

In order to ensure that the cDNA-AFLP experiment conducted was reliable and repeatable, the pre-amplification product at each time point was halved, and two separate selective amplifications were performed. The two selective amplification products were run alongside each other and compared. Similar profiles were obtained indicating the reliability of the selective amplification (Figure 3.6 A and B). This is an indication of the reliability of the selective amplification. Band intensities obtained from the AFLP Quantar*Pro* software (KeyGene products B. V., Wageningen, The Netherlands) analysis of the cDNA-AFLP images were used to draw bar graphs depicting expression of individual TDFs as shown in Figure 3.6 C. Figure 3.6 C further shows that the selected TDFs were expressed differentially in wheat at different time points post infestation with RWA. A biological replicate (i.e., RNA extracted from another set of infested plants over time) was also done. There was 97% repeatability between the experimental replicates and 84% repeatability between the biological replicates (data not shown). The obtained results indicate high reliability between technical and biological repeats.

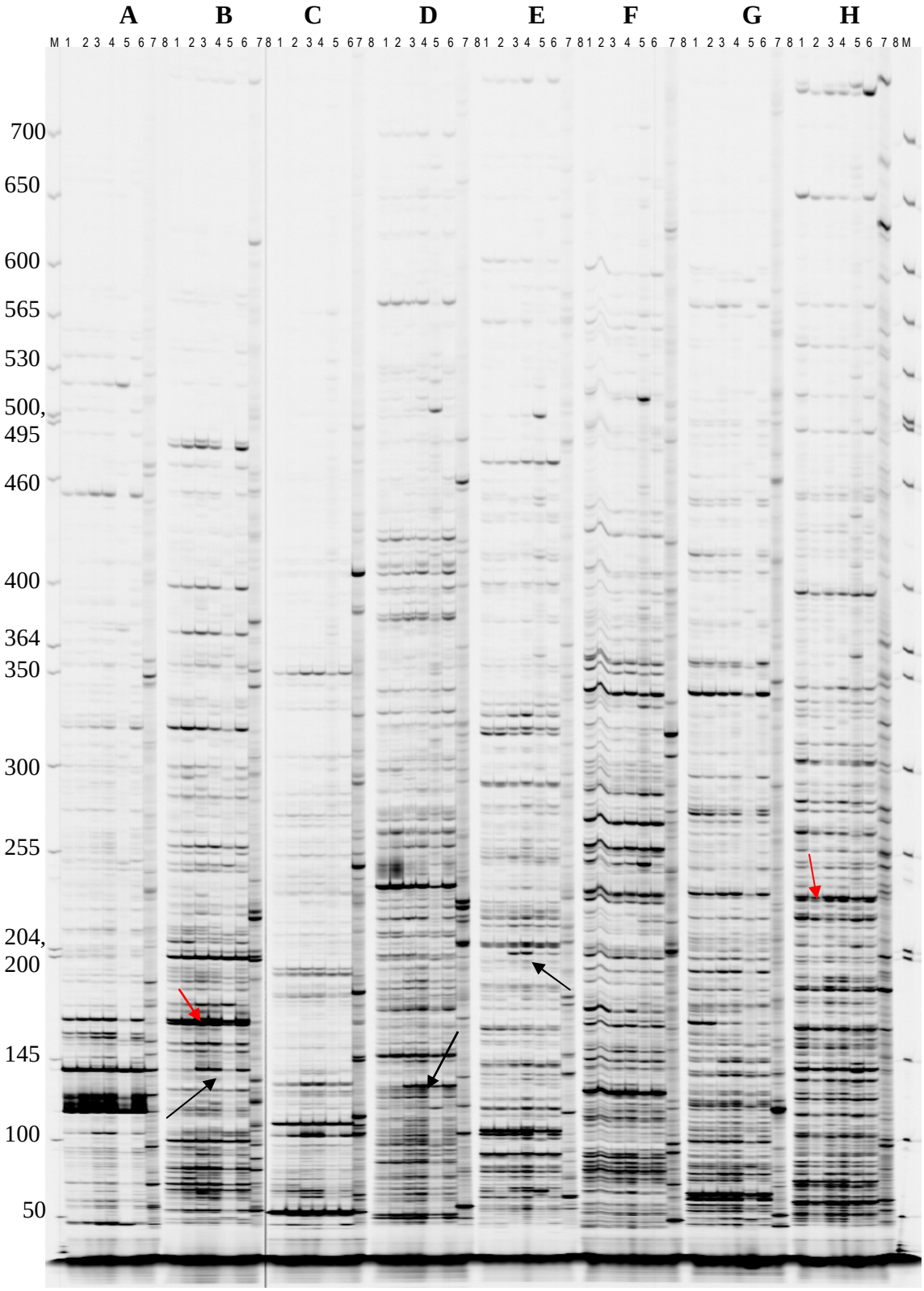


Figure 3.5 LongRanger polyacrylamide gel (8% w/v) image of selective primer combination screening. cDNA derived from 'Tugela DN' leaves collected at 0, 48 and 120-hpi was used to screen different selective primer combinations. Each block (A – H) of eight lanes represents a set of a specific primer combination, where A= M-AC/T-CA, B= M-AC/T-GT, C= M-AC/T-TC, D= M-AC/T-TG, E= M-AC/T-CT, F= M-AC/T-CA, and G= M-AC/T-AG and H= M-AC/T-AC. In each block lanes 1 and 2= 0 hpi, 3 and 4= 48 hpi, 5 and 6= 120 hpi, 7= maize gDNA (positive control), and 8= no DNA (negative control). Lanes M= IRDye700 molecular weight marker. Black arrows point at differentially expressed transcripts and the red arrows show the constitutively expressed transcripts.

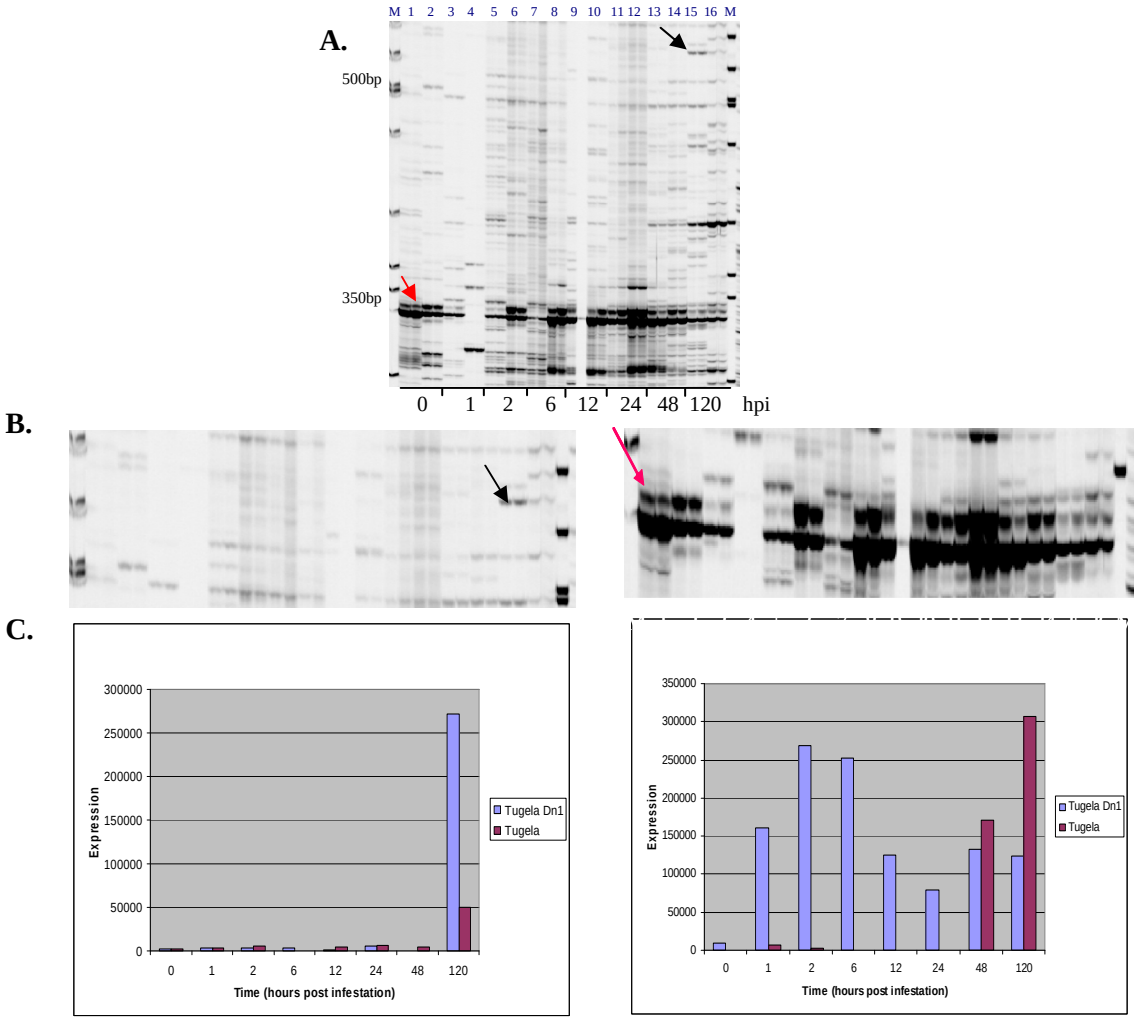


Figure 3.6 LongRanger polyacrylamide gel (8% w/v) image of selective amplification of wheat cDNA fragments with the M-AC/T-CA primer combination. Lanes 1, 3, 5, 7, 9, 11, 13, and 15 (in both A and B) represent 'Tugela' (susceptible) samples and 2, 4, 6, 8, 10, 12, 14, and 16 'Tugela DN' (resistant) samples in these images. The infestation time trial runs from left to right, as outlined below the image (A). Figure B is an image enlargement of fragments indicated in A by the red and black arrow. These are examples of differentially expressed TDFs. Figure C is a representation of the band intensities of the fragments indicated in B.

3.3 cDNA-AFLP mediated minisequencing

Transcripts that showed differential expression over the time trial were targeted for excision and sequence characterization. For most of the primer combinations selected, there were on average 80 and above TDFs generated, resulting in fragments that were very compacted and not properly resolved in the gels. To resolve this problem, cDNA-AFLP mediated minisequencing (Brugmans et al., 2003) with MSe1+3 and MSe1+4 primers was done. The AFLP-mediated minisequencing

relies on the principle that after the first round of selective amplification with a primer combination (TaqI+2/ MseI+2), a secondary selective amplification to this initial one with TaqI+2/ MseI+3 is done. In theory, the secondary selection will generate fewer bands per lane, as only a subset from the already selected pool of fragments will be amplified. This is then followed by a tertiary amplification with TaqI+2/ MseI+4 primer combinations targeted to reduce the number of TDFs even further. For this purpose, M-NNA, M-NNC, M-NNNA and M-NNNC degenerate primers were designed (where N= A/C/G/T). A primary selective amplification with the T-CA/M-AG primer combination was done and the product of this amplification was used as template for the secondary and tertiaty amplification with T-AC/M-NNA, T-AC/M-NNC, T-AC/M-NNNA and T-AC/M-NNNC primer combinations. The products of both the primary, secondary and tertiary selections were run on an 8% LongRanger polyacrylamide for analysis (Figure 3.7).

The numbers of fragments per lane were not reduced to a significant degree following the minisequencing procedure. This was due to the fact that the minisequencing selection was done only from one end of the fragments (Figure 3.7). It was therefore decided that since the most fragment compacting in the images was with the fragments below 200 bases, the TDF isolation will be done from the +2/+2 gels, and only fragments from 200 bp or more will be selected.

An important observation in Figure 3.7 is that the amplified TDFs in the secondary and tertiary reactions were more intense than those after the primary reaction. This is most probably due to the fact that there are less fragments, even though not highly significant, amplified per reaction resulting in less competition for PCR reagents, and therefore an improved yield in the final PCR product.

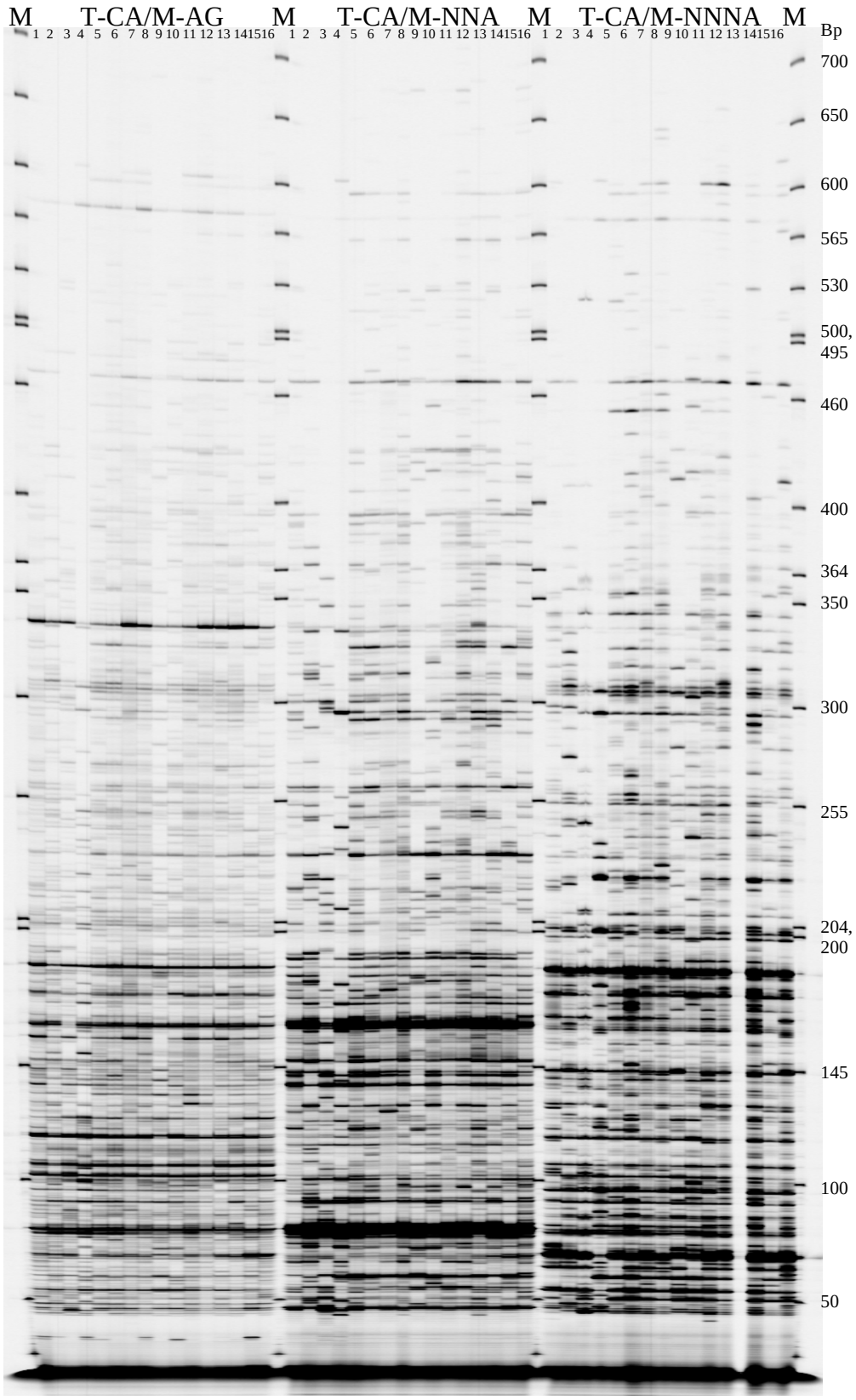


Figure 3.7 cDNA-AFLP mediated minisequencing of TDFs. The selective amplification product of the primer combination T-CA/M-AG was used in secondary and tertiary selections with the primer combinations T-CA/M-NNA and T-CA/M-NNNA. Odd numbered lanes represent 'Tugela DN' TDFs collected at 0, 1, 2, 6, 12, 24, 48 and 120 hpi while the even numbered lanes represent 'Tugela' TDFs at similar intervals. Lanes M= LiCor IRDye700 MW marker.

3.4 Optimization of TDF recovery

For the recovery of target TDFs from the PAGE gels it was necessary to have a system that allowed for an accurate alignment of the gel with the specific image printout. The alignment is important because the fragments on the gel are not visible to the naked eye and therefore a scanned gel image provides the platform to target fragments of interest. The selective products were separated on PAGE gels to allow good separation of the fragments larger than or equal to 200 bp and scanned. The scanned printout was aligned to the gel with the help of markings for accurate alignment that allowed the excision of the targets. Gels were re-scanned following band excision to verify that the correct targets were obtained. Excised fragments were then re-amplified after removal from the cut gel fragments with Taq1+0 and Mse1+0 primers (Figure 3.8).

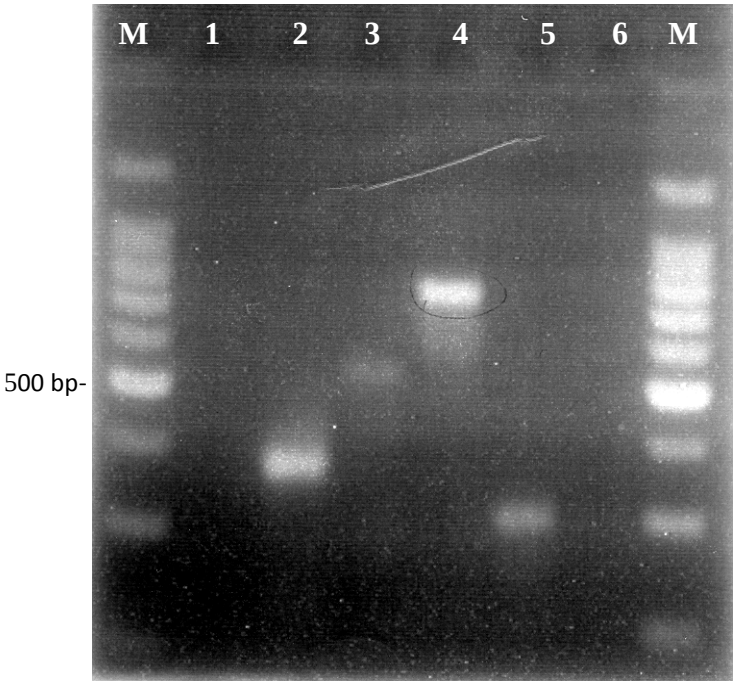


Figure 3.8 EtBr/Agarose gel (3% w/v) analysis of recovered TDFs amplified with *Taq1*+0 and *Mse1*+0 primers. Lane M= Promega 100 bp ladder, lane 1= TDF 31, lane 2= TDF 44, Lane 3= TDF 46, lane 4= TDF 64, lane 5= TDF 95, Lane 6= TDF 102. TDFs 31 and 102 failed to reamplify. The re-amplified TDFs correspond to clones AMOMTM1 (TDF 44), AMOMTM18 (TDF 46), AMOMTM2 (TDF 64) and AMOMTM15 (TDF 95).

3.5 TDF recovery and assignment of putative functions

One hundred and sixty (160) TDFs were excised from the gels and only 132 of these were successfully re-amplified. Of these, only 50 fragments were randomly selected for cloning and sequencing. Nine of the 50 fragments did not give readable sequences and were therefore discarded. After analysis of DNA sequence data, they were grouped into functional categories based on the highest similarity to characterized proteins or genes listed in GenBank (Table 3.4). The putative identities were determined by performing BLASTx and BLASTn searches with the obtained sequences (Altschul et al., 1990).

The functional groups identified were: protein synthesis (18%), charperone (2%), protein degradation (2%), sugar metabolism (5%), carbohydrate metabolism (2%), energy related (7%), signalling (7%), defense related (9%) and uncharacterized or unknown (48%, Figure 3.9). The defense related/ signaling functional group contained TDFs homologous to a *Triticum aestivum* GDP-fucose protein-O-fucosyltransferase 1, a kinase related protein, a serine/threonine protein kinase, a seven transmembrane protein Mlo8 and three senescence-associated proteins (Table 3.4).

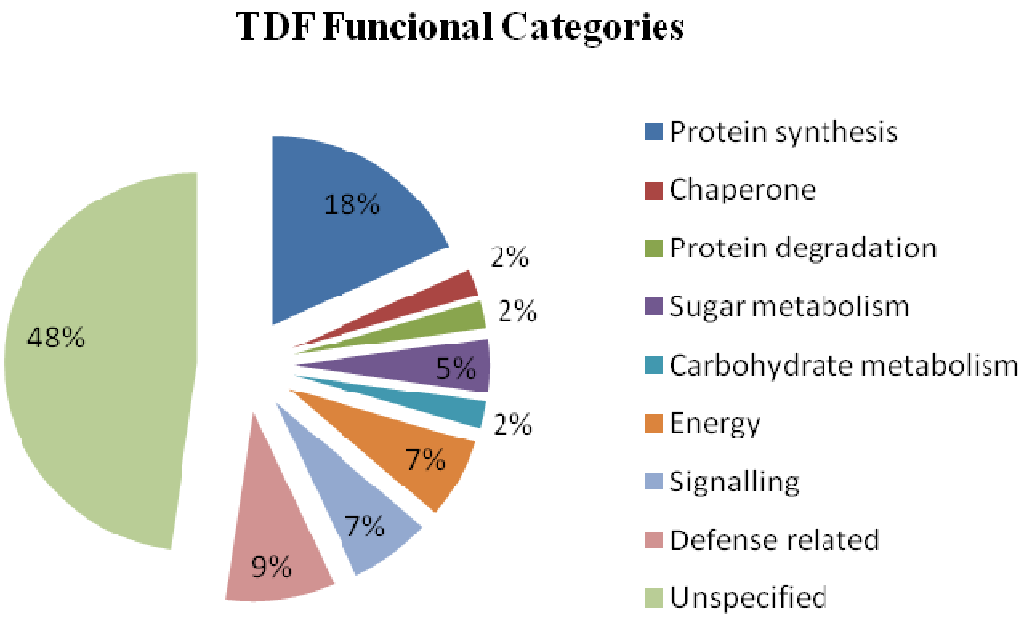


Figure 3.9 Exploded pie chart representation of the TDF functional groups identified following sequencing of 41 isolated and cloned TDFs excised from the cDNA-AFLP gels.

Genes such as acetyl CoA and O-acetylserine (thiol) lyases were identified and classified under the energy functional group. Genes for protein synthesis, folding and degradation were also identified. These included the 26S and 18S rRNA genes, a putative peptidyl-prolyl cys-trans isomerase and polyubiquitin. The functional groups sugar and carbohydrate metabolism included the Rubisco and fructan 1-exohydrolase w1 genes, respectively. The majority of the identified TDFs however showed no significant similarity to any described genes and were classified in the uncharacterized functional group. These TDFs were similar to plant genes that have not been assigned any function or associated to any function. Seven TDFs with no significant similarity to any sequence in Genbank following BLASTn and BLASTx searches were also obtained. These were deposited to Genbank and were assigned the accession numbers ES697585; ES697586; ES697587; ES697588; ES697589; ES697590; ES697591 (Appendix A).

3.6 Transcript profiling

Hierarchical clustering of TDF expression profiles

In this study, fragments ranging from 50 bp to about 750 bp were visualized and scored. One thousand four hundred and eighty nine (1489) TDFs were scored for all ten primer combinations employed across eight time intervals. Bands were scored for absence and presence as well as changes in relative abundance within and between the two test lines. The expression data for all the primer combinations (data in band intensities) were combined and were imported into the Cluster program for the generation of a hierarchical cluster of the TDFs. This cluster was then viewed in the TreeView program. Figure 3.10 shows the hierarchical cluster that was generated. The results indicate eighteen clusters of regulation. In order to obtain a better view of the expression pattern that occurs in each of the clusters, the band intensities of the TDFs of the two test wheat lines were separated from each cluster. Average expression values (band intensities) were obtained and these were plotted to represent the average expression patterns for each of the clusters (Appendix C Figure 1 A - Q, showing the enlarged images of the 18 expression clusters

Table 3.4 BlastN and BlastX results of sequenced differentially expressed TDFs identified by cDNA-AFLP.

Category	TDF	Clone	BLASTn	E-Value*	BLASTx	E-value*
Protein synthesis	M-AC/T-CA	AMOMTM1	*H. vulgare* mRNA for elongation factor 1-alpha	E= 2e-84	Elongation factor 1-alpha (ef-1-alpha)	E= 3e-27
	M-AG/T-GT	AMOMTM41	*H. vulgare* mRNA for elongation factor 1-alpha	E= 1e-91	elongation factor 1-alpha [*Hordeum vulgare* subsp. *vulgare*]	E= 7e-32
	M-AG/T-CT	AMOMTM5	Rye 26S rRNA 3' end and 18S rRNA 5' end	E= e-118	putative senescence-associated protein [*Pyrus communis*]	E= 1e-33
	M-AC/T-AC	AMOMTM7	Wheat rDNA 25S-18S intergenic region *EcoR*I-*BamH*I fragment	E= 0.0	No significant homology found	
	M-AC/T-GT	AMOMTM26	Rye 26S rRNA 3' end and 18S rRNA 5' end	E= e-101	putative senescence-associated protein [*Pyrus communis*]	E= 1e-27
	M-AC/T-AC	AMOMTM2	*Triticum aestivum* (L.) partial chloroplast 16S rRNA gene	E= 0.0	Orf122 [*Chlorobium tepidum*]	E= 1e-27
	M-AC/T-CT	AMOMTM32	Rye 26S rRNA 3' end and 18S rRNA 5' end	E= e-119	putative senescence-associated protein [*Pyrus communis*]	E= 2e-31
	M-AC/T-GT	AMOMTM31	*Oryza sativa* (japonica cultivar-group) cDNA clone:002-101-F08 full insert sequence	E= 9e-39	30S ribosomal protein S16-like [*Oryza sativa* (japonica cultivar-group)]	E= 1e-24
Chaperone	M-AG/T-CA	AMOMTM27	*Oryza sativa* putative peptidyl-prolyl cis-trans isomerase, chloroplast precursor	E= 5e-62	putative peptidyl-prolyl cis-trans isomerase, chloroplast precursor [*Oryza sativa*]	E= 2e-30
Protein degradation	M-AC/T-CA	AMOMTM18	*O. sativa* rub1 mRNA for polyubiquitin	E= e-127	polyubiquitin [*Sporobolus stapfianus*]	E=2e-60
Sugar metabolism	M-AG/T-CT	AMOMTM13	*Triticum aestivum* ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (rbcl) mRNA complete cds; chloroplast gene	E= 0.0	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	E= 2e-70
	M-AC/T-AC	AMOMTM20	*Triticum aestivum* Rubisco	E= e-176	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit,	E= 3e-46
Carbohydrate metabolism	M-AC/T-AC	AMOMTM19	*Triticum aestivum* mRNA for fructan 1-exohydrolase w1 precursor (1-FEH w1 gene)	E= 8e-40	fructan 1-exohydrolase w1 precursor [*Triticum aestivum*]	E= 5e-10
Energy	M-AG/T-TG	AMOMTM17	*Triticum aestivum* mRNA for O-acetylserine (thiol) lyase	E= e-170	Cysteine synthase (O-acetylserine sulfhydrylase) (O-acetylserine (Thiol)-lyase) (CSase A) (OAS-TL A),	E= 5e-43
	M-AC/T-AG	AMOMTM38	*Triticum aestivum* mRNA for O-acetylserine (thiol) lyase	E= 2e-43	No significant homology found	

* The cut-off for significant homology is e-10.

Table 3.4 (Cont.)

	M-AG/T-TG	AMOMTM6	*Oryza sativa* cDNA clone:J013116F19, full insert sequence	E= 1e-69	acetyl-CoA synthetase [*Solanum tuberosum*]	E= 1e-39
Signalling	M-AG/T-TG	AMOMTM22	*Triticum aestivum* partial mRNA for GDP-fucose protein-O-fucosyltransferase 1 (fut12) gene	E= 8e-52	GDP-fucose protein-O-fucosyltransferase 1 [*Triticum aestivum*]	E= 7e-16
	M-AG/T-GT	AMOMTM9	*Oryza sativa* P0671D01.27 (P0671D01.27), mRNA	E= 6e-41	kinase-related [*Arabidopsis thaliana*]	E= 3e-13
	M-AG/T-GT	AMOMTM33	*S. oleracea* mRNA for protein kinase	E= 3e-25	serine/threonine protein kinase (EC 2.7.1.-), nonphototropic hypocotyl protein 1-like [similarity] - spinach	E= 9e-69
			Oryza sativa cDNA clone: J013098G17, full insert sequence	E= e-142		
Defense related	M-AC/T-GT	AMOMTM30	*Triticum aestivum* clone wlm96.pk046.j8:fis, full insert mRNA sequence	E= 4e-90	seven transmembrane protein Mlo8 [*Zea mays*]	E= 2e-15
	M-AC/T-CT	AMOMTM32	Rye 26S rRNA 3' end and 18S rRNA, 5' end	E= e-119	putative senescence-associated protein [*Pyrus communis*]	E= 2e-31
	M-AG/T-CT	AMOMTM5	Rye 26S rRNA 3' end and 18S rRNA, 5' end	E= e-118	putative senescence-associated protein [*Pyrus communis*]	E= 1e-33
	M-AC/T-GT	AMOMTM26	Rye 26S rRNA 3' end and 18S rRNA, 5' end	E= e-101	putative senescence-associated protein [*Pyrus communis*]	E= 1e-27
Unspecified	M-AC/T-AC	AMOMTM3	*Hordeum vulgare* partial mRNA; clone cMWG0645	E= 1e-63	No significant homology found	
	M-AG-T-CT	AMOMTM4	*Oryza sativa* cDNA clone: J013102K12, full insert sequence	E= 2e-56	unknown protein [*Oryza sativa* (japonica cultivar-group)]	E= 2e-37
	M-AG/T-GT	AMOMTM8	*Oryza sativa* cDNA clone: J013107E18, full insert sequence	E= 9e-77	unknown [*Arabidopsis thaliana*]	E= 7e-35
	M-AC/T-AG	AMOMTM10	*Oryza sativa* hypothetical protein	E= 7e-15	hypothetical protein [Oryza sativa]	E= 2e-11
	M-AG/T-AC	AMOMTM11	*Oryza sativa* chromosome 10, section 68 of 77 of the complete sequence.	E= e-170	hypothetical protein Avar020175 [*Anabaena variabilis* ATCC 29413]	E= 2e-11
	M-AG/T-AC	AMOMTM12	*Oryza sativa* genomic DNA, chromosome 4, BAC clone: OSJNBa0042F21	E= e-169	hypothetical protein Avar020175 [*Anabaena variabilis* ATCC 29413]	E= 2e-11
	M-AC/T-AC	AMOMTM14	No significant homology found		No significant homology found	
	M-AC/T-AG	AMOMTM15	*Oryza sativa* chromosome 3 clone OSJNBa0039F10, complete sequence	E= 5e-29	small nuclear ribonucleoprotein U2B" - potato	E= 1e-11

* The cut-off for significant homology is e-10.

Table 3.4 (cont.)

M-AG/T-GT	AMOMTM16	*Oryza sativa* chromosome 3 clone OSJNBa0016I15, complete sequence	E= 3e-17	Expressed protein [*Arabidopsis thaliana*]	E= 2e-16
M-AC/T-AC	AMOMTM21	*Triticum aestivum* (L.) partial chloroplast 16S rRNA gene	E= 0.0	Orf122 [*Chlorobium tepidum*],	E= 2e-25
M-AG/T-CT	AMOMTM23	No significant homology found		No significant homology found	
M-AG/T-CT	AMOMTM24	No significant homology found		No significant homology found	
M-AC/T-GT	AMOMTM25	No significant homology found		No significant homology found	
M-AG/T-CT	AMOMTM28	*Zea mays* PCO148683 mRNA sequence	E= 4e-10	Nosignificant homology found	
M-AC/T-AG	AMOMTM29	No significant homology found		No significant homology found	
M-AG/T-GT	AMOMTM34	*Triticum aestivum* clone wlm1.pk0018.b5:fis, full insert mRNA sequence	E= 2e-89	No significant homology found	
M-AG/T-GT	AMOMTM35	*Triticum aestivum* clone wlm1.pk0018.b5:fis, full insert mRNA sequence	E= 4e-97	No significant homology found	
M-AC/T-CT	AMOMTM36	*Haynaldia villosa* clone kong32 mRNA	E= 2e-99	No significant homology found	
M-AG/T-CT	AMOMTM37	No significant homology found		No significant homology found	
M-AC/T-AC	AMOMTM39	No significant homology found		No significant homology found	
M-AG/T-GT	AMOMTM40	*Oryza sativa* genomic DNA, chromosome 4, BAC clone: OSJNBa0042F21	E= 2e-30	No significant homology found	

* The cut-off for significant homology is e-10.

in Figure 3.10 along with the line graphs representing average expression in each cluster of 'Tugela DN' wheat line vs 'Tugela' wheat line).

Cluster 1 showed down regulation of the TDFs within the first hour of infestation with the RWA in 'Tugela DN' (Appendix C Figure 1 A). The low expression was then maintained throughout the time course of the experiment. In 'Tugela' however, there was no significant regulation of expression. An example of a TDF belonging to this cluster is TDF #124 (AMOMTM32).

In cluster 2, the TDFs in 'Tugela DN' showed an initial decrease in expression within the first hour of infestation that was followed by an increase in the expression to the initial level (Appendix C Figure 1 B). Expression of the TDFs was then maintained at this level until 48 hpi but had dropped drastically by 120 hpi. The similar pattern was observed in Tugela with the exception that the initial decrease was less and at 48 hpi, the genes were down regulated and this continued even at 120 hpi.

In cluster 3 (Appendix C Figure 1 C), TDFs in 'Tugela DN' showed little regulation upon infestation until 24 hpi, where after TDFs were up regulated. In 'Tugela' TDFs were up regulated between 6 and 24 hpi. Expression then returned to the initial levels at 48 and 120 hpi. TDFs 46 (AMOMTM18), 138 (AMOMTM16) and 144 (AMOMTM8) belong to this cluster. Cluster 4 (Appendix C Figure 1 D) showed TDFs that have no regulation from 0 to 24 hpi and were highly up regulated at 48 hpi but by 120 hpi expression had returned to normal in 'Tugela DN'. There was no regulation of TDFs in 'Tugela' during the time trial. Examples of TDFs belonging to this cluster are TDF 141a and 141b (AMOMTM34 and AMOMTM35, respectively).

TDFs in cluster 5 (Appendix C Figure 1 E) were up regulated between 2 and 6 hours post infestation with the RWA and there was no regulation for the rest of the time trial in 'Tugela DN'.

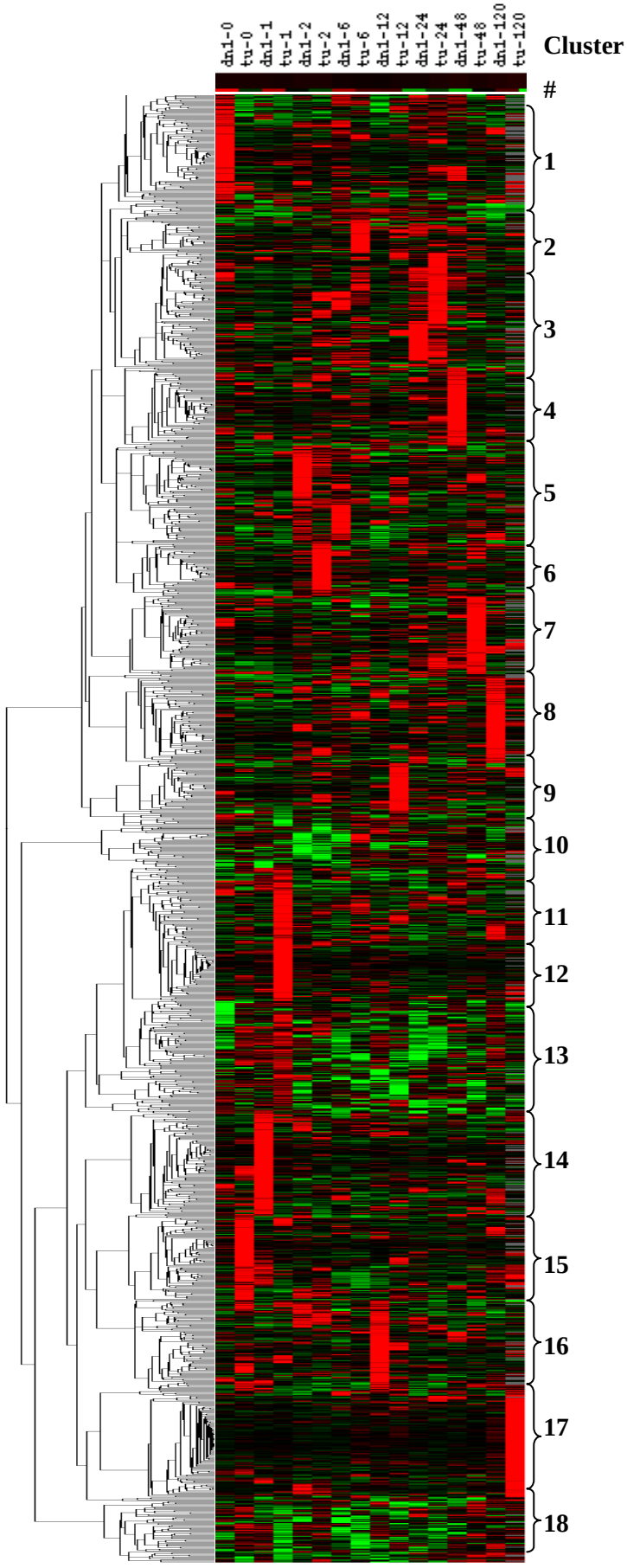


Figure 3.10 Gene expression clusters generated by Cluster and TreeView programmes. Band intensities were exported from AFLP QuantarPro programme and were used to generate a hierarchal cluster of the different TDFs generated by cDNA-AFLP. Right braces mark the borders of each of the clusters labelled 1 – 18. Red = up regulation/ high expression, green = down regulation/ low expression and black = no expression/ switched off.

A similar pattern was observed for TDFs from 'Tugela' however, the up regulation was only observed at 2 hpi. Examples of TDFs in cluster 5 are TDFs 56 (AMOMTM19) and 64 (AMOMTM2). 'Tugela DN' TDFs in Cluster 6 (Appendix C Figure 1 F) did not show any regulation during infestation with the RWA except for a very slight increase in expression levels at 48 hpi. 'Tugela' TDFs on the other hand showed no regulation until 24 hpi and a very strong up regulation by 48 hpi. This was followed by down regulation to expression levels below the initial expression value by 120 hpi.

'Tugela DN' TDFs in cluster 7 (Appendix C Figure 1 G) showed no regulation from 0 – 48 hpi and a significant up regulation at 120 hpi. 'Tugela' TDFs in cluster 7 showed little regulation throughout the time trial of infestation. TDFs 74 (AMOMTM23), 90 (AMOMTM10) and 93 (AMOMTM38) belong to this cluster. TDFs in cluster 8 (Appendix C Figure 1 H) showed no regulation in 'Tugela DN' during infestation with the RWA. In 'Tugela' however there was a significant up regulation only at 12 hpi and no regulation at all the other intervals. An example of a TDF in cluster 8 is TDF 119 (AMOMTM26).

The 'Tugela DN' TDFs in cluster 9 (Appendix C Figure 1 I) showed a slight down regulation following infestation but by 120 hpi the level of expression had returned to normal. In 'Tugela' however, there was major down regulation of expression of the TDFs within the first hour of infestation followed by an increase in expression to a peak at 12 hpi. Expression of these TDFs was then slightly decreased and maintained at the same level for the rest of the time trial. TDF 77 (AMOMTM5) was included in this cluster. In cluster 10 (Appendix C Figure 1 J) there was no significant regulation in both 'Tugela' and 'Tugela DN'.

'Tugela DN' TDFs in cluster 11 (Appendix C Figure 1 K) showed no regulation from the time of infestation until 24 hpi from where there was a slight increase in expression at 48 and 120 hpi. TDFs in 'Tugela' were highly up regulated in the first hour of infestation but then expression was maintained at lower levels throughout the remainder of the time trial.

In cluster 12 (Appendix C Figure 1 K), there was up and down regulation of TDF expression in 'Tugela DN' within the same boundary during infestation, though the initial expression value was lower that the final value. 'Tugela' TDFs on the other hand showed down regulation between the 2nd and 6th hour of infestation from where on the expression was maintained at the low level.

The expression of TDFs from 'Tugela DN' in cluster 13 (Appendix C Figure 1 L) showed little variation during infestation with down regulation of the TDFs at 12 hpi. In 'Tugela' there was an initial increase of TDF expression which was followed by a drop to the initial level where after it was maintained throughout. Expression of TDFs in cluster 14 (Appendix C Figure 1 M) show a major up regulation in 'Tugela DN' within the first hour of infestation which was followed by a drop to the initial level that was maintained at this level until 120 hpi. 'Tugela' genes were kept at low levels throughout the infestation trial. TDFs 27 (AMOMTM22), 131a and 131b (AMOMTM40 and AMOMTM41, respectively) were grouped into this cluster.

In cluster 15 (Appendix C Figure 1 N), there was very little regulation of expression with the levels of TDF expression remaining very low in 'Tugela DN' throughout the infestation period. 'Tugela' TDFs in this cluster showed a high initial expression value that is significantly down regulated to similar levels as in 'Tugela DN' within the first hour of infestation and were maintained at these low levels throughout. An example of a TDF in this cluster is TDF 81 (AMOMTM13).

In cluster 16 (Appendix C Figure 1 O), TDFs in 'Tugela DN' showed a slight increase in expression following infestion within the first two hours followed by a drop at 6 hpi. At 12 hpi TDF expression peaked to very high levels but decreased again at 24 hpi through to 120 hpi. 'Tugela' TDF expression in cluster 16 showed a slight reduction in the expression levels and this was maintained throughout.

Expression in cluster 17 (Appendix C Figure 1 P) was very low and there is no regulation in both 'Tugela DN' and 'Tugela' genes with the exception that at 120 hpi the genes in 'Tugela' were highly up regulated. Examples of TDFs belonging to this cluster are TDFs 59 (AMOMTM15) and 106 (AMOMTM30). In cluster 18 (Appendix C Figure 1 Q) very little regulation was observed in 'Tugela DN' TDFs but a slight increase in TDF expression between 6 and 120 hpi was observed. 'Tugela' TDFs showed significant down regulation during the first hour of infestation, but TDF expression returned to the initial value by the second hour. This was then followed by a slight reduction at 6 hpi succeeded by a slight increase during the rest of the infestation.

3.7 Microarray hybridizations

Images that were scanned from the hybridized microarray slides (Figure 3.11) as well as the data of CyDye intensities that was captured from the Axon GenePix 4000 Microarray scanner and GenePix acquisition software (Axon Instruments, Inc., USA), respectively, were analyzed as described in Appendix B. Images like the one in Figure 3.11 show how immobilized ESTs are regulated in the materials used. Green spots represent the immobilized transcripts on the microarray slide that hybridized to transcripts in the *Cy3*-labeled probe, showing that the target was expressed in the probe sample. The red spots show the target ESTs that hybridized to the *Cy5*-labelled probe and the yellow spots are ones where transcripts in both the *Cy3*- and *Cy5*-labelled probes hybridized equally to the immobilized clones. Yellow spots indicate that the target EST on the microarray was being equally expressed in the two samples contrasted (Naidoo et al., 2005). *CyDye* intensities obtained from the microarray slides were used to determine the expression (log2 fold change and net log*P*) of spotted clones using a general analysis of variance (ANOVA) (Dudoit et al., 2001). The expression of target ESTs in RWA induced leaves are given in Appendix C Table 1.

Figure 3.11 Image of a scanned microarray slide following dual hybridization of *CyDye* RWA induced cDNA probes. The green spots are where only the *Cy3*-labeled probe hybridized to the particular immobilized EST. The red spots indicate ESTs where only the *Cy5*-labled probe hybridized, and the yellow spots indicate that both the *Cy3*- and *Cy5*- labeled probes hybridized equally.

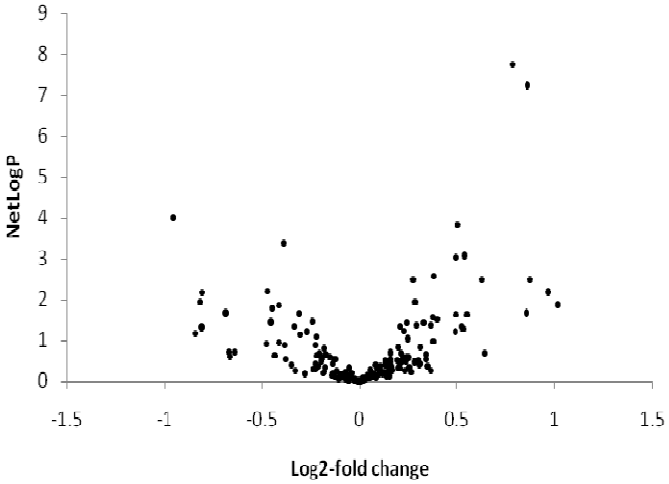


Figure 3.12 Significance plot for wheat EST clones dataset. Volcano plot was generated from *CyDye* intensities obtained following dual hybridizations of *CyDye*-labelled cDNA probes derived from RWA induced and control wheat leaves. The y-axis reprents NetLog*P* for contrast between treatments while the x-axis indicates log2-fold change in the *CyDye* intensities of individual clones. Negative log2 fold changes indicate down regulation while positive values show up- regulation of transcripts.

Log2 fold change values (Appendix C, Table 1) were used to classify the expression of spotted cDNAs as being highly abundant (log2 fold change ≥ 0.1), less abundant (log2 fold change ≤ -0.1) and equal abundant in control and treated material ($-0.1 >$ log2 fold change < 0.1). Under these criteria, 28.5% of cDNA clones were less abundant, 31% were equally abundant and 40.5% were more abundant in RWA induced wheat. A volcano plot was also constructed (Figure

3.12) to indicate the distribution of the clones following ANOVA statistical analysis (Dudoit et al., 2001).

CyDye intensities were further analysed for the significance of expression regulation using the mixed model approach for statistical analysis by Wolfinger and colleagues (2001). Spotted clones (reduced to 29) that showed significant regulation (see Appendix B Figure 1 and Table 2) –i.e. had a threshold log2 expression ratio of 1.5 and $P \leq 0.05$ - were classified into the following functional categories: cell structure and maintenance, photosynthesis related, defense related and clones of unknown function. Figure 3.13 shows an exploded chart representation of these clones.

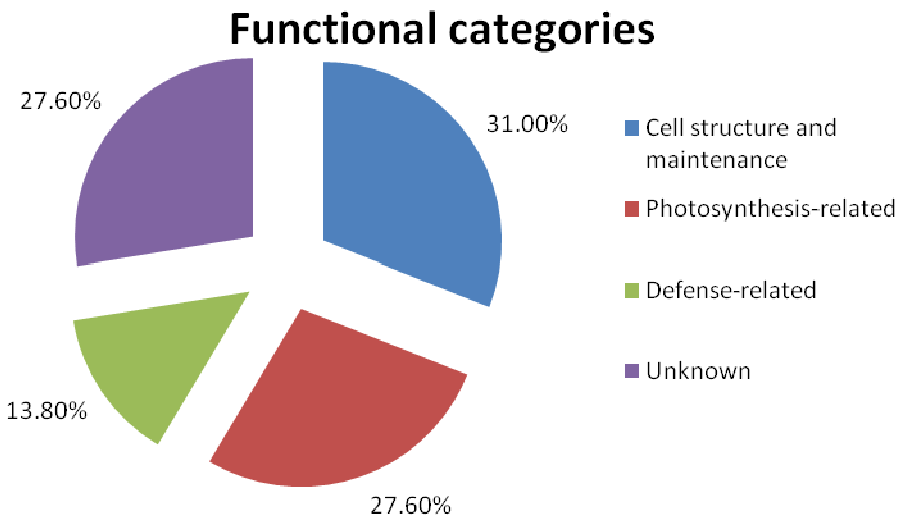


Figure 3.13 Exploded pie chart representing the functional categories of clones that showed significant regulation (log2 ratio ≤ 1.5 and $P \leq 0.05$) following statistical analysis of the spot intensities using the mixed model by Wolfinger et al., (2001).

CHAPTER 4

DISCUSSION AND CONCLUSIONS

4.1 Discussion

Identification and characterization of novel genes involved in defensive responses is a priority for wheat and other important cereal crops. Accordingly, we focused on studying the patterns of gene regulation in wheat during infestation with the RWA using cDNA-AFLP and microarray technology. Genes that showed differential expression within and between wheat near-isogenic lines 'Tugela DN' and 'Tugela' during infestation were identified and characterized.

cDNA-AFLP analysis

This study was preceded by the optimization of the cDNA-AFLP procedure for the display of expression patterns in wheat. Ensuring that the extracted RNA is of good quality, free all contaminating DNA, is the first crucial step before performing a cDNA-AFLP experiment. Enriching for poly A mRNA reduces the amount of false positives in the results. High quality mRNA also results in a good cDNA preparation, which also adds an advantage of yielding reliable and repeatable expression profiles. Bachem and his colleagues also stressed the importance of a high quality mRNA extract in their report (Bachem et al., 1998).

cDNA-AFLP has been previously employed in rapid identification of differentially expressed genes in plant developmental stages as well as in the defensive responses against biotic and abiotic stresses (Bachem et al., 1996; Durrant et al., 2000). This technique allows expression to be studied in a high-throughput manner. Using the LI-COR DNA analyzers and the Odyssey flat-based scanner allowed us to excise bands without having to stain the gels following electrophoresis. The LI-COR systems also generated images that could be imported into the AFLP-Quantar*Pro* software for a highly accurate and rapid quantification of the TDFs (Myburg et al., 2001).

A potential problem with cDNA-AFLP expression profiling is that it is based on PCR, and conventional PCR is not quantitative, however, this is easily overcome by the employment of the quantitative real time PCR on the identified differentially expressed TDFs (Zaayman et al., 2009).

Plants require well-evolved defense responses in order to survive in a world full of pathogen and pest threats. The RWA-resistant wheat cultivar 'Tugela DN' has been found to express antibiotic resistance towards the Russian wheat aphid. This implies that the fecundity of the aphids gets reduced following feeding on these plants (Du Toit, 1989; van Eck, 2007).

HR is a good example of such a defense where cells around the point of infection rapidly die and are also filled by antimicrobial substances (Dangl and Jones, 2001). Because HR is a very expensive method in terms of energy requirements, it is necessary for the plant to be able to recognize when an invasion has occurred. The plant uses *R*-gene products that bind various elicitors from the invading pathogen (*Avr*) as surveillance (Dangl and Jones, 2001). Upon recognition of a pathogen, a burst of ROS that eventually results in PCD occurs. This form of HR is effective against secondary herbivores (Dangle and Jones, 2001) such as the Russian wheat aphid, which is a phloem-sucking insect.

Previous studies suggest that the response to the RWA resembles ROS dependent HR (Van der Westhuizen et al., 1998b; Botha et al., 2006) and we focused on looking at gene regulation in wheat within the first 24 hours of infestation. The hypersensitive response usually elicits a more long-term general response that is spread throughout the entire plant. This resistance is called the SAR (Botha et al., 2005). We therefore included leaf collection at 48 and 120 hours post infestation to identify genes differentially expressed at the onset of SAR.

TDF expression level quantification by cDNA-AFLP indicated the number of genes to be differentially expressed within and between the two wheat NILs during infestation with the RWA. Differential expression is indicated to occur even within the first hour of infestation.

Aphids feed by firstly probing the leaf several times with a stylet in search of best feeding sites (Walters et al., 1980) and this probing induces some defenses in the plants.

Hierarchical clustering of the quantified TDFs resulted in 18 clusters, of which, seven exhibited similarity in regulation between the resistant and the susceptible line. This observation is not very surprising since the two wheat lines possess similar genomes that only differ by a small percentage that carries the resistance gene *Dn1* (Myburg et al., 1998). Another interesting observation that in the clusters with similar regulation modes, the TDFs in the resistant line seem to have higher band intensities than in the susceptible line. Perhaps these are what allow the resistance in 'Tugela DN'. This could mean that there is better surveillance in 'Tugela DN', that allows an improved signaling to other genes involved in the defense response (Botha et al., 2006). This is however very speculative since we are only referring to PCR results which are not quantitative.

Sequenced TDFs were classified according to protein function based on sequence homology to characterized protein sequences in the database. The bulk of the clones hybridized to uncharacterized proteins or had no significant homology any sequences in GenBank (Table 3.4). This is due to the fact that the whole wheat genome has not been sequenced. These might have a potential of being novel transcripts involved in the response of wheat against RWA attack (or general defense). The involvement of these transcripts in RWA defense will have to be researched further.

The signal perception and signaling functional class had two representatives, a seven-transmembrane protein Mlo8 of *Zea mays* and *Triticum aestivum* GDP-fucose protein-*O*-fucosyl transferase 1. MLO is found in the plasma membrane. It consists of seven membrane-spanning domains, with the N-terminus to the extracellular side of the membrane and the C-terminus to the intracellular side (Devoto et al., 1999). The MLO8 protein has been implicated in increased resistance to fungal pathogens (Bushchges et al., 1997). This protein is probably first in the line

of defense when looking at the gene-for-gene interaction model, as was suggested by Botha et al., in 2006 whereby a pathogen *avr* gene product interacts with the host *R*-gene product and elicits a defense response (Dangl and Jones, 2001). In our study however, the protein was elicited in Tugela at 120 hpi and this could indicate its involvement in late respose signals as well. In human cells, *O*-fucose glycans play a significant role in ligand-induced receptor signaling and the *fut1* gene product works on adding O-fucose to glycans (Wang et al., 2001), perhaps the enzyme GDP-fucose protein-*O*-fucosyl transferase 1 plays a similar role in wheat.

Protein phosphorylation has been shown to be an important role in the response of plants to attack. The role of these proteins is well known, e.g. *Pto*, which codes for a serine/threonine protein kinase that has proven effective against *P. syringae* attack in tomato (Zhou *et al*., 1997). The protein phosphorylation in this case is triggered by a direct interaction of AvrPto and Pto (reviewed by Dangl and Jones, 2001). Mitogen activated protein kinases have also been implicated to be activated by *R*-gene pathways (reviewed by Martin, 1999)

One of the effects of aphid feeding on the wheat plants is the reduction in effective leaf area for photosynthesis to occur (Fouché *et al*., 1984). It is therefore not surprising that Rubisco (key enzyme in carbon fixation) is one of the genes that show differential expression in this study. Van der Westhuizen and Botha (1993) found a major induction of Rubisco protein expression in 'Tugela DN' following RWA infestation. The expression profile of this gene shows a decrease over the 24 hour post infestation period and the response in the resistant line is slower than in the susceptible line. This can be seen as evidence of a decrease in photosynthetic capacity of the wheat leaves and also an indication that 'Tugela DN' is able to tolerate the aphids better because it does not lose it effective leaf area as fast as the susceptible line. Botha and colleagues (2006) suggested that maintance of photosynthetic capacity was important for plants to be able to survive the stress associated with RWA feeding. Rubisco activase enzyme has been shown to act as a heat shock protein in spinach leaves, protecting thylakoid-bound ribosomes and thereby indirectly protecting thylakoid associated protein synthesis systems from being degraded (Rokka

et al., 2001). Perhaps Rubisco activase plays some protective role in infested wheat leaves as well.

Inorganic sulphur (S) plays a major role in the resistance of crops to diseases and pests. It is firstly incorporated into cysteine, which can then subsequently be transformed into other S-containing compounds such as glutathione (GSH). GSH acts as an antioxidant in stress responses and is also an important storage and transport form of reduced S (Noctor et al., 1998). *O*-acetyl-$_L$-serine(thiol)lyase (OAS-TL) is the enzyme responsible for incorporating inorganic S into cysteine. The sulphur reduction pathway in plants starts with the assimilation of sulphate followed by the reduction to sulphite and then to sulphide and ends with the coupling of sulphide to *O*-acetyl-resine (OAS) by OAS-TL (reviewed by Youssefian et al., 2001). Cysteine concentrations may actively control the rate of GSH synthesis as well as modify plant responses to oxidative stresses. On the other hand, the rate of cysteine synthesis may be regulated by the demand for it to synthesise GSH (Bloem et al., 2004; Noctor et al., 1998; Youssefian et al., 2001).

The fact that OAS-TL A was detected (Table 3.4) as a differentially expressed gene in this study may be further evidence in support of the suggestion that the response of wheat to RWA attack is a ROS dependent HR (Van der Westhuizen et al., 1998b; Botha et al., 2006). Oxidative burst would result in the demand for GSH to counter the effect of ROS, which in turn would mean that more cysteine would be required to meet the demand for GSH, and thus the activation of OAS-TL expression.

Fructans are one of the main carbohydrate reserves in higher plants. They are classified into different forms based on the glycosidic bonds they possess. In monocots, gramminan type fructans are found. These are levan type fractans with β-(2,6) linked fructofuranosyl units and the mixed-levan type fructans with both β-(2,1) and β-(2,6) linked fructofuranosyl units (Van den Ende et al., 2005). A large amount of this gramminan-type fructan has been found to

accumulate in the vegetative wheat tissues during winter and is associated with the ability to tolerate freezing conditions in wheat (Yukawa et al., 1994). Breakdown of gramminan-type fructans is mainly by two types of fructan exohydrolase (FEH) enzymes, 1-FEHs and 6-FEHs.

FEH activity has been shown to be regulated during second phase cold hardening of winter oat (Livingston and Henson, 1998). FEHs were also reported to be induced following defoliation in witloof chicory roots by Van den Ende and his colleagues in 2001. In 2004, Van Ende and his colleagues suggested that the discovery of FEHs in non-fructan plants, could indicate the role of these enzymes in the defense against fructan producing pathogens. During infestation of wheat by the RWA, the wheat leaf loses its photosynthetic capacity (Fouche et al., 1984), and this might therefore mean that in order to tolerate the attack, then the plant expresses the FEHs to tap into the energy reserves. Perhaps fructan is one of the constituents of the saliva that is deposited by the Russian wheat aphid into the wheat leaves during feeding, and the 1-FEH w1 precursor is expressed as a defense against the RWA.

Elongation factor 1-alpha is an essential component of protein elongation. It functions to carry an aminoacyl-tRNA to the A-site of the ribosome-mRNA-peptidyl complex (van't Klooster et al., 2000). During stress, a number of genes are induced, and as a result a lot of energy goes into synthesizing new proteins, and therefore the expression of EF1-α will be regulated according to the need. Some proteins are expected to be degraded as part of switching off some gene functions to reserve energy for the defense response. Polyubiquitin becomes important to tag proteins that are destined for degradation (reviewed by Chen, 2005).

cDNA-microarray analysis

Fluorescently labeled cDNA probes were successfully synthesized and employed in hybridization experiments. The microarray slides that were prepared for these studies were biased to resistance response because the spotted cDNA fragments were obtained from RWA

induced wheat material using SSH technology (Lacock and Botha, 2003; van Niekerk and Botha, 2003) (256 wheat ESTs), as well as from other treated plants (50 flax and banana clones) via RDA analysis (Appendix C Table 1). Initial log2 fold change values (obtained using a general analysis of variance, ANOVA) indicated that 28.5% of the spotted clones were down regulated, 31% of the clones were not regulated while 40.5% of the clones were upregulated. Further statistical analysis of spot intensities using the mixed model approach by Wolfinger et al., (2001), reduced the number of significantly regulated clones to 29 as explained in Appendix B.

A small number of clones classified as defense-related which included clones similar to *Triticum monococcum* putative resistance protein (RGA-2) and *Aegilops tauschii* leucine-rich-like protein gene were also shown to be regulated during infestation (Appendix C, Table 1). The RGA-2 homologs were mainly upregulated, while some of the leucine-rich-like protein cDNA clones were up- regulated as well as down regulated as determined by ANOVA. Additional to the microarray results, research by Swanepoel and colleagues (2003) provided evidence that NBS-LRR as well as RGA-2 like proteins are involved in RWA induced defense response.

It has been reported previously that RWA feeding on susceptible wheat plants results in the reduction of photosynthetic capacity by the destruction of cell membranes and chloroplasts (Fouche et al., 1984). In this study, a number of clones that showed homology to known photosynthesis related proteins were differentially expressed following the ANOVA analysis of fluorescent intensities (Appendix C, Table 1 and Figure 3.14). These included clones that were similar to wheat chloroplast ATP synthase, clones similar to genes encoding chloroplast proteins as well as for photosystem I P700 apoprotein A1. These genes were mainly upregulated during the infestation of wheat with RWA. Eight of the photosynthesis-related clones were also shown to be significantly regulated using the mixed model approach. The isolation of cDNA clones similar to photosynthesis related proteins suggests the importance of maintaining the photosynthesis machinery during stress, in order to supply enough energy for the plant to tolerate the stress.

cDNA-AFLP technology has been shown in this study as a very useful tool for rapid identification of transcript that are potentially involved in the response in wheat to the RWA attack. The involvement of these transcripts could further be analysed through other technologies like microarrays and revervese transcriptase quantitave PCR to elucidate the pathways through which they effect resistance.

CHAPTER 5

LITERATURE CITED

Aalberg YK, Van der Westhuizen MC and Hewitt PH (1989). Characteristics of the population build-up of the Russian wheat aphid *Diuraphis noxia* and the effect on wheat yield in the eastern Orange Free State. Ann. Appl. Biol. *114*, 231-242.

Altschul SF, Gish W, Miller W, Myers EW and Lipman DJ (1990). Basic local alignment search tool. J. Mol. Biol. *215*, 403-410.

Anderson GR, Papa D, Peng J, Tahir M and Lapitan NLV (2003). Genetic mapping of *Dn7*, a rye gene conferring resistance to the Russian wheat aphid in wheat. Theor. Appl. Genet. *107*, 1297-1303.

Arumuganathan K and Earle ED (1991). Nuclear DNA content of some important plant species. Plant Mol. Biol. Rep. *9*, 208-218.

Ashfield T, Ong LE, Nobuta K, Schneider CM and Innes RW (2004). Convergent evolution of disease resistance gene specificity in two flowering plant families. Plant Cell *16*, 309-318.

Bachem CWB, van der Hoeven RS, de Bruijn SM, Vreugdenhil D, Zabeau M and Visser RGF (1996). Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: analysis of gene expression during potato tuber development. Plant J. 9, 754-753.

Bachem CBW, Oomen RJF and Visser RGF (1998). Transcript imaging with cDNA-AFLP: a step-by-step protocol. Plant Mol. Biol. Rep. *16*, 157-173.

Basky Z (2003). Biotypic and pest status differences between Hungarian and South African populations of Russian wheat aphid, *Diuraphis noxia* (Kurdjumov) (Homoptera: Aphididae). Pest Manag. Sci. *59*, 1152-1158.

Bloem E, Riemenschneider A, Volker J, Papenbrock J, Schmidt A, Salac I, Haneklaus S and Schnug E (2004). Sulphur supply and infection with *Pyrenopeziza brassicae* influence in L-cysteine desulphhydrase activity in *Brassica napus* L. J. Exp. Bot. *55*, 2305-2312.

Boller T (1995). Chemoperception of microbial signals in plant procells. Annu. Rev. Plant Physiol. Plant Mol. Biol. *46*, 189–214.

Borrás-Hidalgo O, Thomma BPHJ, Carmona E, Borroto CJ, Pujol M, Arencibia A and Lopez J (2005). Identification of sugarcane genes induced in disease-resistant somaclones upon inoculation with *Ustilago scitaminea* or *Bipolaris sacchari*. Plant Physiol. Biochem. *43*, 1115-1121.

Boshoff W and Du Toit F (2006). 'n deurbraak in weerstand. Pitkos *3*, 3.

Botha A-M, Nagel MAC, Van der Westhuizen AJ and Botha FC (1998). Chitinase isoenzymes in near-isogenic wheat lines challenged with Russian wheat aphid, exogenous ethylene, and mechanical wounding. Bot. Bull. Acad. Sin. *39*, 99-106.

Botha A-M, Li Y and Lapitan NLV (2005). Cereal host interactions with Russian wheat aphid: A review. J. Plant Inter. *1*, 211-222.

Botha A-M, Lacock L, van Niekerk C, Matsioloko M T, Du Preez FB, Loots S, Venter E, Kunert KJ and Cullis CA (2006). Is photosynthetic Transcriptional regulation in *Triticum aestivum* L. Cv. 'Tugela DN' a contributing factor for tolerance to *Diuraphis noxia* (Homoptera: Aphididae)? Plant cell Reports 25 (1), 41-54.

Boyes DC, Nam J and Dangl JL (1998). The *Arabidopsis thaliana* RPM1 disease resistance gene product is a peripheral plasma membrane protein that is degraded coincident with the hypersensitive response. Proc. Natl. Acad. Sci. USA *95*, 15849-15854.

Breyne P, Dreesen R, Connot B, Rombaut D, Vandepoele K, Rombauts S, Vanderhaegen R, Inzé D and Zabeau M (2003). Quantitative cDNA-AFLP analysis for genome-wide expression studies. Mol. Genet. Genom. 269, 173-179.

Brugmans B, van der Hulst RGM, Visser RGF, Lindhout P and van Eck HJ (2003). A new and versatile method for the successful conversion of AFLP markers into simple single locus markers. Nucleic Acids Res. *31*, e55.

Budak S, Quisenberry SS and Ni X (1999). Comparison of *Diuraphis noxia* resistance in wheat isolines and plant introduction lines. Entomol. Experi. Appl. *92*, 157-164.

Bushchges R, Hollricher K, Panstruger R, Simons G, Wolter M, Friejer A, van Daelen R, van der Lee T, Diergaarde P, Groendijk J and Topsch S (1997). The barley *Mlo* gene: a novel control element of plant pathogen resistance. Cell *88*, 695-705.

Cao H, Scott A, Bowling A, Gordon S and Dong X (1994). Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell *6*, 1583-1592.

Chen CY and Heath MC (1991). Cytological studies of the hypersensitive death of cowpea epidermal cells induced by basidiospores-derived infection by the cowpea fungus. Can. J. Bot. *69*, 1199-1206.

Chen GP, Ma WS, Huang ZJ, Xu T, Xue YB and Shen YZ (2003). Isolation and characterization of *TaGSK1* involved in wheat salt tolerance. Plant Sci. *165*, 1369-1375.

Chen ZJ (2005). Ubiquitin signalling in the NF-κB pathway. Nat. Cell Biol. *7*, 758-765.

Chomczynski P and Sacchi N (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. *162*, 156-159.

Chu T-M, Weir B and Wolfinger RD (2002). A systematic statistical linear modeling approach to oligonucleotide array experiments. Math Biosci. *176*, 35-51.

Dangl J (1998). Plants just say NO to pathogens. Nature *394*, 525-527.

Dangl JL and Jones JDG (2001). Plant pathogens and integrated defense responses to infection. Nature *411*, 826-833.

De Diego JG, Rodriguez FD, Rodrigues Lorenzo JL, Grappin P and Cervantes E (2006). cDNA-AFLP analysis of seed germination in *Arabidopsis thaliana* identifies transposons and new genomic sequences. J. Plant Physiol. *163*, 452-462.

Delaney TP, Uknes S, Vernooij B, Friedrich L, Weymann K, Negrotto D, Gaffney T, Gut-Rella M, Kessmann H, Ward E and Ryals S (1994). A central role of salicylic acid in plant disease resistance. Science *266*, 1247-1250.

Deol GS, Wilde GE and Gill BS (1995). Host plant resistance in some wild wheats to the Russian wheat aphid, *Diuraphis noxia* (Mordvilko) (Homoptera: Aphididae). Plant Breeding *114*, 545-546.

Del Río LA, Corpas FJ, Sandalio LM, Palma JM, Go´mez M and Barosso JB (2002). Reactive oxygen species, antioxidant systems and nitric oxide in peroxisomes. J. Exp. Bot. *53*, 1255-1272.

De Maagd RA, Bosch D and Stiekema W (1999). *Bacillus thurungiensis* toxin-mediated insect resistance in plants. Trends Plant Sci. *4*, 9-13.

Devoto A, Piffanelli P, Nilsson I, Wallin E, Panstruga R, Von Heijne G and Schulze-Lefert P (1999). Topology, subcellular localization, and sequence diversity of the *Mlo* family in plants. J. Biol. Chem. *274*, 34993-35004.

Dong H and Quick JS (1995). Inheritance and allelism of resistances to the Russian wheat aphid in seven wheat lines. Euphytica *81*, 299-303.

Dong H, Quick JK and Zhang Y (1997). Inheritance and allelism of Russian wheat aphid resistance in several wheat lines. Plant Breeding *116*, 449-453.

Dudoit S, Yaug YH, Callow MJ and Speed TP (2001). Statistical methods for identifying differentially expressed genes in replicated cDNA microarray experiments. http://www.stat.berkeley.edu/users/terry/zarray/Html/papersindexhtml.

Durrant WE, Rowland O, Piedras P, Hammond-Kosack KE and Jones JDG (2000). cDNA reveals striking overlaps in race-specific resistance and wound response gene expression profiles. Plant Cell *12*, 963-977.

Du Toit F (1986). Economic thresholds for *Diuraphis noxia* (Hemiptera: Aphididae) on winter wheat in the eastern Orange Free State. Phytophyl. *18*, 107-109.

Du Toit F (1987). Resistance in wheat (*Triticum eastivum*) to *Diuraphis noxia* (Hemiptera: Aphididae). Cereal Res. Commun. *15*, 175-179.

Du Toit F (1989). Inheritance of resistance in two *Triticum aestivum* lines to Russian wheat aphid (Homoptera: Aphididae). J. Econ. Entomol. *82*, 1251-1253.

Du Toit F (1992). Russian wheat aphid resistance in a wheat line from the Caspian Sea area. Cereal Res. Commun. *20*, 55-61.

Flor HH (1942). Inheritance of pathogenecity in a cross between physiologic races 22 and 24 of *Melampsora lini*. Phytopath. *32*, 653-669.

Formosoh ES, Wilde GE, Hatchett JH and Collins RD (1994). Resistance to the Russian wheat aphid (Homoptera: Aphididae) in wheat and wheat related hybrids. J. Econ. Entomol. *87*, 241-244.

Fouché A, Verhoeven RL, Hewitt PH, Walters MC, Kriel CF and de Jager J (1984). Russian aphid (*Diuraphis noxia*) feeding damage on wheat, related cereals and a *Bromus* grass species. Walters M. C. [ed.], Progress in Russian wheat aphid (*Diuraphis noxia* Mord.) research in the Republic of South Africa. Technical Communication, Depart. Agric. Pret. Rep. SA *191*, 22-33.

Gaffney T, Friedrich L, Vernooij B, Negrotto D, Nye G, Uknes S, Ward E, Kessmann H and Ryals J (1993). Requirement of salicylic acid for the induction of systemic acquired resistance. Science *261*, 754-756.

Gill BS, Apples R, Botha-Oberholster A-M, Buell RC, Bennetzen JL, Chumely F, Dvorak J, Iwanaga M, Keller B, Li W, McCombie WR, Quetier F and Sasaki T (2004). A workshop report on wheat genome sequencing: The International Genome Reseach on Wheat IGROW Consortium. Genetics *168*, 1087-1096.

Gill KS, Gill BS, Endo TR and Mukai Y (1993). Fine physical mapping of *Ph1*, a chromosome pairing regulator gene in polyploidy wheat. Genetics *134*, 1231-1236.

Girard C, Metayer ML, Bonade-Bottino M, Pham-Deleague M-H and Jouanin L (1998). High level of resistance to proteinase inhibitors may be conferred by proteolytic cleavage in beetle larvae. Insect. Bioch. Mol. Biol. *28*, 229-237.

Gray ME, Hein GL and Walgenbach DD (1990). Effects of Russian wheat aphid (Homoptera: Aphididae) on winter and spring wheat infested during different plant growth stages under greenhouse conditions. J. Econ. Entomol. *83*, 2434-2442.

Griffiths S, Sharp R, Foote TN, Bertin I, Wanous M, Reader S, Colas I and Moore G (2006). Molecular characterization of *Ph1* as a major chromosome pairing locus in polyploidy wheat. Nature *439*, 749-752.

Haley SD, Peairs FB, Walker CB, Rudolph JB and Randolph TL (2004). Occurrence of a new Russian wheat aphid biotype in Colorado. Crop Sci. *44*, 1589-1592.

Havill NP and Raffa KF (2000). Compound effects of induced plant responses on insect herbivores and parasitoids: implications for tritrophic interactions. Eco. Entomol. *25*, 171-179.

Heil M (1999). Systemic acquired resistance: available information and open ecological questions. J. Ecol. *87*, 341-346.

Hoffman T, Schmidt JS, Zheng X and Bent AF (1999). Isolation of ethylene-insensitive soybean mutants that are altered in pathogen susceptibility and gene-for-gene disease resistance. Plant Physiol. *119*, 935–949.

Jones CS, Davies HV and Taylor MA (2000). Profiling changes in gene expression during raspberry (*Rubus idaeus*) fruit ripening by application of RNA fingerprinting techniques. Planta *211*, 708-714.

Jongsma MA (2004). Novel genes for control and deterrence of sucking insect pests. ISB News Rep. http://www.isb.vt.edu/news/2004/artspdf/nov0401.pdf

Keen NT (1990). Gene-for-gene complementarity in plant-pathogen interactions. Ann. Rev. of Gen. *24*, 447-463.

Kessmann H, Staub T, Hofmann C, Maetzke T, Herzog J, Ward E, Uknes S and Ryals J (1994). Induction of systemic acquired disease resistance in plants by chemicals. Ann. Rev. Phytopath. *32*, 439-459.

Kimber C and Riley R (1963). The relationship of the diploid progenitors of hexaploid wheat. Can. J. Genet. Cytol. *5*, 83-88.

Kindler SD, Springer, TL and Jensen KB (1995). Detection and characterization of the mechanisms of resistance to Russian wheat aphid (Homoptera: Aphididae) in tall wheatgrass. J. Econ. Entomol. *88*, 1503-1509.

Kobe B and Deisenhover J (1995). Proteins with leucine-rich repeats. Cur. Opin. Struc. Biol. *5*, 409-416.

Labuschagne M and Maartens H (1998). The use of low molecular weight glutenin subunits to distinguish between wheat cultivars with and without resistance to the Russian wheat aphid, *Diuraphis noxia*. Plant Breeding *118*, 91-92.

Lacock L and Botha A-M (2003). Suppression Subtractive hybridization (SSH) employed to investigate gene expression after Russian wheat aphid infestation. Proceedings of the 10th International Wheat Genetics Symposium, Paestum, Italy (1-6 September 2003). Volume *3*, p1187-1189.

Lacock L, van Niekerk C, Loots S, du Preez FB and Botha A-M (2003). Functional and comparative analysis of expressed sequences from *Diuraphis noxia* infested wheat obtained utilizing the nucleotide binding site conserved motif. Afr. J. Biotech. *2*, 75-81.

Lam E (2004). Controlled cell death, plant survival and development. Nat. Rev. Mol. Cell Biol. *5*, 305–315.

Lamb CJ, Lawton MA, Dron M and Dixon RA (1989). Signals and transduction mechanisms for activation of plant defenses against microbial attack. Cell *56*, 215-224.

Laudencia-Chingcuanco D, Stamova BS, You FM, Lazo GR, Beckles DM and Anderson OD (2007). Transcriptional profiling of wheat carypsis development using cDNA microarrays. Plant. Mol. Biol. *63*, 651-668.

Lecardonnel A, Chauvin L, Jouanin L, Beaujean A, Prevost G and Sangwan-Norreel B (1998). Effects of rice cystatin I expression in transgenic potato on Colorado potato beetle larvae. Plant Sci. *140*, 87-98.

Lee JH, Elliott NC, Kindler SD, French BW, Walker CB and Eikenbary RD (2005). Natural enemy impact on the Russian wheat aphid in Southeastern Colorado. Environ. Entomol. *34*, 115-123.

Liang P and Pardee AB (1992). Differential display of eukaryotic messenger RNA by means of polymerase chain reaction. Science *257*, 967-971.

Liang P, Averboukh L and Pardee AB (1993). Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimization. Nucleic Acids Res. *21*, 3269-3275

Lievens S, Goormachtig S and Holsters M (2001). A critical evaluation of differential display as a tool to identify genes involved in legume nodulation: looking back and looking forward. Nucl. Acid. Res. *29*, 3459-3468.

Liu XM, Smith CM, Gill BS and Tolmay V (2001). Microsatelite markers linked to six Russian wheat aphid resistance genes in wheat. Theor. Appl. Genet. *102*, 504-510.

Liu XM, Smith CM, Friebe BR and Gill BS (2005). Molecular mapping and allelic relationships of Russian wheat aphid-resistance genes. Crop Sci. *45*, 2273-2280.

Livingston DP and Henson CA (1998) Apoplastic sugars, fructans, fructan exohydrolase, and invertase in winter oat: responses to second-phase cold hardening. Plant Physiol. *116*, 403-408.

Ma Z-Q, Saidi A, Quick JS and Lapitan NLV (1998). Genetic mapping of Russian wheat aphid resistance genes *Dn2* and *Dn4* in wheat. Genome *41*, 303-306.

Marais GF and Du Toit F (1993). A monosomic analysis of Russian wheat aphid resistance in the common wheat PI 294994. Plant Breeding *111*, 246-248.

Marais GF, Horn M and Du Toit F (1994). Intergenic transfer (rye to wheat) of a gene(s) for Russian wheat aphid resistance. Plant Breed. *113*, 265-271.

Marais GF, Wessels WG, Horn M and Du Toit F (1998). Association of stem rust resistance gene (*Sr45*) and two Russian wheat aphid resistance genes (*Dn5* and *Dn7*) with mapped structural loci in common wheat. S. Afr. J. Plant Soil *15*, 67-71.

Martin GB (1999). Functional analysis of plant disease resistance genes and their downstream effectors. Curr. Opin. Plant Biol. *2*, 273-279.

McConn M, Creelman RA, Bell E, Mullet JE and Browse J (1997). Jasmonate is essential for insect defense in *Arabidopsis*. Proc. Natl. Acad. Sci. USA *94*, 5473-5477.

McDowell JM and Woffenden BJ (2003). Plant disease resistance genes: recent insights and potential applications. Trends Biotechnol. *21*, 178-183.

McDowell JM and Dangl JL (2000). Signal transduction in the plant immune response. Trends Biochem. Sci. *25*, 79–82.

McGurl B, Oronzo-Carnenas M, Pearce G and Ryan CA (1994). Overexpression of the prosystemin gene in transgenic tomato plants generates a systemic signal that

constitutively induces proteinase inhibitor synthesis. Proc. Natl. Acad. Sci. USA. *91*, 9799-9802.

Mello MO and Silva-Filho MC (2002). Plant insect interactions: an evolutionary arms race between two distinct defense mechanisms. Braz. J. Plant Physiol *14*, 71-81.

Menke FLH, van Pelt JA, Pieterse CMJ and Klessig DF (2004). Silencing of the mitogen-activated protein kinase MPK6 compromises disease resistance in *Arabidopsis*. Plant Cell *16*, 897-907.

Michaud D, Bernenier-Vadnais N and Yelle S (1995). Constitutive expression of digestive cystein proteinase forms during development of the Colorado potato beetle. Insect Biochem. Molec. Boil. *25*, 1041-1048.

Miles PW (1999). Aphid saliva. Biol. Rev. 74:41-85.

Mittler R, Lam E, Shulaev V and Cohen M (1999). Signals controlling the expression of cytosolic ascorbate peroxidase during pathogen-induced programmed cell death in tobacco. Plant Mol. Biol. *39*, 1025-1035.

Moloi MJ and Van der Westhuizen AJ (2006). The reactive oxygen species are involved in resistance responses of wheat to the Russian wheat aphid. J. Plant Physiol. *163*, 1118-1125.

Morel J-B and Dangl JL (1997). The hypersensitive response and the induction of cell death in plants. Cell Death Diff. *4*, 671-683.

Myburg AA, Cawood M, Wingfield BD and Botha A-M (1998). Development of RAPD and SCAR markers linked to the Russian wheat aphid resistance gene *Dn2* in wheat. Theor. Appl. Genet. *96*, 1162-1169.

Myburg AA, Remington DL, O'Malley DM, Sederoff RR and Whetten RW (2001). High-throughput AFLP analysis using infrared dye labelled primers and an automated DNA sequencer. Biotechniques *30*, 348-357.

Naidoo S, Denby KJ and Berger DK (2005). Microarray experiments: considerations for experimental design. S. A. J. Sci. *101*, 347-354.

Naranjo T and Corredor E (2004). Clustering of centromeres preceeds bivalent chromosome pairing of polyploid wheats. Trends Plant Sci. *9*, 214-217.

Nkongolo KK, Quick JS, Peairs FB and Meyer WL (1991). Inheritance of resistance of PI 372129 wheat to the Russian wheat aphid. Crop Sci. *31*, 905-907.

Noctor G, Arici ACM, Jouanin L, Kunert KJ, Renneberg H and Foyer CH (1998). Glutathione: biosynthesis, metabolism and relationship to stress tolerance explored in transformed plants. J. Exp. Bot. *49*, 623-647.

Nyamsuren O, Coldits F, Rosendahl S, Tamasloukht M, Bekel T, Meyer F, Kuester H, Franken P and Krajinski F (2003). Transcriptional profiling of *Medicago truncatula* roots after infection with *Aphanomyces euteiches* (oomycota) identifies novel genes upregulated during this pathogenic interaction. Physiol. Mol. Plant Pathol. *63*, 17-26.

Pieterse CMJ, Ton J and van Loon LC (2001). Cross-talk between defense signalling pathways: boost or burden? AgBiotechNet *3*,1-8.

Puterka GJ, Burd JD and Burton RL (1992). Biotypic variation in a world wide collection of Russian wheat aphid (Homoptera: Aphididae). J. Econ. Entomol. *85*, 1497-1506.

Puterka GJ, Burd JD, Mornhinweg DW, Haley SD and Peairs FB (2006). Response of resistant and susceptible barley to infestation of five *Diuraphis noxia* (Homoptera: Aphididae) biotypes. J. Econ. Entomol. *99*, 2151-2155.

Rabbani MA, Maruyama K, Abe H, Khan MA, Katsura K, Ito Y, Yoshiwara K, Seki M, Shinozaki K and Yamaguchi-Shinozaki K (2003). Monitoring expression profiles of rice genes under cold, drought and high-salinity stresses and abscisic acid application using cDNA microarray and RNA gel-blot analyses. Plant Physiol. *133*, 1755-1767

Rafi MM, Zemetra RS and Quisenberry SS (1996). Interaction between Russian wheat aphid (Homoptera: Aphididae) and resistant and susceptible genotypes of wheat. J. Econ. Entomol. *89*, 239-246.

Rafi MM, Zemetra RS and Quisenberry SS (1997). Feeding damage of Russian wheat aphid on resistant and susceptible wheat genotypes. Cereal Res. Commun. *25*, 63-68.

Rathjen JP and Moffetty P (2003). Early signal transduction events in specific plant disease resistance. Curr. Opin. Plant Biol. *6*, 300-306.

Robinson J (1992). Russian wheat aphid: a growing problem for small-grain farmers. Outl. Agric. *21*, 57-62.

Rokka A, Zhang L and Aro E-M (2001). Rubisco activase: an enzyme with a temperature-dependent dual function? Plant J. *25*, 463-471.

Romies T, Piedras P and Jones JDG (2000). Resistance gene-dependent activation of a calcium-dependent protein kinase in the plant defence response. Plant Cell *12*, 803-815.

Ryals J, Lawton KA, Delaney TP, Friedrich L, Kessmann H, Neuenschwander U, Uknes S, Vernooij B and Weymann K (1995). Signal transduction in systemic acquired resistance. Proc. Nat. Acad. Sci. USA. *92*, 4202-4205.

Ryals JA, Neuenschwander UH, Willits MG, Molina A, Steiner HY and Hunt MD (1996). Systemic acquired resistance. Plant Cell *8*, 1809-1819.

Saidi A, and Quick JS (1996). Inheritance and allelic relationships among Russian wheat aphid resistance genes in winter wheat. Crop Sci. *36*, 256-258.

Sambrook J, Fritsch EF and Maniatis T (1989). Molecular cloning: A laboratory manual. Sec. Ed. Cold Spring Habour Lab, NY.

Sardesai N, Nemacheck JA, Subramanyam S and Williams CE (2005). Identification and mapping of *H32*, a new wheat gene conferring resistance to Hessian fly. Theor. Appl. Genet. *111*, 1167-1173.

Seo S, Okamoto H, Seto H, Ishizuka K, Sano H and Ohashi Y (1995). Tobacco MAP kinase: A possible mediator in wound signal transduction pathways. Science *270*,1988-1992.

Sessa G and Martin GB (2000). Signal transduction mediated by the tomato Pto kinase: a paradigm of innate immunity in plants. Microbes Infec. *2*, 1591-1597.

Smith CM (1989). Plant resistance to insects: A fundamental approach. John Wiley and Sons, NY, pp 268.

Smith CM, Schotzko D, Zemetra RS, Souza EJ and Teeter SS (1991). Identification of Russian wheat aphid (Homoptera: Aphididae) resistance in wheat. J. Econ. Entomol. *84*, 328-332.

Smith CM, Tesfay B, Stauffer C, Stary P, Kumbeckova I and Starkey S (2004). Identification of Russian wheat aphid (Homoptera: Aphididae) populations virulent to the *Dn4* resistance gene. J. Econ. Entomol. *97*, 1112-1117.

Smith CM, Liu X, Wang LJ, Liu X, Chen M-S, Starkey S and Bai J (2010). Aphid feeding activates expression of a transcriptome of oxylipin-based defense signals in wheat involved in resistanse to herbivory. J. Chem. Ecol. *36*, 260-276.

Song F and Goodman RM (2002). *OsBIMK1*, a rice MAP kinase gene involved in disease resistance responses. Planta *215*, 997-1005.

Sticher L, Mauch-Mani B and Métraux JP (1997). Systemic acquired resistance. Ann. Rev. Phytopath. *35*, 235-270.

Stotz HU, Kroymann J and Mitchell-Olds T (1999). Plant-insect interactions. Curr. Opin. Plant Biol. *2*, 268-272.

Swanepoel E, Lacock L, Myburg AA and Botha AM (2003). A leucine-rich homolog to *Aegilops tauschii* from bread wheat line PI137739 obtained by suppression subtractive hybridization shows linkage to Russian wheat aphid resistance gene *Dn1*. Proceedings of the 10th International Wheat genetics Symposium, Paestum, Italy (1-6 September 2003), Vol 3, p1263-1265.

Tanignoshi KL, Pike KS, Miller RH, Miller TD and Allison D (1995). Search for, and release of, parasitoids for the biological control of Russian wheat aphids in Washington State (USA). Agric. Ecosys. Environ. *52*, 25-30.

Thie NMR and Houseman JG (1990). Identification of cathepsin B, D and H in the larval midgut of Colorado potato beetle, *Leptinotarsa decemlineata* Say (Coleoptera:Chrysomelidae). Insect Biochem. *20*, 313-318.

Thompson GA and Goggin FL (2006). Transcriptomics and functional genomics of plant defense induction by phloem-feeding insects. J Exp. Bot. *57*, 755-766.

Tolmay VL, Lindeque RC and Prinsloo GJ (2007). Preliminary evidence of a resistance-breaking biotype of the Russian wheat aphid, *Diuraphis noxia* (Kurdjumov) (Homoptera: Aphididae), in South Africa. African Entomol. *15*, 228-230.

Van den Ende W, Michiels A, van Wontergem D, Clerens S, De Roover J and van Laere A (2001). Defoliation induces 1-exohydrolase IIb in witloof chicory roots. Cloning and purification of two isoforms (1-FEH IIa and 1-FEH IIb). Mass fingerprint of the 1-FEH II enzymes. Plant Physiol. *126*, 1186-1195.

Van den Ende W, De Coninck B and van Laere A (2004). Plant Fructan exohydrolases: Potential role in signalling and defense? Trends Plant Sci. *9*, 523-528.

Van den EndeW, Yoshida M, Clerence S, Vergauwen R and Kawakami A (2005). Cloning, characterization and functional analysis of novel 6-ketose exohydrolases (6-KEHs) from wheat (*Triticum aestivum*). New Phytol. *166*, 917-932.

Van der Westhuizen AJ and Botha FC (1993). Effect of the Russian wheat aphid on the composition and synthesis of water soluble proteins in the resistant and susceptible wheat. J. Agron. Crop Sci. *170*, 322-326.

Van der Westhuizen AJ and Pretorius Z (1996). Protein composition of wheat apoplastic fluid and resistance to the Russian wheat aphid. Aust. J. Plant Physiol. *23*, 645-648.

Van der Westhuizen AJ, Qian X-M and Botha A-M (1998a). *β*-1,3-Glucanases in wheat and resistance to the Russian wheat aphid. Physiol. Plant. *103*, 125-131.

Van der Westhuizen AJ, Qian X-M and Botha A-M (1998b). Differential induction of apoplastic peroxidase and chitinase activities in susceptible and resistant wheat cultivars by Russian wheat aphid infestation. Plant Cell Rep. *18*, 132-137.

Van der Westhuizen AJ, Qian X-M, Wilding M and Botha A-M (2002). Purification and immunocytochemical localization of a wheat b-1,3-glucanase induced by Russian wheat aphid infestation. S. Afr. J. Sci. *98*, 197-202.

Van Eck L (2007). Aphid-induced transcriptional regulation in near-isogenic wheat. M.Sc. Dissertation. University of Pretoria, South Africa, pp 145.

Van Niekerk C and Botha A-M (2003). Using suppression subtractive hybridization (SSH) to screen for novel sequences expressed in response to Russian wheat aphid feeding. Proceedings of the 10th International Wheat Genetics Symposium, Paestum, Italy (1-6 September 2003). Volume *3*, p1281-1283.

Van't Klooster JW, Van den Berg-Velthuis G, Van West P and Govers F (2000). Tef1 *Phytophthora infestans* gene encoding translation elongation factor 1 alpha. Gene *249*, 145-151.

Van Zyl RA (2007). Elucidation of possible virulence factors present in Russian wheat aphid (*Diuraphis noxia*) biotypes´ saliva. MSc. Dissertation, University of Pretoria, South Africa, pp 118.

Walters MC, Penn F, Du Toit F, Botha TC, Aalsberg K, Hewitt PH and Broodryk SW (1980). The Russian wheat aphid. Farming S. Afr. Leafl. Ser. Wheat *G.3*, 1-6.

Wan JS, Sharp SJ, Poirier GM-C, Wagaman PC, Chambers J, Pyiyati J, Hom Y-L, Galindo JE, Huvar A, Peterson PA, Jackson MR and Erlander MG (1996). Cloning differentially expressed mRNAs. Nat. Biotechnol. 14, 1685-1691.

Wang Y, Shaao L, Shi S, Harris RJ, Spellman MW, Stanley P and Haltiwanger RS (2001). Modification of epidermal growth factor-like repeats with *O*-fucose. Molecular cloning and expression of a novel GDP-fucose protein *O*-fucosyl transferase. J. Biol. Chem. *43*, 40338-40345.

Warren RF, Henk A, Mowery P, Holub E and Innes RW (1998). A mutation within the leucine-rich repeat domain of the *Arabidopsis* disease resistance gene family *RPS5*

partially suppresses multiple bacterial and downy mildew resistance genes. Plant Cell *10*, 1439-1452.

Webster JA, Starks KJ and Burton RL (1987). Plant resistance studies with *Diuraphis noxia* (Homoptera: Aphididae), a new United States wheat pest. J. Econ. Entomol. *80*, 944-949.

Webster JA and Amosson S (1994). Economic impact of the Russian wheat aphid in the western United State, 1992-1993. A report by the Russian wheat aphid task force to the Great Plains Agricultural Council, December 1994. Great Plains Agricultural Council Publication *152*.

Williams CE, Collier CC, Sardesai N, Ohm HW and Cambron SE (2003). Phenotypic assessment and mapped markers for *H31*, a new wheat gene conferring resistance to Hessian fly (Diptera: Cecidomyiidae). Theor. Appl. Genet. *107*, 1516-1523.

Wolfinger RD, Gibson G, Wolfinger ED, Bennet L, Hamadeh H, Bushel P, Afshari C and Paules RS (2001). Assessing gene significance from cDNA microarray expression data via mixed models. J. Comput. Biol. *8*, 625-637.

Xie Z, Fan B and Chen Z (1998). Induction of PR-1 proteins and potentiation of pathogen signals by salicylic acid exhibit the same dose response and structural specificity in plant cell cultures. Mol. Plant Microbe Int. *11*, 568-571.

Yang L, Zheng B, Mao C, Yi K, Liu F, Wu Y, Tao Q and Wu P (2003). cDNA-AFLP analysis of inducible gene expression in rice seminal root tips under a water deficit. Gene *314*, 141-148.

Youssefian S, Nakamura M, Orudgev E and Kondo N (2001). Increased cysteine biosynthesis capacity of transgenic tobacco overexpressing an *O*-acetylserine(thiol) lyase modifies plant responses to oxidative stress. Plant Physiol. *126*, 1001-1011.

Yukawa T, Watanabe Y and Yamatoto S (1994) Studies on fructan accumulation in wheat (*Triticum aestivum* L.) 2. Changes in degree of polymerisation of fructan under treatment at 1 ˚C in dark. Japanese J. Crop Sci. *63*, 430-435.

Zaayman D, Lapitan NLV and Botha A-M (2009). Dissimilar molecular defense responces are elicited in *Triticum aestivum* after infestation by different *Diuraphis noxia* biotypes. Physiol. Planta. *136*, 209-222.

Zhou L and Thornburg R (1999). Wound-inducible genes in plants. In Inducible gene expression. P.H.S. Reynolds (ed), CAB International. pp. 127-158.

Zhou J, Tang X and Martin GB (1997). The Pto kinase conferring resistance to tomato bacterial speck disease interact with proteins that bind a *cis*-element of pathogenesis-related genes. EMBO J. *16*, 3207-3218.

APPENDIX A

dbEST ENTRIES

APPENDIX A:
dbEST entries

GenBank: ES697585

```
LOCUS       ES697585                193 bp    mRNA    linear   EST 19-JUN-2007
DEFINITION  CG2007_038 cDNA-AFLP of wheat response to Diuraphis noxia in
            pTZ57R/T Triticum aestivum cDNA clone AMOMTM14 5', mRNA sequence.
ACCESSION   ES697585
VERSION     ES697585.1  GI:149118151
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 193)
  AUTHORS   Matsioloko,M.T., Myburg,A.A. and Botha-Oberholster,A.M.
  TITLE     Profiling Diuraphis noxia induced expression in Triticum aestivum
            near-isogenic lines using cDNA microarray and cDNA-AFLP technology
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
            Institute
            Faculty of Agriculture and Natural Science, University of Pretoria
            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3984
            Fax: 27 12 420 3947
            Email: anna.oberholster@up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
            PCR PRimers
            FORWARD: TaqI+0
            BACKWARD: MseI+0
            Insert Length: 193   Std Error: 0.00
            Seq primer: T7
            POLYA=No.
FEATURES             Location/Qualifiers
     source          1..193
                     /organism="Triticum aestivum"
                     /mol_type="mRNA"
                     /cultivar="Tugela"
                     /db_xref="taxon:4565"
                     /clone="AMOMTM14"
                     /dev_stage="2-3 leaf stage"
                     /lab_host="Escherichia coli (JM 109)"
                     /clone_lib="cDNA-AFLP of wheat response to Diuraphis noxia
                     in pTZ57R/T"
                     /note="Organ: leaf; Vector: pTZ57R/T; Site_1: EcoRI;
                     Site_2: HindIII; Transcript-derived fragments generated
                     via cDNA-AFLP of wheat leaf mRNA after induction of
                     resistance response to the Russian wheat aphid, Diuraphis
                     noxia."
ORIGIN
        1 actgtctgta gcctatgtta ctgacgggtg tcatatgact ggtaatttga actgtgaaag
       61 ggtcagttat caggtaggtt tattcaaagg catcatgatt ttctaggtag atgagcctgt
      121 ggtatacttc tctattttca gaactgcatt tatgatggtt tcctagaatg tactcatttt
      181 ttgtgttgtg tgt
//
```

APPENDIX A:

dbEST entries

GenBank: ES697586

```
LOCUS       ES697586                 220 bp    mRNA    linear   EST 19-JUN-2007
DEFINITION  CG2007_039 cDNA-AFLP of wheat response to Diuraphis noxia in
            pTZ57R/T Triticum aestivum cDNA clone AMOMTM23 5', mRNA sequence.
ACCESSION   ES697586
VERSION     ES697586.1  GI:149118153
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 220)
  AUTHORS   Matsioloko,M.T., Myburg,A.A. and Botha-Oberholster,A.M.
  TITLE     Profiling Diuraphis noxia induced expression in Triticum aestivum
            near-isogenic lines using cDNA microarray and cDNA-AFLP technology
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
            Institute
            Faculty of Agriculture and Natural Science, University of Pretoria
            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3984
            Fax: 27 12 420 3947
            Email: anna.oberholster@up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
            PCR PRimers
            FORWARD: Taq1+0
            BACKWARD: Mse1+0
            Insert Length: 220   Std Error: 0.00
            Seq primer: T7
            POLYA=No.
FEATURES             Location/Qualifiers
     source          1..220
                     /organism="Triticum aestivum"
                     /mol_type="mRNA"
                     /cultivar="Tugela"
                     /db_xref="taxon:4565"
                     /clone="AMOMTM23"
                     /dev_stage="2-3 leaf stage"
                     /lab_host="Escherichia coli (JM 109)"
                     /clone_lib="cDNA-AFLP of wheat response to Diuraphis noxia
                     in pTZ57R/T"
                     /note="Organ: leaf; Vector: pTZ57R/T; Site_1: EcoRI;
                     Site_2: HindIII; Transcript-derived fragments generated
                     via cDNA-AFLP of wheat leaf mRNA after induction of
                     resistance response to the Russian wheat aphid, Diuraphis
                     noxia."
ORIGIN
        1 agaatgcaaa tatacaaaca aagatgagga acatggacaa cagacacgag ttcagatttg
       61 tctccatcgt cgcaaaattt tccgagaatt tacaggtacg accatatcca gtacaaagcc
      121 acccaattct cctcttgttt tgttttgttt ttgttttgcc cctataaatg tacacatcta
      181 acaccaccgg aaagcaccct aattcatctt tccccgccag
//
```

APPENDIX A:

dbEST entries

GenBank: ES697587

```
LOCUS       ES697587                229 bp    mRNA    linear   EST 19-JUN-2007
DEFINITION  CG2007_040 cDNA-AFLP of wheat response to Diuraphis noxia in
            pTZ57R/T Triticum aestivum cDNA clone AMOMTM24 5', mRNA sequence.
ACCESSION   ES697587
VERSION     ES697587.1  GI:149118154
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 229)
  AUTHORS   Matsioloko,M.T., Myburg,A.A. and Botha-Oberholster,A.M.
  TITLE     Profiling Diuraphis noxia induced expression in Triticum aestivum
            near-isogenic lines using cDNA microarray and cDNA-AFLP technology
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
            Institute
            Faculty of Agriculture and Natural Science, University of Pretoria
            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3984
            Fax: 27 12 420 3947
            Email: anna.oberholster@up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
            PCR PRimers
            FORWARD: Taq1+0
            BACKWARD: Mse1+0
            Insert Length: 229   Std Error: 0.00
            Seq primer: T7
            POLYA=No.
FEATURES             Location/Qualifiers
     source          1..229
                     /organism="Triticum aestivum"
                     /mol_type="mRNA"
                     /cultivar="Tugela"
                     /db_xref="taxon:4565"
                     /clone="AMOMTM24"
                     /dev_stage="2-3 leaf stage"
                     /lab_host="Escherichia coli (JM 109)"
                     /clone_lib="cDNA-AFLP of wheat response to Diuraphis noxia
                     in pTZ57R/T"
                     /note="Organ: leaf; Vector: pTZ57R/T; Site_1: EcoRI;
                     Site_2: HindIII; Transcript-derived fragments generated
                     via cDNA-AFLP of wheat leaf mRNA after induction of
                     resistance response to the Russian wheat aphid, Diuraphis
                     noxia."
ORIGIN
        1 agagcatgct gacctgtatg ccagttgaag cactccacta cgtgtacctt tttccttcgg
       61 cttggtacac ctctggacca agtttcagta gtgaagcaaa gggctgctta gcatttccca
      121 aaatattgtg ccaggagact ttatttgagt gaagagagat gcggcattat ggtttattat
      181 cactgccgtg tttagattca gggttttgca aagtaacttc cctatgaag
//
```

APPENDIX A:

dbEST entries

GenBank: ES697588

```
LOCUS       ES697588                200 bp    mRNA    linear   EST 19-JUN-2007
DEFINITION  CG2007_041 cDNA-AFLP of wheat response to Diuraphis noxia in
            pTZ57R/T Triticum aestivum cDNA clone AMOMTM25 5', mRNA sequence.
ACCESSION   ES697588
VERSION     ES697588.1  GI:149118155
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 200)
  AUTHORS   Matsioloko,M.T., Myburg,A.A. and Botha-Oberholster,A.M.
  TITLE     Profiling Diuraphis noxia induced expression in Triticum aestivum
            near-isogenic lines using cDNA microarray and cDNA-AFLP technology
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
            Institute
            Faculty of Agriculture and Natural Science, University of Pretoria
            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3984
            Fax: 27 12 420 3947
            Email: anna.oberholster@up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
            PCR PRimers
            FORWARD: Taq1+0
            BACKWARD: Mse1+0
            Insert Length: 200   Std Error: 0.00
            Seq primer: T7
            POLYA=No.
FEATURES             Location/Qualifiers
     source          1..200
                     /organism="Triticum aestivum"
                     /mol_type="mRNA"
                     /cultivar="Tugela"
                     /db_xref="taxon:4565"
                     /clone="AMOMTM25"
                     /dev_stage="2-3 leaf stage"
                     /lab_host="Escherichia coli (JM 109)"
                     /clone_lib="cDNA-AFLP of wheat response to Diuraphis noxia
                     in pTZ57R/T"
                     /note="Organ: leaf; Vector: pTZ57R/T; Site_1: EcoRI;
                     Site_2: HindIII; Transcript-derived fragments generated
                     via cDNA-AFLP of wheat leaf mRNA after induction of
                     resistance response to the Russian wheat aphid, Diuraphis
                     noxia."
ORIGIN
        1 acgccagaag aagcctggca gctctttgcc gctgccaaaa atcatatcgg tgctgtttca
       61 gtgatgcttt ggtatactac aacataagaa gaaatcacgt gatctacgcc gtcaggatcc
      121 gtatgccttc atcgctggct tgcatgctag cacttggtga ggatctctgc gccggtcctg
      181 gtgatgagtc ctgagtaaac
//
```

APPENDIX A:

dbEST entries

GenBank: ES697589

```
LOCUS       ES697589                172 bp    mRNA    linear   EST 19-JUN-2007
DEFINITION  CG2007_042 cDNA-AFLP of wheat response to Diuraphis noxia in
            pTZ57R/T Triticum aestivum cDNA clone AMOMTM29 5', mRNA sequence.
ACCESSION   ES697589
VERSION     ES697589.1  GI:149118156
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 172)
  AUTHORS   Matsioloko,M.T., Myburg,A.A. and Botha-Oberholster,A.M.
  TITLE     Profiling Diuraphis noxia induced expression in Triticum aestivum
            near-isogenic lines using cDNA microarray and cDNA-AFLP technology
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
            Institute
            Faculty of Agriculture and Natural Science, University of Pretoria
            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3984
            Fax: 27 12 420 3947
            Email: anna.oberholster@up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
            PCR PRimers
            FORWARD: Taq1+0
            BACKWARD: Mse1+0
            Insert Length: 172   Std Error: 0.00
            Seq primer: T7
            POLYA=No.
FEATURES             Location/Qualifiers
     source          1..172
                     /organism="Triticum aestivum"
                     /mol_type="mRNA"
                     /cultivar="Tugela"
                     /db_xref="taxon:4565"
                     /clone="AMOMTM29"
                     /dev_stage="2-3 leaf stage"
                     /lab_host="Escherichia coli (JM 109)"
                     /clone_lib="cDNA-AFLP of wheat response to Diuraphis noxia
                     in pTZ57R/T"
                     /note="Organ: leaf; Vector: pTZ57R/T; Site_1: EcoRI;
                     Site_2: HindIII; Transcript-derived fragments generated
                     via cDNA-AFLP of wheat leaf mRNA after induction of
                     resistance response to the Russian wheat aphid, Diuraphis
                     noxia."
ORIGIN
        1 aagtactgca gcgaagtttc tgccaagatg ctactaacaa aaatggaaac aaagggcatt
       61 agctgtgatc aaggtggaac gtcgtccttg agtacctggc ttcagtattc aatacaagtc
      121 atgtgcttat tgtagtgatc agtactccct ccgtaaagaa atataagagc gt
//
```

APPENDIX A:

dbEST entries

GenBank: ES697590

```
LOCUS       ES697590                274 bp    mRNA    linear   EST 19-JUN-2007
DEFINITION  CG2007_043 cDNA-AFLP of wheat response to Diuraphis noxia in
            pTZ57R/T Triticum aestivum cDNA clone AMOMTM37 5', mRNA sequence.
ACCESSION   ES697590
VERSION     ES697590.1  GI:149118157
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 274)
  AUTHORS   Matsioloko,M.T., Myburg,A.A. and Botha-Oberholster,A.M.
  TITLE     Profiling Diuraphis noxia induced expression in Triticum aestivum
            near-isogenic lines using cDNA microarray and cDNA-AFLP technology
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
            Institute
            Faculty of Agriculture and Natural Science, University of Pretoria
            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3984
            Fax: 27 12 420 3947
            Email: anna.oberholster@up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
            PCR PRimers
            FORWARD: Taq1+0
            BACKWARD: Mse1+0
            Insert Length: 274   Std Error: 0.00
            Seq primer: M13
            POLYA=No.
FEATURES             Location/Qualifiers
     source          1..274
                     /organism="Triticum aestivum"
                     /mol_type="mRNA"
                     /cultivar="Tugela"
                     /db_xref="taxon:4565"
                     /clone="AMOMTM37"
                     /dev_stage="2-3 leaf stage"
                     /lab_host="Escherichia coli (JM 109)"
                     /clone_lib="cDNA-AFLP of wheat response to Diuraphis noxia
                     in pTZ57R/T"
                     /note="Organ: leaf; Vector: pTZ57R/T; Site_1: EcoRI;
                     Site_2: HindIII; Transcript-derived fragments generated
                     via cDNA-AFLP of wheat leaf mRNA after induction of
                     resistance response to the Russian wheat aphid, Diuraphis
                     noxia."
ORIGIN
        1 agaaatgaac tgcccttgcg gattgggtta ttgttcaagc acttagtaga caaatagagg
       61 gtttatacta gaactttaca acaactgaat ttctgaatgc tctgccaagg ttgcgtgatc
      121 tcttattaca gactgtgtaa acctatagaa gtggtcaatt gatcaatctg gcgtgtcagc
      181 ggcagctaca ataccttcca gcccgtatat ctatatgtgt acaagtatag ctattttact
      241 gtatgtataa cggcgtggct atctgcttct ctcc
//
```

APPENDIX A:

dbEST entries

GenBank: ES697591

```
LOCUS       ES697591                222 bp    mRNA    linear   EST 19-JUN-2007
DEFINITION  CG2007_044 cDNA-AFLP of wheat response to Diuraphis noxia in
            pTZ57R/T Triticum aestivum cDNA clone AMOMTM39 5', mRNA sequence.
ACCESSION   ES697591
VERSION     ES697591.1  GI:149118158
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 222)
  AUTHORS   Matsioloko,M.T., Myburg,A.A. and Botha-Oberholster,A.M.
  TITLE     Profiling Diuraphis noxia induced expression in Triticum aestivum
            near-isogenic lines using cDNA microarray and cDNA-AFLP technology
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
            Institute
            Faculty of Agriculture and Natural Science, University of Pretoria
            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3984
            Fax: 27 12 420 3947
            Email: anna.oberholster@up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
            PCR PRimers
            FORWARD: Taq1+0
            BACKWARD: Mse1+0
            Insert Length: 222   Std Error: 0.00
            Seq primer: T7
            POLYA=No.
FEATURES             Location/Qualifiers
     source          1..222
                     /organism="Triticum aestivum"
                     /mol_type="mRNA"
                     /cultivar="Tugela"
                     /db_xref="taxon:4565"
                     /clone="AMOMTM39"
                     /dev_stage="2-3 leaf stage"
                     /lab_host="Escherichia coli (JM 109)"
                     /clone_lib="cDNA-AFLP of wheat response to Diuraphis noxia
                     in pTZ57R/T"
                     /note="Organ: leaf; Vector: pTZ57R/T; Site_1: EcoRI;
                     Site_2: HindIII; Transcript-derived fragments generated
                     via cDNA-AFLP of wheat leaf mRNA after induction of
                     resistance response to the Russian wheat aphid, Diuraphis
                     noxia."
ORIGIN
        1 gttcgcaacg gacctccgga aaccgaattg tatgagtcct gagtaaacac gcacaaatgt
       61 ctggtttgca tatttttgcc tagccctatt actagcagtg tatttttttgc cgtaagaaag
      121 acaacaatgc tttatttctt tgacttttttg accgtgtcta atttctggta aaagagagga
      181 atttcggaga gatagacaac ctggagagct ttgaatggtt gt
//
```

APPENDIX B

IS PHOTOSYNTHETIC TRANSCRIPTIONAL REGULATION IN *TRITICUM AESTIVUM* L. CV. 'TUGELA DN' A CONTRIBUTING FACTOR FOR TOLERANCE TO *DIURAPHIS NOXIA* (HOMOPTERA: APHIDIDAE) ?

APPENDIX B:

Is photosynthetic transcriptional regulation in *Triticum aestivum* L. cv. 'Tugela DN' a contributing factor for tolerance to *Diuraphis noxia* (Homoptera: Aphididae) ?

Plant Cell Rep (2006) 25: 41–54
DOI 10.1007/s00299-005-0001-9

GENETICS AND GENOMICS

Anna-Maria Botha · Lynelle Lacock · Chantal van Niekerk · M. Thuto Matsioloko · Franco B. du Preez · Shilo Loots · Eduard Venter · Karl J. Kunert · Christopher A. Cullis

Is photosynthetic transcriptional regulation in *Triticum aestivum* L. cv. 'TugelaDN' a contributing factor for tolerance to *Diuraphis noxia* (Homoptera: Aphididae)?

Received: 31 October 2004 / Revised: 17 March 2005 / Accepted: 23 March 2005 / Published online: 18 November 2005

Abstract *Diuraphis noxia* (Russian wheat aphid, RWA) is a major pest on wheat in South Africa and most other wheat growing countries. Being a probing-sucking insect, RWAs insert their stylets into the phloem sieve elements and feed on the phloem sap. This feeding causes necrotic lesions in resistant varieties, or decoloration of leaves and death in susceptible varieties. In an effort to broaden our understanding on the response of the plant to RWA feeding, we synthesized and analyzed expressed sequence tags (ESTs) from suppression subtractive hybridization (SSH) libraries. These libraries were constructed using near isogenic wheat lines susceptible 'Tugela' and resistant 'TugelaDN'(*Dn1*) to RWA, as well as accession lines PI137739 (*Dn1*) and PI294994 (*Dn5*). Analysis of 200 ESTs from the libraries revealed the involvement of transcripts encoding genes involved in cell maintenance, growth and regulation, plant defense and signaling, photosynthesis and energy production, and of unknown function. A selection of these ESTs, in combination with clones obtained from other sources, were used on a custom array to study the expression profiles of 256 candidate wheat sequences putatively involved in plant defense against RWA. The selected sequences included wheat genomic clones with putative nucleotide binding site (NBS) motifs, rapid amplification of cDNA ends PCR (RACE-PCR), and cDNA clones from RWA induced libraries. Genomic banana and flax clones that were obtained using representative difference analysis (RDA), and suspected to be involved in abiotic stress responses, were also spotted onto the microarray slides. The spotted custom arrays were then hybridized against cDNA isolated from a resistant cultivar 'Tugela DN' on 0, 2, 5, and 8 days after infestation, post-labeled with Cy3- or Cy5-fluorescent dyes. The subsequent expression profiling using DNA microarray, RT-PCR, and Northern Blot analysis identified 29 transcripts associated with the feeding response. These transcripts encoded proteins functioning in direct defense and signaling, oxidative burst, cell wall degradation, cell maintenance, photosynthesis, and energy production. Results indicate that plants co-ordinately regulate gene expression when attacked by RWA. It is hypothesized that the NBS-LRR proteins are important in receptor recognition and signaling, which enable the plant to overcome the stresses inflicted by RWA feeding. It is further suggested that the ability to maintain photosynthetic function with resultant energy production is one of the determining factors ensuring the survival of the resistant varieties when coping with the RWA feeding.

Keywords Chloroplast ATP synthase · *Diuraphis noxia* · Microarray · NBS-LRR · Photosynthesis · Plant–insect interaction · RGA-2 · Signal transduction · SSH

A.-M. Botha (✉) · L. Lacock · C. van Niekerk · M. T. Matsioloko · F. B. du Preez · S. Loots
Department of Genetics, Forestry and Agriculture Biotechnology Institute, University of Pretoria, Hillcrest, Pretoria, ZA0002 South Africa
e-mail: AMBothao@postino.up.ac.za or AMBothaO@lamar.colostate.edu
Tel.: +970-491-5335
Fax: +970-491-0564

E. Venter
Plant-Pathogen Interaction Programme, Scottish Crop Research Institute, Invergowrie, DD2 5DA, UK

K. J. Kunert
Department of Botany, Forestry and Agriculture Biotechnology Institute, University of Pretoria, Hillcrest, Pretoria, ZA0002 South Africa

C. A. Cullis
Department of Biology, Case Western Reserve University, Cleveland, Ohio, USA

A.-M. Botha
Department of Soil and Crop Sciences, 1170 Campus Delivery, Colorado State University, Fort Collins, CO 80523-1170, USA

Introduction

The molecular basis of plant–aphid interactions remains poorly understood, despite the fact that aphids are the

APPENDIX B:

Is photosynthetic transcriptional regulation in *Triticum aestivum* L. cv. 'Tugela DN' a contributing factor for tolerance to *Diuraphis noxia* (Homoptera: Aphididae) ?

largest group of phloem-feeding insects. A common feature among the reported studies are the expression of genes known to be involved in defense against bacterial and fungal pathogens (Fidantsef et al. 1999; Moran and Thompson 2001; Moran et al. 2002). Such responses are generally not associated with chewing insects (Reymond et al. 2000). As with plant–pathogen interactions, the defense against the Russian wheat aphid (RWA) (*Diuraphis noxia* Mordvilko) is accompanied by the production of reactive oxygen species (ROS), including H_2O_2. ROS induce the accumulation of cellular salicylic acid (SA) concentrations and trigger the expression of pathogenesis related (PR) proteins. Several defense-related products have been shown to accumulate in the apoplast of resistant wheat cultivars, including the PR proteins β-1,3-glucanases, chitinases, and peroxidases (Van der Westhuizen et al. 1998a, b). Resistance is not constitutively expressed, but is induced by *D. noxia* infestation. Furthermore, the level of the response varies in different resistant cultivars. Also, the genetic background in which the *Dn*-gene is bred plays a role in the effectiveness of the resistance response (Van der Westhuizen et al. 1998a).

Studies conducted on the intercellular washing fluid of wheat (*Triticum aestivum* L. cv. 'Tugela DN') resistant to the RWA, showed that proteins were induced within 6 days of infestation. Changes in ethylene production were observed within the first 24 h after infestation by the RWA in resistant cultivar 'Tugela DN', but not in the susceptible near isogenic line (NIL) 'Tugela' (Botha et al. 1998). Coincidently, changes in transcript expression occur and these comprise of two phases, an immediate response (i.e. hypersensitive response, HR) 24 h after infestation with RWA (Botha et al. 1998; Matsioloko and Botha 2003), and a second prolonged response that prevails in the tissue for an extended period of time, i.e. systemic acquired resistance (SAR) (Van der Westhuizen et al. 1998a, b).

A study on feeding behavior of RWA confirmed that the RWA probe more and feed less on resistant cultivars, resulting in the formation of more lesions in the resistant cultivars compared to susceptible varieties (Bahlmann et al. 2003). The damage inflicted by probing and feeding of *D. noxia* prevents new leaves from unrolling. These rolled leaves shelter the aphids from chemical and biological control methods (Smith et al. 1992). The prevention of proper unfolding of new leaves and reduction in leaf size caused by *D. noxia* feeding apparently results from the reduction of leaf turgor below the threshold for elongation and cell wall extensibility. Furthermore, feeding on the flag leaf results in the developing grain head becoming trapped and interferes with self-pollination and grain-filling (Van der Westhuizen et al. 1998a).

Symptoms of RWA feeding on susceptible cultivars include longitudinal streaking, which is typified by longitudinal white and yellow streaking and stunted growth (Burd and Elliot 1996), which under severe infestation leads to a drastic reduction in effective leaf area (Walters et al. 1980). The damage occurs mainly because of a phytotoxin injected by the aphids during feeding. This toxin causes chloroplast and cellular membrane breakdown in susceptible plants. Chlorophyll deficiency, due to infestation, reduces yields by up to 50% (Burd and Elliot 1996). Ultrastructural studies showed limited chloroplast breakdown of the leaf cells of resistant cultivars after feeding. *D. noxia*-infested leaves show more collapsed auto-fluorescent cells typical of a hypersensitive cell death response (Van der Westhuizen et al. 1998a). It has been suggested that insect feeding impacted negatively on the stacked region of the thylakoid membranes, most probably the light harvesting complex II, although the exact site of damage is still unknown. It is clear that resistant varieties can compensate with aphid feeding in a yet unknown manner (Heng-Moss et al. 2003). However, for the plant to compensate with the stress load, it needs an effective defense/stress strategy and signaling pathway to produce the gene products that will activate the mechanisms for stress tolerance.

The presence of nucleotide-binding site (NBS) and leucine-rich repeat (LRR) regions is consistent with the protein products playing a significant role in signal transduction and having a putative role in protein–protein interactions (Whitham et al. 1994; Jackson and Taylor 1996; Pan et al. 2000; Cannon et al. 2002). While NBS-LRR proteins may recognize the presence of an invader directly or indirectly, specific recognition of multiple pathogens could necessitate the activity of numerous *R* genes. The guard hypothesis proposes that NBS-LRR proteins guard plant targets against pathogen effector proteins. In this scenario, the pathogen products act as virulence factors to enhance the susceptibility of the host plant in the absence of recognition (Van der Biezen and Jones 1998).

The objectives of this study were, to identify genes that are regulated in response to RWA feeding, and then to study the expression profile of these genes. Further objectives were to establish a potential role for the NBS-LRRs in the RWA resistance response, and to confirm the importance of photosynthetic associated genes and the unknown ESTs. The expression profiles of these gene sequences were characterized through hybridization of the microarray against cDNA isolated from leaves of the RWA resistant cultivar 'Tugela DN' pre- (day 0) and post-infestation (p.i.)(days 2, 5, and 8), in an effort to identify gene sequences with significance to the RWA defense response.

Material and methods

Plant material

Wheat NILs 'Tugela' (RWA susceptible) and 'Tugela DN' (Tugela*5/SA1684, *Dn1*, RWA resistant), and RWA resistant wheat lines PI137739 (*Dn1*) and PI294994 (*Dn5*) were grown in pots under greenhouse conditions with prevailing day and night cycles at the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. The temperature was maintained at about 24°C, and the plants were watered daily. Half of the wheat seedlings were infested with RWA (10 aphids per plant) at the 3–4-leaf growth stage (Botha et al. 1998). The second and third leaves from uninfested (day 0) and infested

APPENDIX B:

Is photosynthetic transcriptional regulation in *Triticum aestivum* L. cv. 'Tugela DN' a contributing factor for tolerance to *Diuraphis noxia* (Homoptera: Aphididae) ?

plants were removed after days 2, 5, and 8 p.i. for analysis. The aphids were removed from the infested leaves prior to RNA isolation

RNA isolation and mRNA purification

RNA was isolated from the second and third leaves of the infested plants, 2–8 days p.i. (Botha et al. 1998). RNA was also extracted from control wheat plants using leaves at similar developmental stages as that of the infested plants. The RWAs were removed from the leaves through rinsing with water. The leaves were ground to powder in liquid nitrogen and RNA extracted according to the guanidine thiocyanate method of Chomczynski and Sacchi (1987), using DEPC-treated equipment and solutions. mRNA was purified from total RNA using Oligo(dT)-cellulose columns supplied with the mRNA Purification Kit (Amersham Pharmacia Biotechnology, UK).

cDNA synthesis

First strand cDNA was synthesized using 1.6 µg tester (infested) and 0.85 µg driver (uninfested) mRNA (DNA Synthesis System, Roche Molecular Biochemicals, Germany), using primer Pr 16 (200 pmol; 5′-TTT-TGT-ACA-AGC-TT_{30} -3′). Second strand cDNA was synthesized, the products were cleaned using the MinElute Reaction Cleanup Kit (Qiagen, USA), and eluted with 40 µl double distilled H_2O.

Suppression subtractive hybridization

SSH reactions were performed on the synthesized cDNA using the PCR Select cDNA subtraction kit according to the manufacturers instructions (BD Biosciences Clontech, USA). cDNA from the 'Tugela DN'/PI137739/PI294994 were used as tester, and the driver consisted of 'Tugela'. The subtraction was performed with a 1:600 and 1:1000 dilution. The efficiency of the subtractions was verified through Southern Blotting (Lacock and Botha 2003; Van Niekerk and Botha 2003).

Cloning and sequencing

The secondary SSH fragments were purified using the Geneclean III Kit (Southern Cross Biotechnology, USA). The fragments were cloned into pGEM-T Easy Vectors (Promega Corporation, USA), and positive transformants were screened through colony PCR (Gussow and Clackson 1989) and used as template in subsequent sequencing reactions using either the SP6 (5′-ATT-CTA-TAG-TGT-CAC-CTA-AAT-3′) or T7 (5′-TAA-TAC-GAC-TCA-CTA-TAG-GG-3′) primers.

Preparation of cDNA clones/ESTs for spotting on the microarray

Total RNA isolation, purification of mRNA, cDNA synthesis, cDNA library construction and sequencing was performed as previously described (Lacock et al. 2003). After sequencing the clones, sequence identities were annotated through BLAST searching and alignment to other published sequences in GenBank (Altschul et al. 1997). Functions were assigned to dbESTs based on the results (E value $< 10^{-5}$) returned from searches using the BLASTX algorithm (Lacock et al. 2003).

Northern Blot analysis and RT-PCR

Northern Blot analyses were performed using total RNA extracted from uninfested (day 0) and infested (day 2) wheat leaves and stems, as well as leaves infested at day 0, 2, and 5 p.i. with the RWA. RNA (200 ng) was transferred onto nylon membranes (Roche Diagnostic Corporation, Germany) and UV-cross linked to the membranes (Sambrook et al. 1989).

DNAs (50 ng) were labeled using the *Gene Images* Random Prime Labeling module (Amersham Pharmacia Biotechnology, UK), and hybridized to the filters according to the CDP-*Star* protocol (Amersham Pharmacia Biotechnology, UK).

Quantitative PCR was performed using 70 ng first strand cDNA from selected total RNA as required, 10 pmol forward and reverse primers, 3 mM $MgCl_2$, and the LightCycler–FastStart DNA Master SYBR Green 1 Mix (Roche Diagnostics Corporation, Germany) in a 20 µl reaction, according to manufacturer's procedures (LightCycler–FastStart DNA Master SYBR Green 1 Manual, Roche Applied Science, Germany). The cycling parameters consisted of 1 cycle at 95°C for 10 min; 40 cycles starting with 1 cycle at 95°C for 10 s, primer specific annealing T°C for 5 s, 72°C for 10 s; followed by the melting curve analysis (95°C for 0 s, 65°C for 15 s, 95°C for 0 s), and cooling (40°C for 30 s). A minimum of seven reactions was done for each fragment analyzed, standard curves were generated using dilution series (1:1, 1:10, 1:100, 1:1000) and repeated. Results obtained were analyzed using LightCycler Software version 3.5 (Roche Applied Science, Germany).

Microarray preparation

Target DNA (256 wheat ESTs, 50 flax and banana genomic clones and control genes), for spotting on the microarray, were amplified using standard PCR procedures (40 cycles; annealing at 64°C; 2 ng plasmid template). PCR products were purified using Multiscreen purification plates as prescribed by the manufacturer (Millipore, Germany). PCR products were quantified by electrophoresis on 0.8% (w/v) agarose gels and visualized by ethidium bromide staining. Microarrays were printed using a BioRobotics Generation II Arrayer according to the manufacturer's instructions. Arrays were printed on aminosaline slides and each target DNA spotted eight times. Negative controls on the array included blank spots, Lucidea Universal Scorecard (ratios 1:1, 1:3, 1:10, 3:1, 10:1), constitutively expressed and stress responsive genes.

APPENDIX B:

Is photosynthetic transcriptional regulation in *Triticum aestivum* L. cv. 'Tugela DN' a contributing factor for tolerance to *Diuraphis noxia* (Homoptera: Aphididae) ?

Fluorescent probe preparation and hybridization

For Cy3- and Cy5-labeled cDNA, total RNA was isolated from wheat leaves on days 0, 2, 5, and 8 p.i. by the RWA, as previously described (Matsioloko and Botha 2003). Poly A^+ RNA was purified from total RNA using Oligotex mRNA spin-columns (Qiagen Inc., USA). Purified mRNA (100 ng) was used for the preparation of Cy3- and Cy5-labeled cDNA for microarray hybridization using the Cyscribe Post-labeling kit according to the manufacturer's instructions (Amersham Biosciences, Little Chalfont, UK). Unincorporated label nucleotides were removed from the prepared probe using the MinElute cleanup kit according to the manufacturer's protocol (Qiagen Inc., USA).

Microarrays were pre-hybridized by adding 35 µl pre-hybridization solution (3.5 × SSC, 0.2% (w/v) SDS, 1% (w/v) BSA) for 20 min at 60°C using a humidified hybridization-cassette. Slides were washed in ddH_2O for 1 min and air-dried using nitrogen gas. For hybridization, 30 pmol of each probe per slide was dried in a 0.5 ml eppendorf and resuspended in 35 µl hybridization solution [50% (v/v) formamide, 25% (v/v) hybridization buffer; 25% (v/v) deionized water] and denatured (98°C for 2 min). The slides were hybridized for 12–18 h at 42°C. After hybridization, slides were washed once in 1× SSC, 0.2% (w/v) SDS at 37°C for 4 min, twice in 0.1× SSC, 0.2% (w/v) SDS at 37°C for 4 min, twice in 0.1× SSC at room temperature for 1 min, and then rinsed in deionized water for 2 s. Slides were dried using N_2 gas.Hybridizations were repeated over biological material, made use of direct comparisons, and a reference, 2×2 factorial design (Wang and Speed 2002).

Scanning and data analysis

An Axon GenePix 4000A Microarray scanner and GenePix acquisition software (Axon Instruments, Inc., USA) were used according to the manufacturer's instructions regarding dye emission to capture the data. Normalization between Cy3- and Cy5-fluorescent dye emission intensities was achieved by adjusting the level of the photomultiplier gains ('global normalization'). After scanning and capturing of data using the GenePix 3.0 software, the raw data was imported into Microsoft Excel for further analysis. Background fluorescence values were automatically calculated by the GenePix program and subtracted from all feature intensities before further calculations were performed. Genes of interest were identified by computational analysis using ANOVA as proposed by Dudoit and coworkers (Dudoit et al. 2001) and the mixed model approach (Wolfinger et al. 2001; Chu et al. 2002) and SAS/STAT software version 8 (SAS Institute Inc. 1999).

Chlorophyll concentration

Total chlorophyll concentrations were determined in wheat NILs 'Tugela' (susceptible) and 'Tugela DN' (resistant) after RWA feeding. The plants were infested as previously described and chlorophyll was extracted 0, 8, and 16 days p.i. using the biochemical extraction methods as described by Arnon (1949) and Vernon (1960).

Results

Isolation of stress/defense-related genes in wheat

In an effort to obtain the molecular profiles of the wheat transcriptome elucidated in response to RWA, a combination of suppression subtractive and microarray technologies was used. After suppression subtraction of cDNA from different RWA resistant varieties, a total of 200 non-redundant ESTs were obtained. Following sequence annotation, the obtained ESTs were classified in groups according to their respective significant sequence homologies with known sequences present in GenBank (Table 1). Of the obtained ESTs, 26.7% sequences showed significant homology to sequences that encode genes related to cell structure and maintenance, 18.9% to genes involved in photosynthesis (i.e. chloroplast genes for LSU, Rubisco *rbcL* gene, red chlorophyll catabolic reductase gene, gene for Photosystem I P700 apoprotein, mRNA for thioredoxin *m*, chloroplast ATP synthase, and chloroplast gene), 1% to nitrogen fixation (i.e. Noduline-like protein), 1% to the group classified as the reactive oxygen species (i.e. Glutathion-S-transferase). A further 11.5% of the sequences had significant homology to genes putatively involved in signaling or defense (i.e. RGA-2, Leucine rich-like protein, LZ-LRR-NBS, *Mla* locus, and the *Meloidogyne*-induced giant cell protein), whilst 41% had significant homology with genes with no known or ascribed function, or had no significant homology to any known sequence in GenBank, and thus were classified as unknown.

Expression profiling of transcripts

Expression in post-RWA infested tissue was monitored using cDNA microarray slides that contained 380 spots, including 256 ESTs previously isolated from RWA induced cDNA libraries (Lacock et al. 2003), 50 *Musa* (banana) and *Linum usitatissinum* (flax) clones, and controls (e.g. genes known to be regulated under stress and Lucidea Universal Score Card). Our focus was on a comparison between pre- and post-infestation events. The analysis of the fluorescence data from the microarray slides using a general analysis of variance (ANOVA) as suggested by Dudoit et al. (2001) indicated that 27% ESTs were down-regulated, 28% up-regulated, and 45% displayed no change (not shown). This was an unexpectedly large fraction and we argued that many false positives may be present. The ANOVA model is based on the normalization of log ratios and then permutation-based *t*-statistics for testing the significance of each gene, and *p*-values which are suitably adjusted for multiplicity. Therefore, the data was subjected to the statistically rigorous mixed model approach (Wolfinger et al. 2001) that allows for the identification of false positives, as

APPENDIX B:

Is photosynthetic transcriptional regulation in *Triticum aestivum* L. cv. 'Tugela DN' a contributing factor for tolerance to *Diuraphis noxia* (Homoptera: Aphididae) ?

Table 1. Expressed sequence tags (ESTs) isolated from enriched SSH libraries upon RWA feeding

GenBank accessions[a]	Putative function/Homologues[b]
Cell structure and maintenance	
M11585.1; AJ309824.2	25S rRNA: *Oryza sativa; Zea mays*
AY049040	18S Ribosomal RNA gene: *Triticum aestivum*
AY049041.1; M37231	26S rRNA 3′ end: *Triticum aestivum; Triticale cereale*
	Beta-1,4-endoglucanase precurser: *Heterodera schachtii; Triticum aestivum cv. TugelaDN*
AF326781.1	Actin gene: *Triticum monococcum*
Z17327.1	BARE-1 copia-like retroelement: *Hordeum vulgare*
Z84569.1	BARE-1 long terminal repeat: *Hordeum vulgare*
AJ279072	BARE-2 and BAGY-2 retrotransposon: *Hordeum vulgare*
U40814.1	T-DNA integration target sequence: *Oryza sativa*
Z75576.1	Telomere-associated DNA: *Triticum aestivum*
AF254799.1	Tonoplast intrinsic proteins 1 + 2: *Hordeum vulgare*
AJ241338.1; U88031.1	Ty1-copia retrotransposon: *Hordeum vulgare; Secale cereale*
AF439728.1	Serine hydroxymethyl-transferase mRNA: *Zea mays; Triticum aestivum PI137739*
AY114061.1	Ubiquitin-conjugating enzyme: *Arabidopsis thaliana*
M28059.1; CA798959	Ubiquitin carrier protein mRNA: *Triticum;*[b]*Triticum aestivum PI137739*
X57168.1; AF339051	WIS-2-1A Ty1-copia-like retrotransposon: *Triticum aestivum; Triticum monococcum*
AJ291716.1; AJ291717.1	DNA WIS-2-1A retroelement: *Aegilops squarrosa; S. cereale* × *T. turgidum*
X65875.1	Inverted terminal repeat1 gene: *Hordeum vulgare*
AAL6539.1; BU808659	3-methylcrotonyl CoA carboxylase biotin containing subunit: *Oryza sativa;*[b]*Triticum aestivum cv. TugelaDN*
AF324485	Aminotransferase-like protein: *Oryza sativa*
X03042.2	Ay gene for High molecular weight glutenin: *Triticum aestivum*
AF326781.1	Chromosome condensation factor: *Triticum monococcum*
AJ300268.2; AJ300565.1	Retrotransposon-like element: *Aegilops speltoides; Triticum tauschii*
AF454918.1	Proembryo mRNA: *Oryza sativa*
Photosynthesis related	
D00207; X62117.1	Chloroplast genes for LSU: *Oryza sativa; Triticum aestivum*
L15300.1; Z49845.1	Rubisco *rbcL* gene: *Avena sativa; Hordeum lechleri*
AJ243066.1	Red chlorophyll catabolic reductase gene: *Hordeum vulgare*
BAA83440.1; CA407985	Photosystem I P700 apoprotein: *Anthoceros puctrantus;*[b]*Triticum aestivum cv. TugelaDN*
AJ005840	mRNA for thioredoxin M: *Triticum aestivum PI137739*
M16843.1; CB412238; CB412237; CB412222; CB12206; CB412223; CB412239; CB4122240; CB412217; CB412200; CB412218;	Chloroplast ATP synthase: *Triticum aestivum; Aegilops crassa;*[b]*Triticum aestivum PI137739;*[b]*Triticum aestivum cv. TugelaDN;*
AB042240.3; CB412240; CB412223	Chloroplast gene:[b]*Triticum aestivum*
Nitrogen fixation	
AF326781.1	Noduline-like-like protein: *Triticum monococcum*
Active oxygen related (AOR)	
AY013753.1	Glutathion-S-transferase 1 + 2: *Aegilops tauschii*
Defense-related (i.e. receptors and signal transduction)	
AF326781; CB412258; CB412247	Putative resistance protein (RGA-2): *Triticum monococcum; Aegilops tauschii;*[b]*Triticum aestivum cv. TugelaDN*
AF497474.1; CA798962; CB412215; CB412254	Leucine rich-like protein: *Aegilops tauschii; Triticum aestivum PI137739*

APPENDIX B:

Is photosynthetic transcriptional regulation in *Triticum aestivum* L. cv. 'Tugela DN' a contributing factor for tolerance to *Diuraphis noxia* (Homoptera: Aphididae) ?

Table 1. Continued

GenBank accessions[a]	Putative function/Homologues[b]
AF497474.1; CA798962; CB412215; CB412254	Leucine rich-like protein: *Aegilops tauschii*; *Triticum aestivum PI137739*
AF446141.1; CA798957; CA798960; CA798961	LZ-LRR-NBS: *Aegilops tauschii*;[b]*Triticum aestivum PI137739*
AF427791.1	*Mla* locus: *Hordeum vulgare*
L24012.1	*Meloidogyne*-induced giant cell protein: *Lycopersicon esculentum*
Proteins of unknown function or no sequence homology found	
X12849.1	DNA: non-functional rpl23 homologue: *Triticum aestivum*
AP003017; BU808657	Unknown protein: *Mesorhizobium loti*;[b]*Triticum aestivum cv. TugelaDN*
NP191007.1; BU808659; BU808660	Putative protein: *Arabidopsis thaliana*;[b]*Triticum aestivum PI137739*;[b]*Triticum aestivum cv. TugelaDN*
BU808658; BU808656; CA407985; BU808657; CA407985; CB412174; CB412178; CB412183-84; CB412192; CB412194; CB412196-7; CB412200-1; CB412203; CB412207-9; CB412211; CB412214; CB412216; CB412219; CB412221; CB412223; CB412225; CB412227; CB412232; CB412234-36; CB412238-39; CB412241; CB412243; CB412245-46; CB412254-58	Unknown protein:[b]*Triticum aestivum cv. TugelaDN*;[b]*Triticum aestivum PI137739*
ZP00026480.1; CA407984	Hypothetical protein: *Ralstonia metallidurans*;[b]*Triticum aestivum cv. TugelaDN*
AY065285; NP_190339	Unknown protein: *Arabidopsis thaliana*; *Triticum aestivum PI1294994*;

[a]BLASTN and BLASTX searches were conducted to determine homologous genes and putative function of the ESTs, cDNA, and genomic DNA fragments. The cut-off *e*-value used was 10^{-5}

[b]Sequences with no significant hit were classified as unknown. Sequences have been submitted to GenBank dbEST database (all listed BU, CA, and CB Accession numbers)

well as the selection of genes with significant expression. The latter model centers on two interconnected ANOVA models, namely the normalization model and the gene model. The analysis corrects for spot position, pen position, fluorescence bias, and differences due to experiment design and biological repeats. This reduced the count to only 29 of the spotted gene fragments that had a threshold log-2 expression ratio of 1.5 and $P \leq 0.05$ (Fig. 1). These included several of the ESTs showing significant altered expression patterns (Table 1). The flax homologue to APC/C ubiquitin-protein ligase with known function in cell cycle regulation proved to be regulated if statistically analyzed via ANOVA, but not if analyzed via the Mixed Model approach. This was also true for the clones with significant homology to the *Linum usitatissinum* LIS-1 insertion sequences, even though it was previously shown to be induced, in genotrophs, by the environment (GenBank accession no. AF104351, http://www.ncbi.nlm.nih.gov/entrez/query). The significance of a selected set of clones was verified using RNA Blot and real-time PCR analysis (Fig. 2).

The microarray analysis of the selected RNAs indicated that one EST was unchanged, and four ESTs were up-regulated in response to RWA feeding. The probe for the hybridization of ribosomal RNA was included to demonstrate equal loading, since no significant regulation was observed with this clone. The other clones included a BARE-1 long terminal repeat, a Ty1-copia-like retrotransposon, and two sequences with no significant homology to

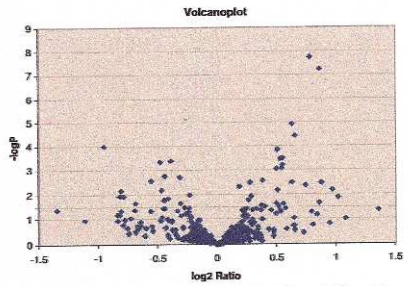


Fig. 1 Gene significance results for RWA infested wheat data. Volcano significance plot for a subset of wheat ESTs. Plotted on the vertical axis is $-\log_{10}$ (p-value) for contrast between treatments. Horizontal axis is $\log_2$ of the estimated fold change, suitably adjusted for other systematic and random effects in the experiment. Broken line indicates the threshold log-2 expression ratio of 1.5 and $P \leq 0.05$

APPENDIX B:

Is photosynthetic transcriptional regulation in *Triticum aestivum* L. cv. 'Tugela DN' a contributing factor for tolerance to *Diuraphis noxia* (Homoptera: Aphididae) ?

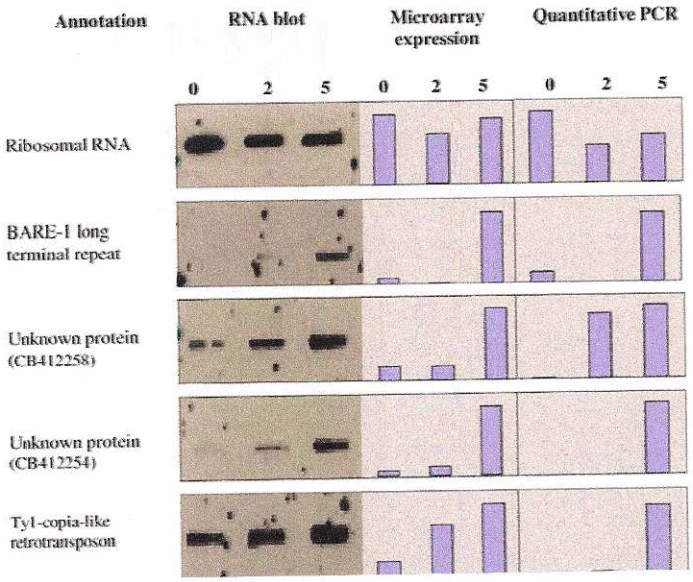


Fig. 2 Comparison of measured changes in transcript expression as quantified by conventional RNA Northern Blot, quantitative PCR analysis, and cDNA microarray

any known sequence in GenBank. Figure 2 confirmed that the data obtained with the microarray were in general agreement between the methods, although, with the RT-PCR the level of up-regulation was much higher due to the sensitivity of the technology. Nevertheless, taken as a whole, the comparison indicated that microarray quantification was reliable.

The ESTs with significant expression were listed under four broad categories (Table 2), namely sequences involved in cell structure and maintenance (i.e. division, growth, and organization), photosynthesis (i.e. chloroplast structure and function), defense related (i.e. receptors and signal transduction), and unclassified or unknown.

Cell maintenance genes

Among numerous cell maintenance genes included on the array, only the wheat homologues to a *Beta nana* Ty1-copia-like retrotransposon, an *Oryza sativa* T-DNA integration factor, *Hordeum vulgare* BARE-1 long terminal repeat, T-DNA integration factor, partial mRNA 3′UTR sequence, serine hydroxymethyl transferase mRNA, and a beta-1,4-glucanase 1 precursor were significantly regulated after RWA feeding. Interestingly, the flax clones that encode for the LIS-1 insertion sequences previously suggested to be under environmental control, as well as the APC/C ubiquitin-protein ligase known to be involved in cell cycle regulation, appeared significantly regulated after RWA infestation of wheat leaf tissue using ANOVA statistical analysis, but not with the Mixed Model (Table 2). The sequences that encode for proteins with homology to T-DNA integration factor and beta-1,4-glucanase 1 precursor were up-regulated throughout the feeding experiment, whilst the APC/C ubiquitin-protein ligase and BARE-1 long terminal repeat were initially down-regulated on days 2 and 5 p.i., but then up-regulated on day 8 p.i. All of the thers were initially down-regulated on day 2 p.i., but then up-regulated for the duration of the experiment.

Photosynthesis-related genes

Eight ESTs listed under the photosynthesis-related group were significantly regulated during the duration of the RWA feeding experiment. These include a banana unknown chloroplast sequence and a chloroplast gene for a chloroplast product, and wheat homologues to chloroplast genes for photosystem I P700 apoprotein, matK pseudogene from chloroplast, maize chloroplast DNA for 4.5S rRNA, and chloroplast ATP synthase (Table 2). The regulation of the ESTs that encode for photosynthesis/chloroplast function followed three expression patterns. Some are initially up-regulated and then down-regulated (e.g. flax chloroplast genes), some were initially down-regulated and then up-regulated (e.g. matK pseudogene, chloroplast genes), and then those that were mostly up-regulated in response to RWA feeding (e.g. sequences that encode for proteins with homology to chloroplast ATP synthases, and the gene for photosystem I P700 apoprotein A1).

APPENDIX B:

Is photosynthetic transcriptional regulation in *Triticum aestivum* L. cv. 'Tugela DN' a contributing factor for tolerance to *Diuraphis noxia* (Homoptera: Aphididae) ?

Table 2 Genes differentially expressed in response to RWA feeding

Putative function/homology[a]	Ratios of signal intensity [b] (Fluorescence units)		
	Day 2/0	Day 5/0	Day 8/0
Cell structure and maintenance			
[cd]APC/C ubiquitin-protein ligase (cell cycle regulation)	−1.661	−0.351	1.812
Partial mRNA 3'URT	1.559	−0.285	0.048
Ty1-copia-like retrotransposon for putative reverse transcriptase	−15.961	0.865	12.369
BARE-1 long terminal repeat-2	−1.652	−0.115	4.156
[cd]LIS-1 insertion sequence	1.13	−0.171	0.988
[cd]LIS-1 insertion sequence in genotrophs induced by the environment	−6.398	1.239	9.017
T-DNA integration factor	1.101	0.014	0.54
Serine hydroxymethyl transferase mRNA	−1.328	0.467	1.627
Beta-1,4-glucanase 1 precursor	0.395	0.299	3.466
Photosynthesis			
Chloroplast gene	−0.933	3.773	−3.479
Chloroplast ATP synthase	1.796	0.209	4.024
Chloroplast gene for photosystem I P700 apoprotein A1	11.163	−1.84	14.849
Dendrobium chrysotoxum trnK, matK pseudogene, chloroplast genes	−2.872	−1.468	14.494
[d]Banana chloroplast gene for chloroplast product	2.007	0.905	−0.12
[d]Banana unknown chloroplast sequence	2.361	−0.107	−2.887
Maize chloroplast DNA for 4.5S rRNA	2.153	1.134	−2.983
Chloroplast genes ATP synthase	224.264	−63.19	463.415
Defense-related (i.e. receptors and signal transduction)			
Leucine-rich-like protein gene (CB412254)	−2.687	1.827	−0.097
Leucine-rich-like protein gene (CB412215)	2.828	1.043	96.983
Putative resistance protein (RGA-2)	22.777	2.133	−45.604
Putative resistance protein (RGA-2) (CB412258)	−46.583	25.112	132.091
[e]*Mla* locus	98.6	942.3	–
Proteins of unknown function or no sequence homology found			
[d]*Linum usitatissimum* clone 5–2	−1.461	0.764	9.264
[d]Banana unknown protein	−1.111	−0.424	2.883
'TugelaDN' unknown protein (CB412254)	8.74	2.903	25.424
'Tugela DN' unknown protein (CB412234)	−2.14	−0.505	−0.331
'Tugela DN' unknown protein (CB412236)	9.804	5.988	−12.688
PI137739 unknown protein (CB412238)	2.853	−1.039	−0.23
PI137739 unknown protein (CB412257)	−1.478	−0.531	−0.237
PI137739 unknown protein (CB412258)	−7.879	−1.383	16.725

[a]BLASTN and BLASTX searches were conducted to determine homologous genes and putative function of the ESTs, cDNA, and genomic DNA fragments. The cut-off *e*-value used was 10^{-5}. Sequences with no significant hit were classified as unknown

[b]Ratios of signal intensity were determined by cDNA microarray as described under Material and Methods. Values are highlighted in red if up-regulated ≥1.5-fold in gene expression by the treatment, or in green if down-regulated ≥ 1.5 fold at a significance level of $P < 0.05$ using the Mixed model algorithm (Wolfinger et al. 2001)

[c]Values at a significance value of $P < 0.05$ using ANOVA (Dudoit et al. 2001)

[d]gDNA clones obtained via RDA analysis from Banana or Flax

[e]Expression values determined by RT-PCR. (−) not determined

Defense-related genes

Wheat homologues to RGA-2 and Leucine-rich-like proteins were mostly up-regulated in response to RWA infestation. The *Mla* locus was not included on the microarray, but the regulation was determined using RT-PCR, and it was also shown to be significantly up-regulated in response to RWA feeding (Table 2).

Other genes that displayed regulation

Seven of the ESTs classified as unknown or without any discernable function were under regulation during RWA feeding. These include all types of regulation, e.g. mainly down-regulated, mainly up-regulated, only down-regulated, only up-regulated, as well as a combination of down-/up-regulation in response to RWA feeding.

APPENDIX B:

Is photosynthetic transcriptional regulation in *Triticum aestivum* L. cv. 'Tugela DN' a contributing factor for tolerance to *Diuraphis noxia* (Homoptera: Aphididae) ?

Tissue specificity and response in RWA susceptible and resistance cultivars

Since it was suggested that RWA feeding adversely affects photosynthetic capacity of susceptible plants (Burd and Elloitt 1996; Heng-Moss et al. 2003; Wang et al. 2004), the chlorophyll content of resistant and susceptible NILs was measured (Fig. 3). It was found that the total chlorophyll content of susceptible 'Tugela' decrease significantly when compared to the resistant 'Tugela DN' over the 16-day period after RWA infestation. RWA further induce decoloration of leaves and chlorotic streaking in the susceptible variety, which is in sharp contrast to the development of necrotic lesions in resistant plants. Therefore, the expression of candidate genes was verified. These candidate genes were selected as they were the highest up-regulated genes, multiple copies were isolated during SSH, and they are putatively involved in photosynthesis and signaling (Fig. 4). The results indicated significantly higher expression of these genes in the resistant 'Tugela DN' when compared to the susceptible 'Tugela' cultivar. As expected, the expression was significantly higher in leaf tissue than in stem tissue.

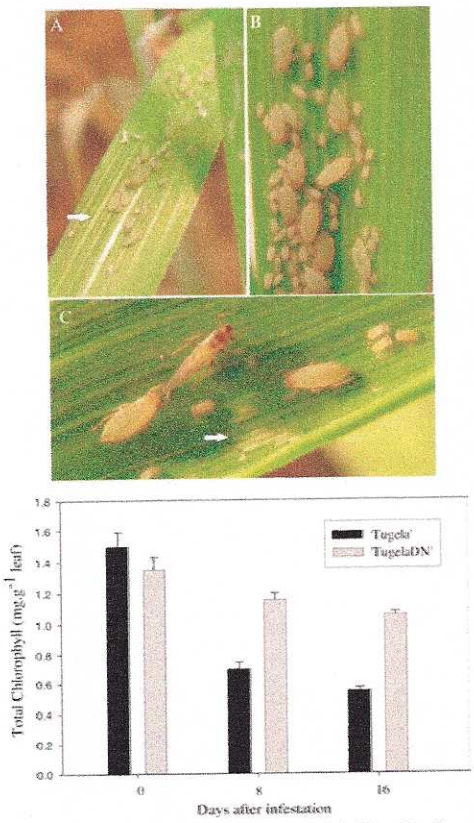


Fig. 3 RWA infestation of susceptible 'Tugela' (A), and resistant 'TugelaDN' (B, C). RWA feeding results in leaf chlorosis and streaking (arrow) in the susceptible NIL (A), while the resistant plant only expresses necrotic lesions (arrow, C). Changes in total chlorophyll content after RWA feeding (D)

Discussion

A sequence with significant homology to the gene that encode the *Meloidogyne*-induced giant cell protein in *Lycopersicon esculentum* was expressed in wheat in response to RWA. Since all the insects were removed prior to extraction, it can only be reminiscent of the feeding process. The release of proteinaceous secretions (i.e. polygalacturonases) is associated with aphid feeding. This helps to predigest plant polysaccharides for nutritional purposes (Miles 1999). However, the formation of giant-cells such, as known to occur in plant-parasitic nematodes interaction (Davies et al. 2004), has not been reported. A successful *Meloidogyne*—host interaction requires molecular signals from the parasite to modify directly or indirectly, plant root cells into elaborate feeding cells, called giant-cells. These cells serve as sole source for the nematode's development and growth (Haung et al. 2004).

Are the expressed transcripts enabling the plant to overcome the stress?

Transposable elements appear to be integral constituents of all the genomes studied so far, and they have been broadly categorized into two classes depending on their mode of transposition. Class I elements or retrotransposons (e.g. LTRs, non-LTRs, LINEs and SINEs) transpose via an RNA intermediate by a "copy-and-paste mechanism", while class II type (bacterial type) elements transpose by a "cut-and-paste mechanism" involving a DNA intermediate (Doolittle et al. 1989; Xiong and Eickbush 1990). Little is known regarding the roles of BARE-1 long terminal repeats, BARE-2 and BAGY-2, WIS-2-1A Ty1-copia-like, and Ty-1-copia-like retrotransposons, apart from their evolutionary significance, and their contributions to genome size differences. They are also important elements in genome organization (Bennetzen 2000; Hanson et al. 2000; Katsiotis et al. 2002), and are activated by stress. Furthermore, retrotransposons are known to cause alterations in transcript processing, by altering the spatial and temporal patterns of gene expression or the structure of the resulting gene product (Flavell et al. 1994; Wessler et al. 1995). More recently, *Panzee*, a copia-like retrotransposon was shown to drive gene expression in pigeon pea (Lall et al. 2002). Significant up-regulation of only the wheat homologues to a *Beta nana* Ty-1-copia-like retrotransposon, an *Oryza sativa* T-DNA integration factor, *Hordeum vulgare* BARE-1 long terminal repeat, T-DNA integration factor occurred after RWA feeding. This may

APPENDIX B:

Is photosynthetic transcriptional regulation in *Triticum aestivum* L. cv. 'Tugela DN' a contributing factor for tolerance to *Diuraphis noxia* (Homoptera: Aphididae) ?

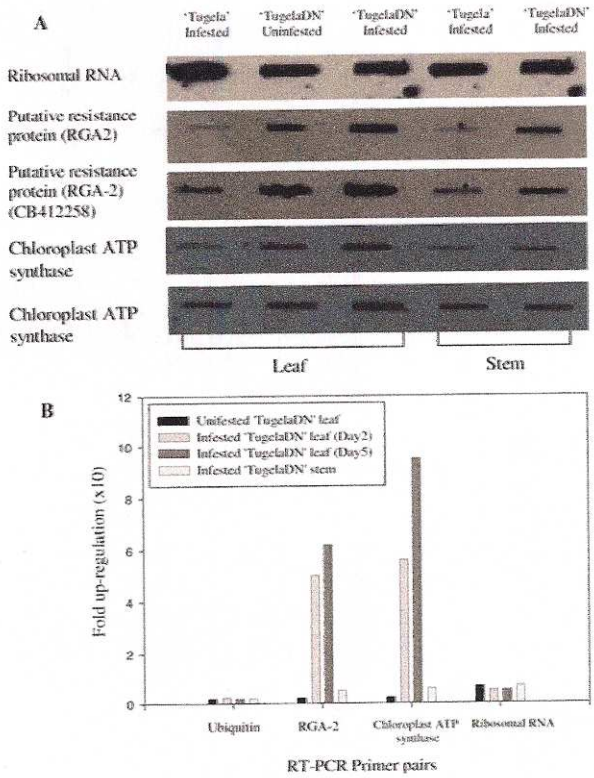


Fig. 4. Tissue specific and patterns of differential expression observed between RWA resistant ('Tugela DN') and susceptible ('Tugela') NILs. Comparison of measured changes in transcript expression as quantified by conventional RNA Northern Blot (A) and quantitative PCR (B). RNA was extracted on day 0 (uninfested), days 2 and 5 (infested) from leaf and stem tissue. Ribosomal RNA indicates equal loading, and ubiquintin was used as standard during quantitative PCR

indicate that they are one of the means whereby the plant changes to adapt to this stressful environment.

The expression of higher plant endo-β-1,4-glucanases, also known as 'cellulases', has long been studied in the events where separation of cells is required (Rose and Bennett 1999). It was only recently, that cellulase expression was associated with fruit development and ripening under auxin control. The involvement of this enzyme in cell wall modification and ethylene up-regulation was also identified (Rose and Bennett 1999). Since there exist two types of responses, one can only speculate about the role of the enzyme during RWA feeding. It may function in response to the hydrolytic enzymes secreted by the RWA during the feeding process, or it is part of the cell wall modification process to circumvent the invasion.

Nucleotide binding site-leucine rich—repeat transcripts may play a role in RWA defense

The recognition of invading pathogens is facilitated through resistance (*R*)-gene products and the activation of responses results in the impediment of pathogen growth (Keen 1990). *R*-gene mediated response is dependent on the expression of a complementary avirulence (*Avr*) gene within the pathogen or pest. If a host *R*-gene product pairs with a specific invader *Avr*-gene product, recognition occurs and an incompatible interaction follows. Incompatibility of this interaction results in a rapid signal cascade, leading to an active defense response. In the absence of either the *R*-gene or the corresponding *Avr*-gene, a compatible interaction occurs, and the pathogen is able to proliferate and cause disease. This genetic relationship between the hosts and pathogens, termed gene-for-gene interaction (Flor 1971), is involved in a wide range of pathogen types including fungi, bacteria, viruses, nematodes, and more recently it was also suggested for insects (Bent 1996; Baker et al. 1997; Dixon 1998; Van der Biezen and Jones 1998).

Resistant genes that function in a gene-for-gene manner generally belong to one of four general classes based on the amino-acid motifs that are found within the encoded protein sequence. Members of the largest class encode for cytoplasmic proteins with a NBS and several LRRs (Whitham et al. 1994). The proteins encoded by the

APPENDIX B:

Is photosynthetic transcriptional regulation in *Triticum aestivum* L. cv. 'Tugela DN' a contributing factor for tolerance to *Diuraphis noxia* (Homoptera: Aphididae) ?

NBS-LRR resistance genes can be further subdivided in either having a coiled-coil (CC) or Toll-interleukin-1 receptor (TIR) homologous domain at the amino terminus. However, only the CC domains occur in wheat. Examples of NBS-LRR genes in wheat complying to the gene-for-gene rule, include the *R*-genes *Lr10* (Feuilett et al. 2003) and *Lr21* (Huang and Gill 2001; Huang et al. 2003), and the *Mla*-locus (Halterman et al. 2001).

R-genes belong to a large gene family where arrays of similar sequences allow for recombination events that can lead to the evolution of the gene for novel recognition specificities (Michelmore and Meyers 1998). Despite selection of divergence, many of these race-specific resistance genes retain the requirement for similar downstream events (Aarts et al. 1998). Considering the function of NBS-LRRs in plant defense, and taking into account that several copies of the same ESTs encoding for proteins with significant homology to NBS-LRRs (i.e. wheat homlogues to RGA-2, leucine rich-like protein, a LZ-LRR-NBS, and *Mla* locus— also a CC-NBS-LRR) were isolated from RWA induced libraries, the significance of these clones during RWA defense was investigated.

All the NBS-LRR-like ESTs were significantly regulated during RWA feeding on the host plants. Linkage to the *Dn1* gene previously confirmed for NBS-LRR and RGA-2 using a segregating F_3 'Tugela' × 'Tugela Fast Grow' population (Swanepoel et al. 2003), further provide supporting evidence for their involvement in RWA defense. RGA-2 is also an NBS-LRR-like protein with a putative receptor-like function, and thus may be involved in signal transduction (Whitham et al. 1994; Jackson and Taylor 1996; Pan et al. 2000; Cannon et al. 2002) and/or invader recognition (Van der Biezen and Jones 1998).

In barley and wheat, it has been demonstrated that the *Mla*-locus confers multiple resistance specificities to the obligate fungal biotroph, *Blumeria graminis* f. sp. *hordei*. This is accomplished by highly regulated CC-NBS-LRR proteins encoded by alleles of the *Mla*-locus, and these proteins can detect similar powdery mildew resistance phenotypes, yet still require distinct downstream signaling agents. It has also been demonstrated that the recognition can only take place if the host and pathogen make intimate membrane-to-membrane contact (Halterman et al. 2001).

The downstream events after infestation by RWA have been studied extensively for the NILs 'Tugela' and 'Tugela DN', and include a general (Van der Westhuizen and Botha 1993; Van der Westhuizen et al. 1998a, b, 2002; Mohase and Van der Westhuizen 2002a, b; Bahlmann et al. 2003; Matsioloko and Botha 2003) and specific defense response (Botha et al. 1998; Botha-Oberholster et al. 2004). Using cDNA-AFLP transcript profiling, it was shown that the response against RWA feeding is rapid, and the first transcript changes happen within 1–2 h of RWA feeding (Matsioloko and Botha 2003). Recently, it was further demonstrated, that NILs of 'Tugela' containing the different *Dn*-genes expressed different transcripts after infestation with RWA (Botha-Oberholster et al. 2004), providing more supporting evidence for a gene-for-gene type of interaction.

Is the ability to maintain functional photosynthetic machinery during feeding the key to RWA tolerance?

Previous studies suggested a decrease in effective leaf area (Walters et al. 1980), as RWA feeding destroys the cell membranes, damages the chloroplasts, and thus, effective photosynthetic capacity declines (Fouché et al. 1984). This decline in chlorophyll is visible and measurable only in the RWA susceptible cultivar 'Tugela' and does not occur in the RWA resistant 'Tugela DN' (Fig. 3). Chlorophyll loss was also observed after feeding of RWA and greenbug (*Schizaphis graminum*) on susceptible TAM107 (Deol et al. 2002). Furthermore, recent studies reported a decrease in total chlorophyll content (i.e. Chl*a* and Chl*b*), and carotenoids in susceptible wheat cultivars, but not in resistant NILs after RWA/greenbug feeding (Heng-Moss et al. 2003; Wang et al. 2004). The observed loss in chlorophyll content after feeding by RWA and greenbug was correlated with a decrease in photosynthetic rate (Nagaraj et al. 2002; Heng-Moss et al. 2003). The absence of chlorophyll loss and reduced photosynthetic rate in resistant plants suggested that the plants can compensate for the insect damage (Heng-Moss et al. 2003).

The mechanism enabling resistant wheat plants to overcome the stress of RWA feeding, as well as the exact site of the damage is still unknown. In a study on the localization of an intercellular ß-1,3-glucanase, gold labeling was observed in the cell walls of different cells, but was denser in the vascular bundle cells (Van der Westhuizen et al. 2002). Gold labeling was also detected in the chloroplasts and thus, it seems that gold accumulated in tissues that were affected most by the feeding aphids (Van der Westhuizen et al. 2002).

A suggested site for RWA damage is proposed to be the light harvesting complex II, where chlorophylls (*a* and *b*) and carotenoids (luteins) play an important role, since a decrease in these components were observed after RWA feeding (Heng-Moss et al. 2003). In a study using knock-out *Arabidopsis* mutants of the genes coding for the Rieske protein (*petc-2*) and the ATP-Synthase δ—subunits (*atpd-1*), respectively, it was established that both the Rieske protein and the δ-subunit of the chloroplast ATPase (cpATPase) are essential for photosynthesis (Maiwald et al. 2003). Several copies of cpATPase were isolated from the subtracted libraries, and it was found to be highly up-regulated upon RWA feeding on the array (Table 2). It was further found to be significantly higher expressed in the resistant 'Tugela DN' when compared to the susceptible 'Tugela' cultivar (Fig. 4). Therefore, it seems that cpATPase is important for maintenance of photosynthetic activity in resistant wheat during RWA feeding.

A reduction of chlorophyll *a* is also indicative of damage to the PSI reaction center, and more specifically damage to the reaction center protein D, which is important for the assembly and stability of PSI. Chemical cross-linking of PSI in the presence of ferrodoxin consistently yields a product of PSI-D and Fd, suggesting that PSI-D has an important function in the docking of Fd (Jordan et al. 2001). *Arabidopsis* knock-out plants lacking the PSI-D subunit of

APPENDIX B:

Is photosynthetic transcriptional regulation in *Triticum aestivum* L. cv. 'Tugela DN' a contributing factor for tolerance to *Diuraphis noxia* (Homoptera: Aphididae) ?

PSI, grow slowly, and experience severe photoinhibition and altered redox homeostasis in the chloroplast stroma. When the levels of PSI-D were reduced, the plants showed leaf chlorosis phenotype, lowered chlorophyll content and P700 levels, and a high nonphotochemical quenhcing suggesting inefficient electron transport (Haldrupt et al. 2003). The lack of PSI-D also affected the redox state of thioredoxin. Thioredoxins are one of the main participants in regulating cellular redox balance by reducing disulfide bridges.The most well-known chloroplast thioredoxins, *f* and *m* regulate the enzymatic activity of certain enzymes involved in photosynthetic carbon metabolism, such as Fru-1,6-bisphosphatase and NADP malate dehydrogenase (Ruelland and Miginiac-Maslow 1999). Normally CF1 of the cpATPase is activated under conditions when carbon fixation is possible, i.e. in the light. However, when the PSI-D in a plant is deficient the PSI reaction center may function poorly, and the oxidation of the thioredoxin *m* might turn cpATPase off (Haldrupt et al. 2003). Also, during the normal light cycle thioredoxin becomes increasingly oxidized, the change in the thiol disulfide redox state may be fatal for plants with a defective PSI (e.g. in PSI-D less plants), because thioredoxin is one of the main switches for the initiation of CO_2 assimilation and photoprotection. However, it is not the low amount of PSI per se that is most lethal to the plant, but the direct cause of damage and decreased growth is the ability to down-regulate the PSII levels accordingly (Haldrupt et al. 2003). This suggests that RWA feeding could reduce protein synthesis making photoinhibition irreversible. In addition to the blockage in the electron transport on the acceptor site of photosystem II reaction center, this causes an over-reduction in the system (Burd and Elliot 1996).

Conclusion

NBS-LRRs proteins were regulated during the RWA defense response, and may be involved either as receptor proteins binding the elicitor (e.g. toxin or other component) or as signaling agents, or both. The results further suggested that maintenance of the chloroplast machinery is one of the determining factors in enabling resistant varieties to overcome the stress during RWA feeding. The chloroplast ATP synthases are instrumental in this through the provision of energy during cell maintenance. They may increase the plant's stress tolerance by keeping the photosynthetic machinery intact. Thus, it is hypothesized that when the RWA probes the leaf surface of resistant varieties for a suitable feeding site, the feeding process both damages the cell and also secretes salival compounds (Fig. 5). These compounds from the deposited sheaf and/or saliva are released into the intercellular washing fluid and bind to the receptor proteins (such as the NBS-LLRs) found on the cell membrane in resistant plants. Through activation of the signaling cascade this results in the onset of the defense response. The defense response occurs within 1–2 h p.i. (Matsioloko and Botha 2003), and the observed response (HR) is later visible as necrotic lesions on the leaves of resistant plants. The HR is then followed by an SAR response that results in

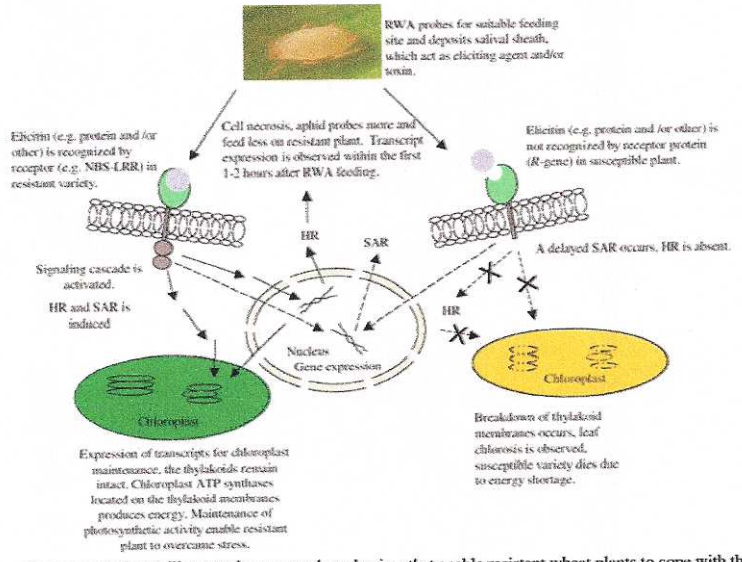


Fig. 5 Diagrammatic representation to illustrate the proposed mechanism that enable resistant wheat plants to cope with the stress inflicted upon them by the feeding Russian wheat aphid, and ensure their survival

APPENDIX B:

Is photosynthetic transcriptional regulation in *Triticum aestivum* L. cv. 'Tugela DN' a contributing factor for tolerance to *Diuraphis noxia* (Homoptera: Aphididae) ?

a prolonged resistance. In contrast, in susceptible varieties the recognition process does not occur, since no observable HR has been reported. This is compounded by delayed activation of the SAR (Van der Westhuizen et al. 1998a, b). Thus, the susceptible plant has no time to activate the appropriate machinery for cell maintenance. This leads to loss of energy production and cell death as a result of chlorophyll breakdown and a decrease in photosynthesis.

Acknowledgements The authors wish to express their sincere gratitude to Mr. Danie Theron (ACTG Microarrayer Facility, UP) for printing of microarrays and technical advice, and to Dr. Zander Myburg for his valuable contributions during the statistical analysis of the data. We greatly acknowledge the Winter Cereal Trust, National Research Foundation (NRF), Technology and Human Resources for Industry Programme (THRIP) of the NRF, and Department of Trade and Industry (DTI) for financial support, and the University of Pretoria for the provision of infrastructure

References

Aarts N, Metz M, Holub M, Staskawicz BJ, Daniels MJ, Parker JE (1998) Different requirements for *eds1* and *NDR1* by disease resistance genes define at least two R-mediated signalling pathways in *Arabidopsis*. Proc Natl Acad Sci USA 95:10306–10311

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Arnon DI (1949) Copper enzymes in isolated chloroplasts. Phenoloxidae in *Beta vulgaris*. Plant Physiol 24:1–15

Bahlmann L, Govender P, Botha A-M (2003) The leaf epicuticular wax ultrastructure and trichome presence on Russian wheat aphid (*Diuraphis noxia*) resistant and susceptible leaves. Afr Entomol 11:59–64

Baker B, Zambryski P, Staskawicz B, Dinesh-Kumar SP (1997) Signalling in plant–microbe interactions. Science 276:726–733

Bennetzen JL (2000) Transposable element contributions to plant gene and genome evolution. Plant Mol Biol 42:251–269

Bent AF (1996) Plant disease resistance: function meets structure. Plant Cell 8:1757–1771

Botha A-M, Nagel MAC, van der Westhuizen AJ, Botha FC (1998) Chitinase isoenzymes in near-isogenic wheat lines challenged with Russian wheat aphid, exogenous ethylene and mechanical wounding. Bot Bull Acad Sin 39:99–106

Botha-Oberholster A-M, Matsioloko MT, Van Eck L, Walters RSG (2004) Russian wheat aphid-mediated elicitation of the wheat defense-transcriptome. Plant Biology 2004 Programme, Lake Buena Vista, Florida, pp 126

Burd JD, Elliott NC (1996) Changes in chlorophyll α fluorescence induction kinetics in cereals infested with Russian wheat aphid (Homoptera: Aphididae). J Econ Entomol 89:1332–1337

Cannon SB, Zhu H, Baumgarten AM, Spangler R, May G, Cook DR, Young ND (2002) Diversity, distribution, and ancient taxonomic relationships within the TIR and non-TIR NBS-LRR resistance gene subfamilies. J Mol Evol 54:548–562

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159

Chu T-M, Weir B, Wolfinger RD (2002) A systematic statistical linear modeling approach to oligonucleotide array experiments. Math Biosci 176:35–51

Davies EL, Hussey RS, Baum TJ (2004) Getting to the roots of parasitism by nematodes. Trends Parasitol 20:134–141

Deol GS, Reese JC, Gill BS, Wilde GE, Campbell LR (2002) Comparative chlorophyll losses in susceptible wheat leaves fed upon by the Russian wheat aphids or greenbugs (Homoptera: Aphididae). J Kans Entomol Soc 74:192–198

Dixon AFG (1998) Aphid ecology: an optimization approach, 2nd edn. Chapman & Hall, London, pp 27–38

Doolittle RF, Feng DF, Johnson MS, McClure MA (1989) Origins and evolutionary relationships of retroviruses. Q Rev Biol 64:1–30

Dudoit S, Yang YH, Callow MJ, Speed TP (2001) Statistical methods for identifying differentially expressed genes in replicated cDNA microarray experiments http://www.stat.berkeley.edu/users/terry/zarray/Html/papersindexhtml

Feuillet C, Travella S, Stein N, Albar L, Nublat A, Keller B (2003) Map-based isolation of the leaf rust disease resistance gene *Lr10* from the hexaploid wheat (*Triticum aestivum* L.) genome. Proc Natl Acad Sci USA 100:15253–15258

Fidantsef AL, Stout MJ, Thaler JS, Duffey SS, Bostock RM (1999) Signal interactions in pathogen and insect attack: expression of lipoxygenase, proteinase inhibitor II, and pathogenesis-related protein P4 in the tomato, *Lycopersicon esculentum*. Physiol Mol Plant Pathol 54:97–114

Flavell AJ, Pearce SR, Kumar A (1994) Plant transposable elements and the genome. Curr Opin Genet Dev 4:838–844

Flor HH (1971) Current status of the gene-for-gene concept. Ann Rev Phytopathol 9:275–296

Fouché A, Verhoeven RL, Hewitt PH, Walters MC, Kriel CF, De Jager J (1984) Russian wheat (*Diuraphis noxia*) feeding damage on wheat, related cereals and a *Bromus* grass species. In: Walters MC (ed) Progress in Russian wheat aphid (*Diuraphis naoxia* Mordv.) research in the Republic of South Africa. Technical communication, Vol 191, pp22–33

Gussow D, Clackson T (1989) Direct clone characterization from plaques and colonies by the polymerase chain reaction. Nucleic Acids Res 17:4000

Haldrupt A, Lunde C, Scheller HV (2003) *Arabidopsis thaliana* plants lacking the PSI-D subunit of photosystem I suffer severe photoinhibition, have unstable photosystem I complexes, and altered redox homeostasis in the chloroplast stroma. J Biol Chem 278:33276–33283

Halterman D, Zhou F, Wei F, Wise RP, Schulze-Lefert P (2001) The MLA coiled-coil, NBS-LRR protein confers *AvrMla6* resistance specificity to *Blumeria graminis* f. sp. *hordei* in barley and wheat. Plant J 25:335–348

Hanson RE, Islam-Faridi MN, Crane CF, Zwick MS, Czeschin DG, Wendel JF, McKnight TD, Price HJ, Stelly DM (2000) Ty-1-copia-retrotransposon behavior in a polyploid cotton. Chromosome Res 8:73–76

Haung G, Dong R, Maier T, Allen R, Davis EL, Baum TJ, Hussey RS (2004) Use of solid-phase subtractive hybridization for the identification of parasitism gene candidates from the root-knot nematode *Meloidogyne incognita*. Mol Plant Pathol 5:217–222

Heng-Moss TM, Ni X, Macedo T, Markwell JP, Baxendale FP, Quisenberry SS, Tolmay V (2003) Comparison of chlorophyll and cartenoid concentrations among Russian wheat aphid (Homoptera: Apididae)-infested wheat isolines. J Econ Entomol 96:475–481

Huang L, Gill BS (2001) An RGA-like marker detects all known *Lr21* leaf rust resistance gene family members in *Aegilops tauschii* and wheat. Theor Appl Genet 103:1007–1013

Huang L, Brooks SA, Li W, Fellers JP, Trick HN, Gill BS (2003) Map-based cloning of leaf rust resistance gene *Lr21* from the large and polyploid genome of bread wheat. Genetics 164:655–664

Jackson AO, Taylor CB (1996) Plant–microbe interactions: life and death at the interface. Plant Cell 8:1651–1668

Jordan P, Fromme P, Witt HT, Klukas O, Saenger W, Krauss N (2001) Three-dimensional structure of cyanobacterial photosystem I at 2.5 Angstrom resolution. Nature 411:909–916

Katsiotis SG, Hagidimitrriou M, Loukas M (2002) Genomic and chromosomal organization of Ty1-copia-like sequences in *Olea europaea* and evolutionary relationships of *Olea* retroelements. Theor Appl Genet 104:926–933

Keen NT (1990) Gene-for-gene complementarity in plant–pathogen interactions. Annu Rev Genet 24:425–429

APPENDIX B:

Is photosynthetic transcriptional regulation in *Triticum aestivum* L. cv. 'Tugela DN' a contributing factor for tolerance to *Diuraphis noxia* (Homoptera: Aphididae) ?

Lacock L, van Niekerk C, Loots S, du Preez FB, Botha A-M (2003) Functional and comparative analysis of expressed sequences from *Diuraphis noxia* infested wheat obtained utilizing the Nucleotide Binding Site conserved motif. Afr J Biotech 2:75–81

Lacock L, Botha A-M (2003) Suppression subtractive hybridization (SSH) employed to investigate gene expression after Russian wheat aphid infestation. Proceedings of the 10th International Wheat Genetics Symposium, Paestum, Italy (1–6 September 2003), Vol 3, pp 1187–1189

Lall IPS, Maneesha KC, Upadhyaya KC (2002) *Panzee*, a copia-like retrotransposon from the grain legume, pigeonpea (*Cajanus cajan* L.). Mol Genet Genomics 267:271–280

Maiwald D, Dietzmann A, Jahns P, Pesaresi P, Joliot P, Joliot A, Levin JZ, Salamini F, Leister D (2003) Knock-out of the genes coding for the Rieske protein and the ATP-Synthase δ-subunit of *Arabidopsis*. Effects on photosynthesis, thylakoid protein composition, and nuclear chloroplast gene expression. Plant Physiol 133:191–202

Matsioloko MT, Botha A-M (2003) cDNA-AFLP profiling in wheat upon Russian wheat aphid feeding. Proceedings of the 10th International Wheat Genetics Symposium, Paestum, Italy (1–6 September 2003), Vol 3, pp 1275–1277

Michelmore RW, Meyers BC (1998) Clusters of resistance genes in plants evolve by divergent selection and a birth-and-death process. Genome Res 8:1113–1130

Miles PW (1999) Aphid saliva. Biol Rev 74:41–85

Mohase L, van der Westhuizen AJ (2002a) Salicylic acid is involved in resistance responses in the Russian wheat aphid–wheat interaction. J Plant Physiol 159:585–590

Mohase L, van der Westhuizen AJ (2002b) Glycoproteins from Russian wheat aphid infested wheat induce defense responses. Z Naturforsh 57c:867–873

Moran PJ, Thompson GA (2001) Molecular responses to aphid feeding in *Arabidopsis* in relation to plant defense pathways. Plant Physiol 125:1074–1085

Moran PJ, Cheng YF, Cassell JL, Thompson GA (2002) Gene expression profiling of *Arabidopsis thaliana* in compatible plant–aphid interactions. Arch Insect Biochem Physiol 51:182–203

Nagaraj N, Reese JC, Kirkham MB, Kofoid K, Campbell LR, Loughin TM (2002) Relationship between chlorophyll loss and photosynthetic rate in greenbug (Homoptera: Aphididae) damaged sorghum. J Kans Entomol Soc 75:101–109

Pan Q, Liu Y-S, Budai-Hadrian O, Sela M, Carmel-Goren L, Zamir D, Fluhr R (2000) Comparative genetics of nucleotide binding site-leucine rich repeat resistance gene homologues in the genomes of two dicotyledons: tomato and *Arabidopsis*. Genetics 155:309–322

Reymond P, Weber HY, Damond M, Farmer EE (2000) Differential gene expression in response to mechanical wounding and insect feeding in *Arabidopsis*. Plant Cell 12:707–720

Rose JKC, Bennet AB (1999) Cooperative disassembly of the cellulose-xyloglucan network of plant cell walls: parallels between cell expansion and fruit ripening. Trends Plant Sci 4:176–183

Ruelland E, Miginiac-Maslow M (1999) Regulation of chloroplast enzyme activities by thioredoxins: activation of relief or inhibition? Trends Plant Sci 4:136–141

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Vol 1, 2, 3. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

SAS Institute Inc. (1999) SAS/STAT Software version 8. SAS Institute, Cary, NC

Smith CM, Schotzko DJ, Zemetra RS, Souza EJ (1992) Categories of resistance in plant introductions of wheat resistant to Russian wheat aphid (Homoptera: Aphididae). J Econ Entomol 96:352–360

Swanepoel E, Lacock L, Myburg AA, Botha AM (2003) A leucine rich homolog to *Aegilops tauschii* from bread wheat line PI137739 obtained by suppression subtractive hybridization shows linkage to Russian wheat aphid resistance gene *Dn1*. Proceedings of the 10th International Wheat Genetics Symposium, Paestum, Italy (1–6 September 2003), Vol 3, p 1263–1265

Van der Biezen EA, Jones JDG (1998) Plant disease resistance proteins and the gene for gene concept. Trends Biochem Sci 23:454–456

Van der Westhuizen AJ, Botha FC (1993) Effect of the Russian wheat aphid on the composition and synthesis of water soluble proteins in resistant and susceptible wheat. J Agron Crop Sci 170:322–326

Van der Westhuizen AJ, Qian X-M, Botha A-M (1998a) Differential induction of apoplastic peroxidase and chitinase activities in susceptible and resistant wheat cultivars by Russian wheat aphid infestation. Plant Cell Rep 8:132–137

Van der Westhuizen AJ, Qian X-M, Botha A-M (1998b) ß-1,3-glucanases in wheat and resistance to the Russian wheat aphid. Physiol Plant 103:125–131

Van der Westhuizen AJ, Qian X-M, Wilding M, Botha A-M (2002) Purification and immunocytochemical localization of a wheat β-1,3-glucanase induced by Russian wheat aphid infestation. SA J Sci 98:197–202

Van Niekerk C, Botha A-M (2003) Using suppression subtractive hybridization (SSH) to screen for novel sequences expressed in response to Russian wheat aphid feeding. Proceedings of the 10th International Wheat Genetics Symposium, Paestum, Italy (1–6 September 2003), Vol 3, p 1281–1283

Vernon LP (1960) Spectrophotometric determination of chlorophylls and pheophytins in plant extracts. Anal Chem 32:1144–1150

Walters MC, Penn F, Du Toit F, Botha TC, Aalbersberg K, Hewitt PH, Broodryk SW (1980) The Russian wheat aphid. Farming in South Africa. Wheat: Winter Rainfall G.3/1980

Wang T, Quisenberry SS, Ni X, Tolmay V (2004) Aphid (Hemiptera: Aphidae) resistance in wheat near-isogenic lines. J Econ Entomol 96:475–481

Wang YH, Speed T (2002) Design of microarray expression experiments. In: Bowtell D, Sambrook J (eds) DNA microarrays: A molecular cloning manual, Part 1. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 513–525

Wessler SR, Burea TE, White SE (1995) LTR-retrotransposons and MITEs: important players in the evolution of plant genomes. Curr Opin Genet Dev 5:814–821

Whitham S, Dinesh-Kumar SP, Choi D, Hehl R, Corr C, Baker B (1994) The product of the tobacco mosaic virus resistance gene N: Similarity to Toll and the interleukin-1 receptor. Cell 78:1101–1115

Wolfinger RD, Gibson G, Wolfinger ED, Bennett L, Hamadeh H, Bushel P, Afshari C, Paules RS (2001) Assessing gene significance from cDNA microarray expression data via mixed models. J Comput Biol 8:625–637

Xiong Y, Eickbush TH (1990) Origin and evolution of retroelements based upon their transverse transcriptase sequences. EMBO J 9:3353–3362

APPENDIX C

RESULTS – SUPPORTING DATA

APPENDIX C

Results – Supporting data

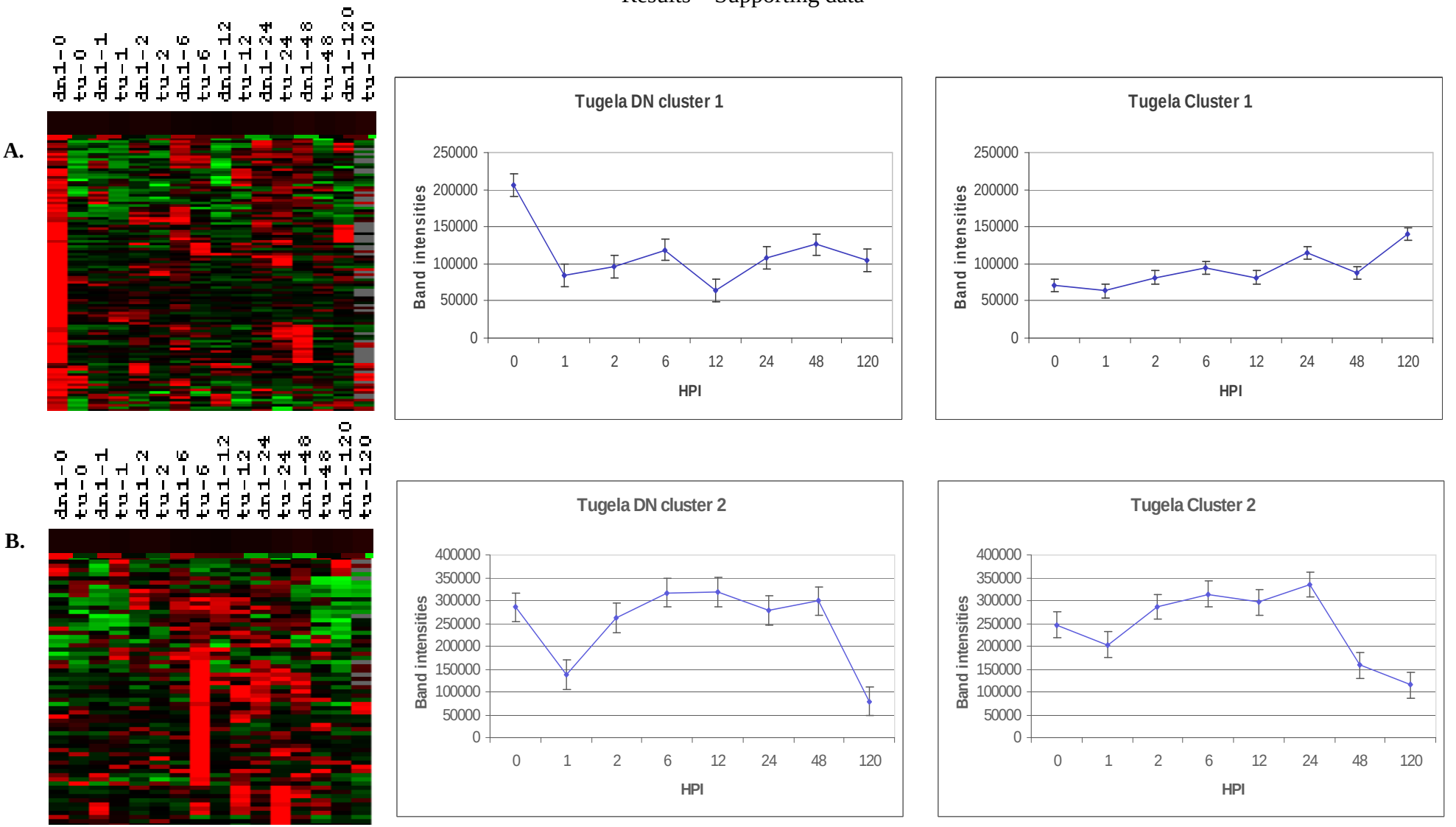
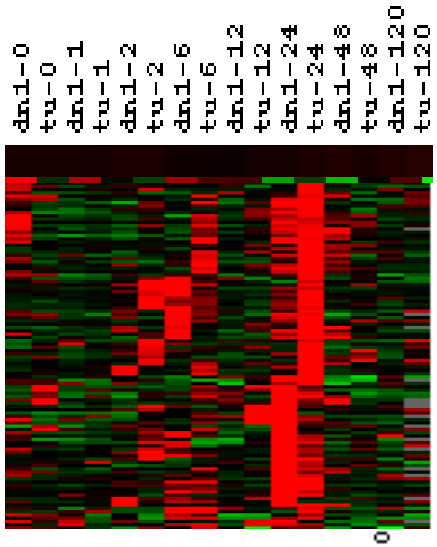


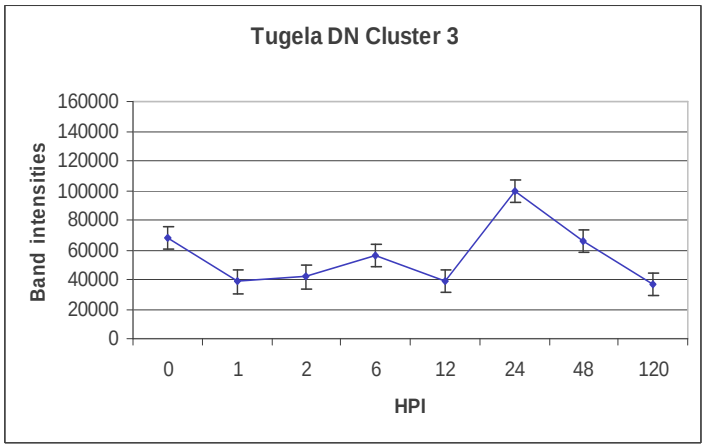
Figure 1 Enlargement of the hierarchical clusters in Figure 3.10. Panels A and B represent clusters 1 and 2, respectively. Each panel is composed of an enlarged section of the respective cluster, followed by line graphs representing the general trends of expression in 'Tugela DN' and 'Tugela' in each cluster. Red = high expression and green = low expression.

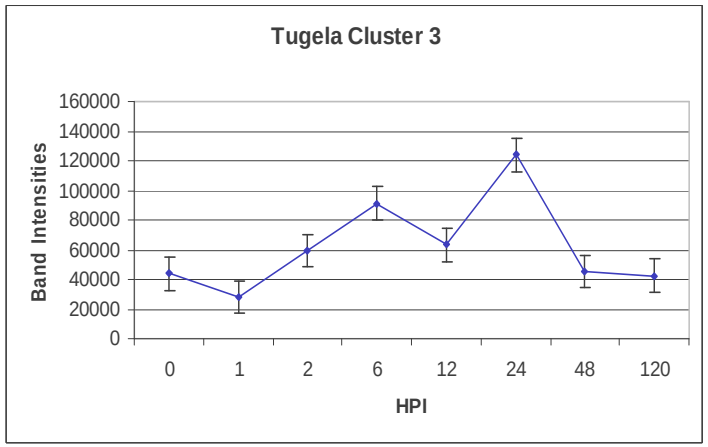
APPENDIX C

Results – Supporting data

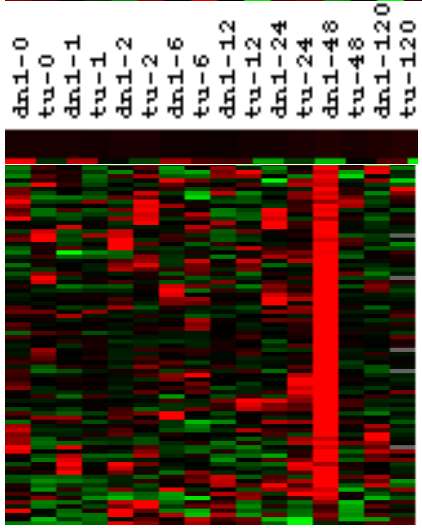
C.

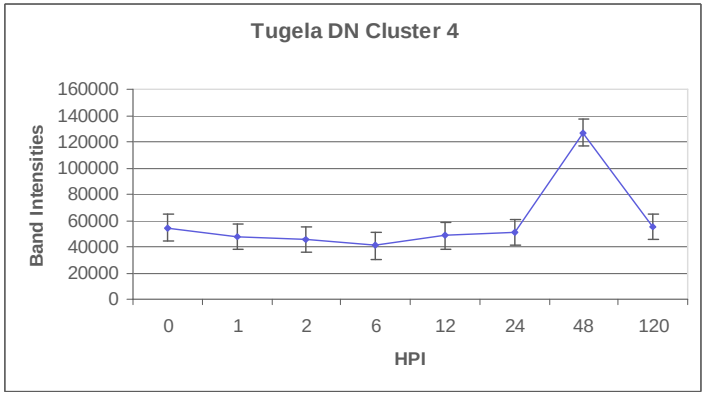






D.





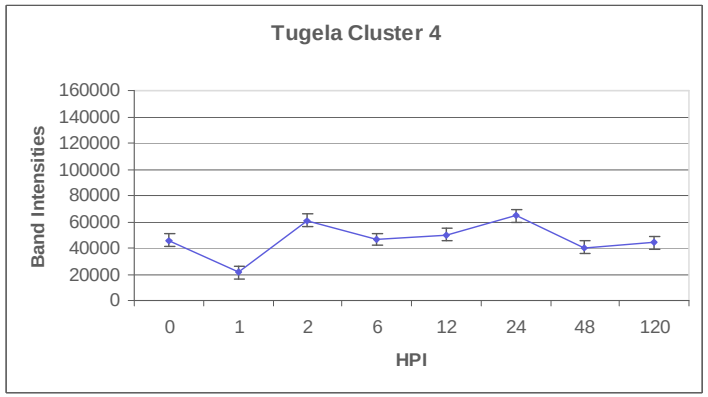


Figure 1 Enlargement of the hierarchical clusters in Figure 3.10. Panels C and D represent clusters 3 and 4, respectively. Each panel is composed of an enlarged section of the respective cluster, followed by line graphs representing the general trends of expression in 'Tugela DN' and 'Tugela' in each cluster. Red = high expression and green = low expression.

APPENDIX C

Results – Supporting data

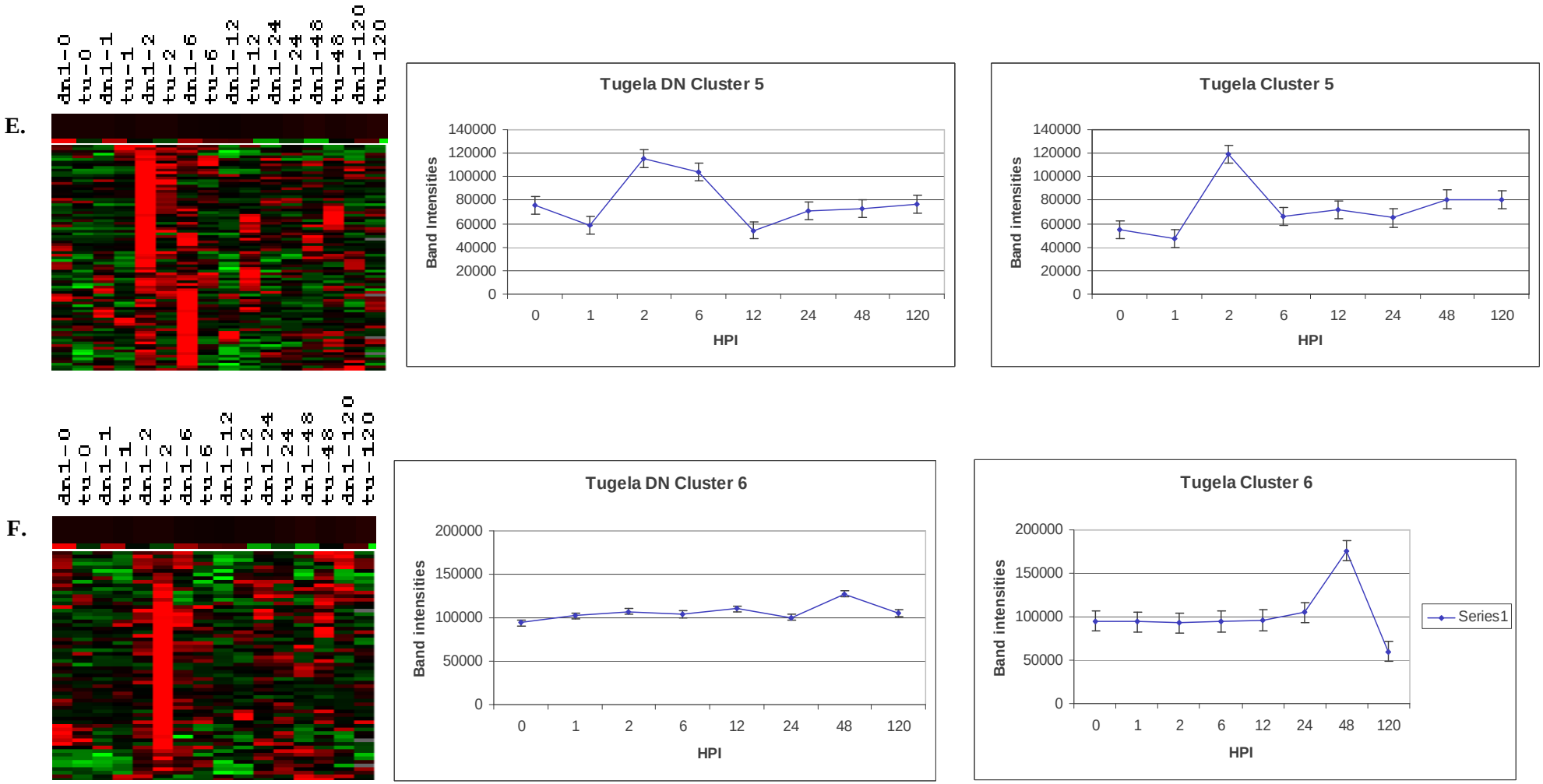


Figure 1 Enlargement of the hierarchical clusters in Figure 3.10. Panels E and F represent clusters 5 and 6, respectively. Each panel is composed of an enlarged section of the respective cluster, followed by line graphs representing the general trends of expression in 'Tugela DN' and 'Tugela' in each cluster. Red = high expression and green = low expression.

APPENDIX C

Results – Supporting data

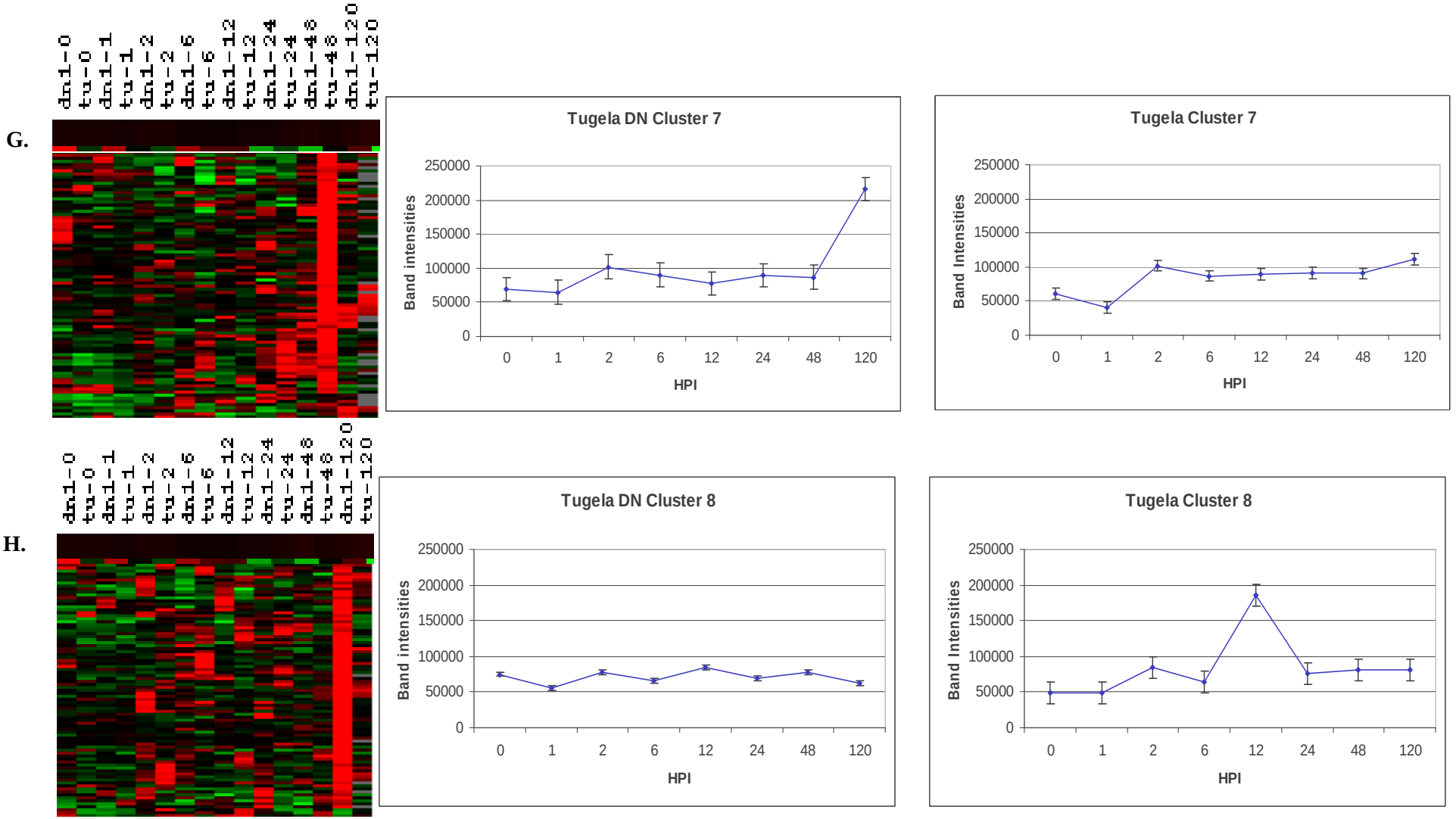
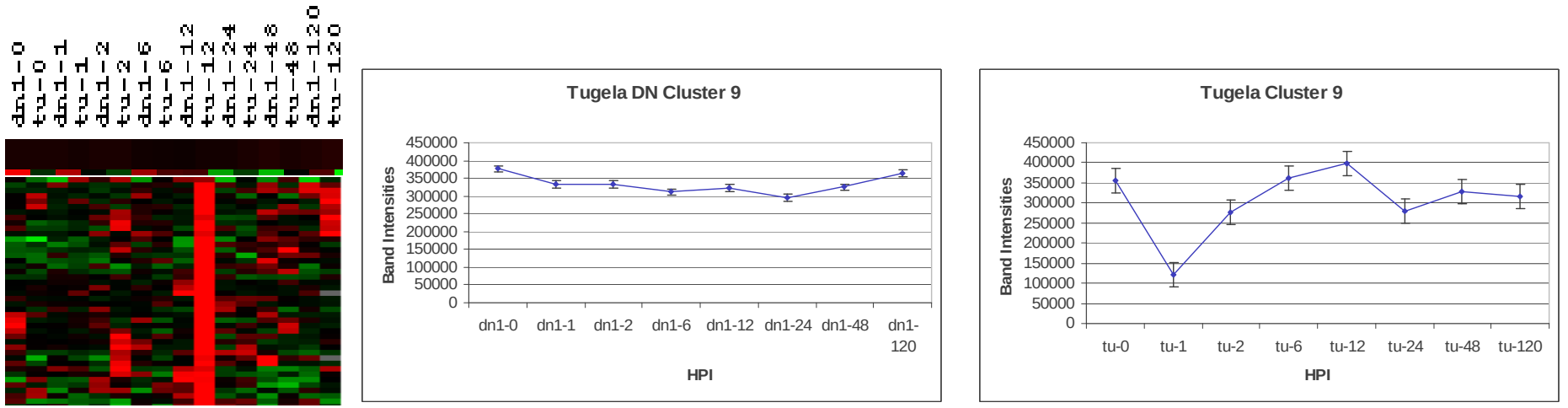


Figure 1 Enlargement of the hierarchical clusters in Figure 3.10. Panels G and H represent clusters 7 and 8, respectively. Each panel is composed of an enlarged section of the respective cluster, followed by line graphs representing the general trends of expression in 'Tugela DN' and 'Tugela' in each cluster. Red = high expression and green = low expression.

APPENDIX C

Results – Supporting data

I.



J.

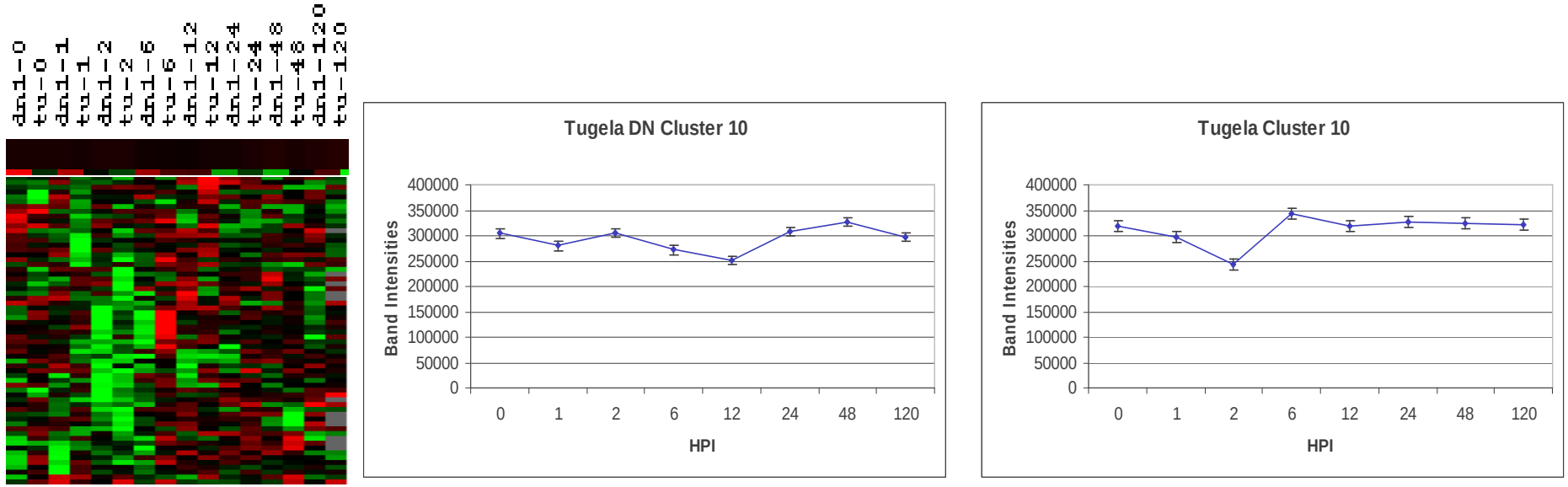


Figure 1 Enlargement of the hierarchical clusters in Figure 3.10. Panels I and J represent clusters 9 and 10, respectively. Each panel is composed of an enlarged section of the respective cluster, followed by line graphs representing the general trends of expression in 'Tugela DN' and 'Tugela' in each cluster. Red = high expression and green = low expression.

APPENDIX C

Results – Supporting data

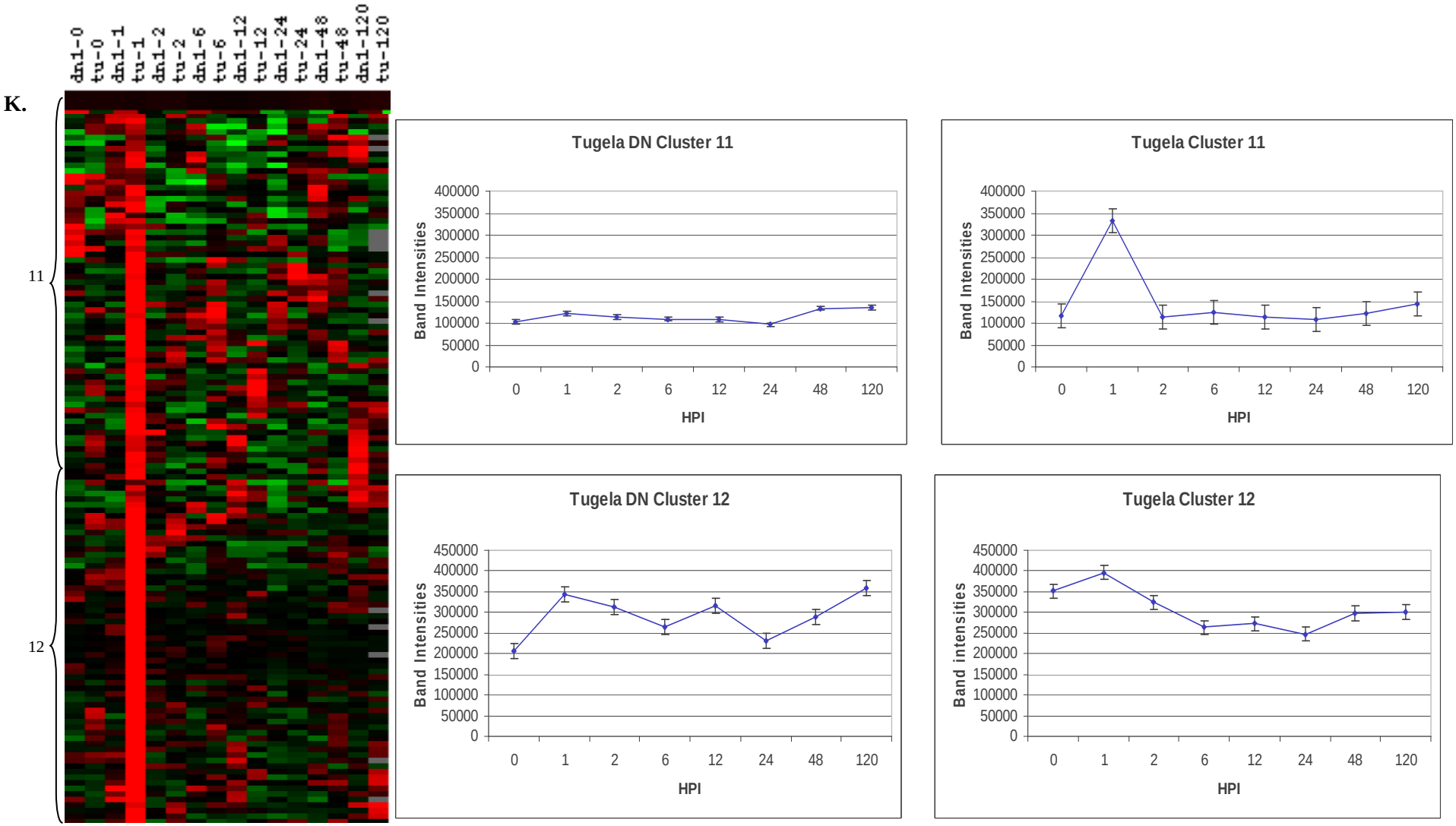
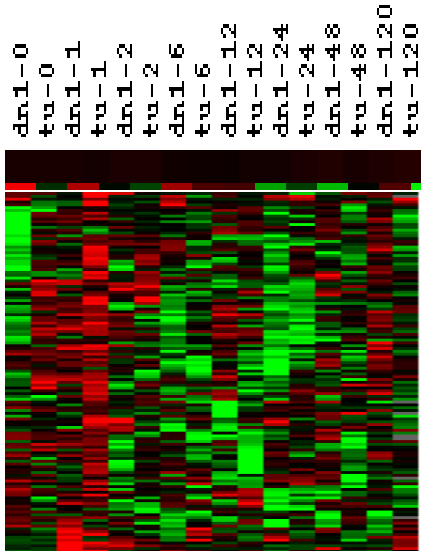


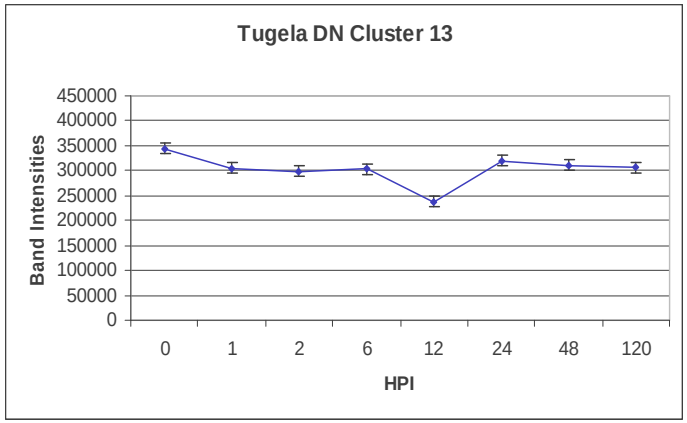
Figure 1 Enlargement of the hierarchical clusters in Figure 3.10. Panel K Represents clusters 11 and 12 as indicated by the left braces. The panel is composed of an enlarged section of the respective clusters, followed by line graphs representing the general trends of expression in 'Tugela DN' and 'Tugela' in each cluster. Red = high expression and green = low expression.

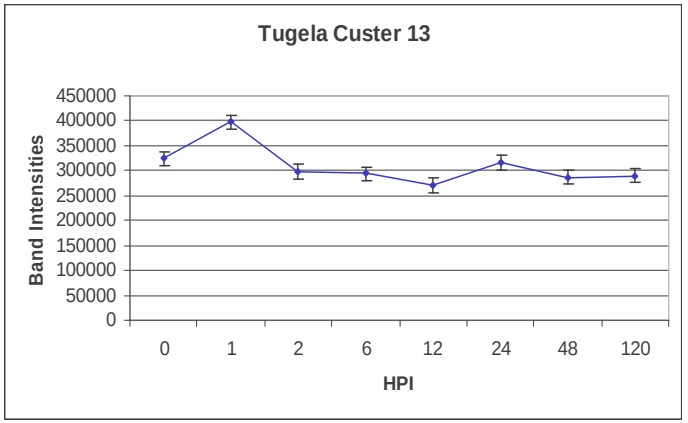
APPENDIX C

Results – Supporting data

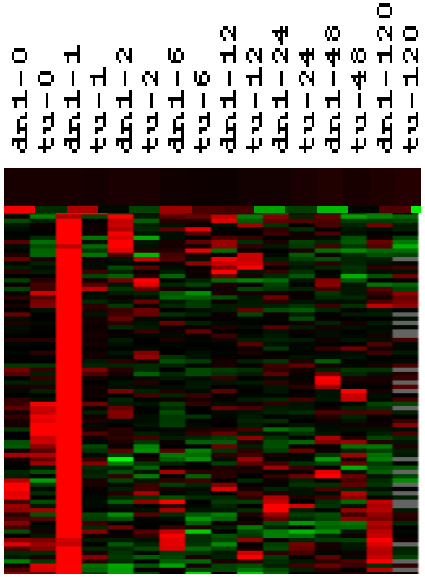
L.

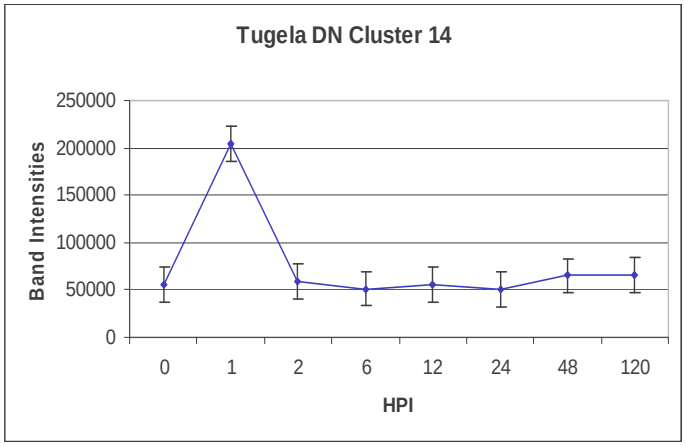






M.





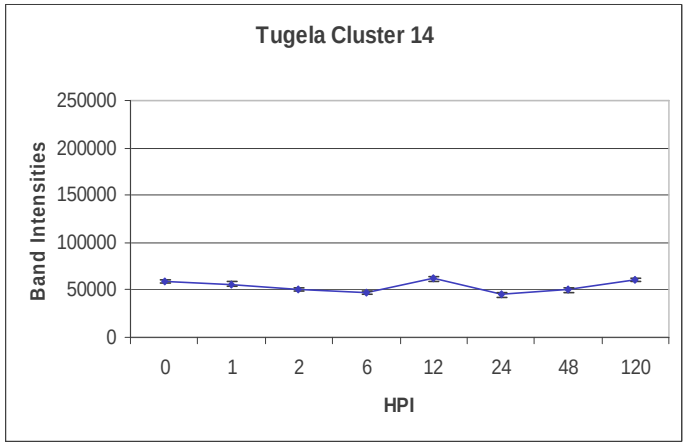


Figure 1 Enlargement of the hierarchical clusters in Figure 3.10. Panels L and M Represent clusters 13 and 14, respectively. The panels are composed of an enlarged section of the respective clusters, followed by line graphs representing the general trends of expression in 'Tugela DN' and 'Tugela' in each cluster. Red = high expression and green = low expression.

APPENDIX C

Results – Supporting data

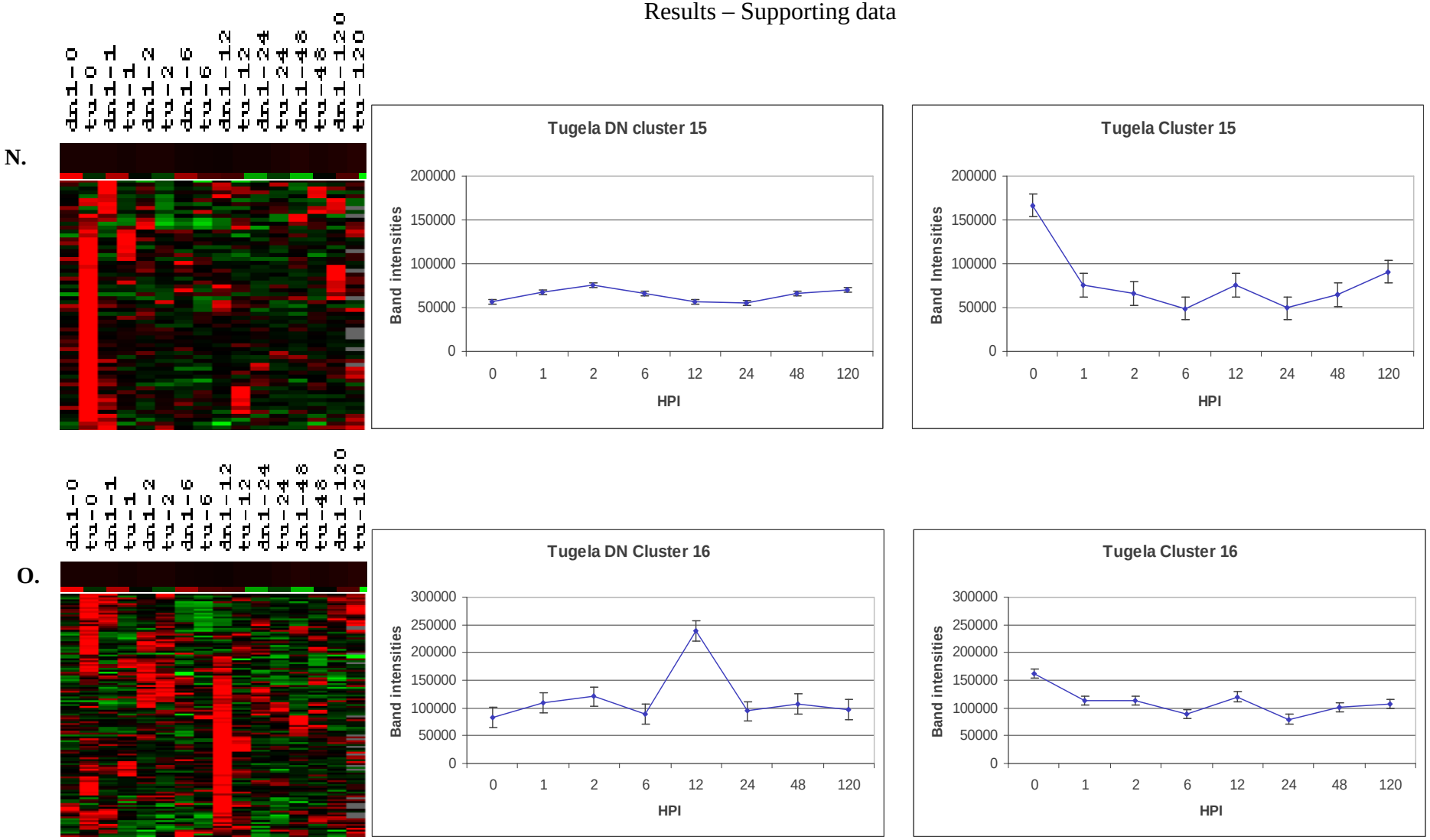
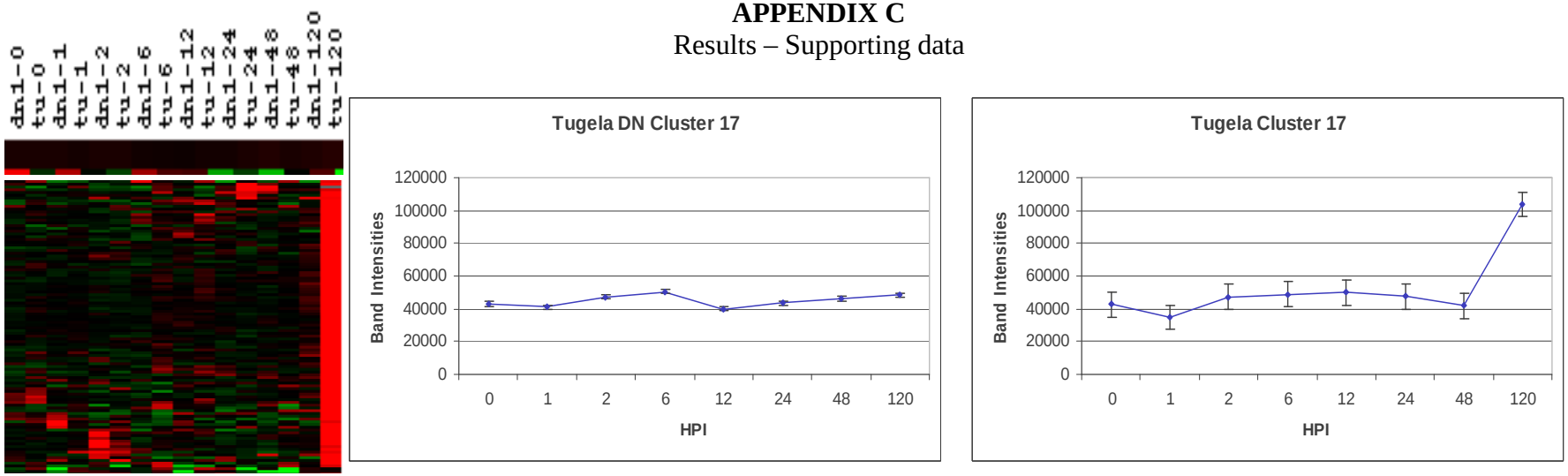


Figure 1 Enlargement of the hierarchical clusters in Figure 3.10. Panels N and O Represent clusters 15 and 16, respectively. The panels are composed of an enlarged section of the respective clusters, followed by line graphs representing the general trends of expression in ‘Tugela DN’ and ‘Tugela’ in each cluster. Red = high expression and green = low expression.

APPENDIX C

Results – Supporting data

P.



Q.

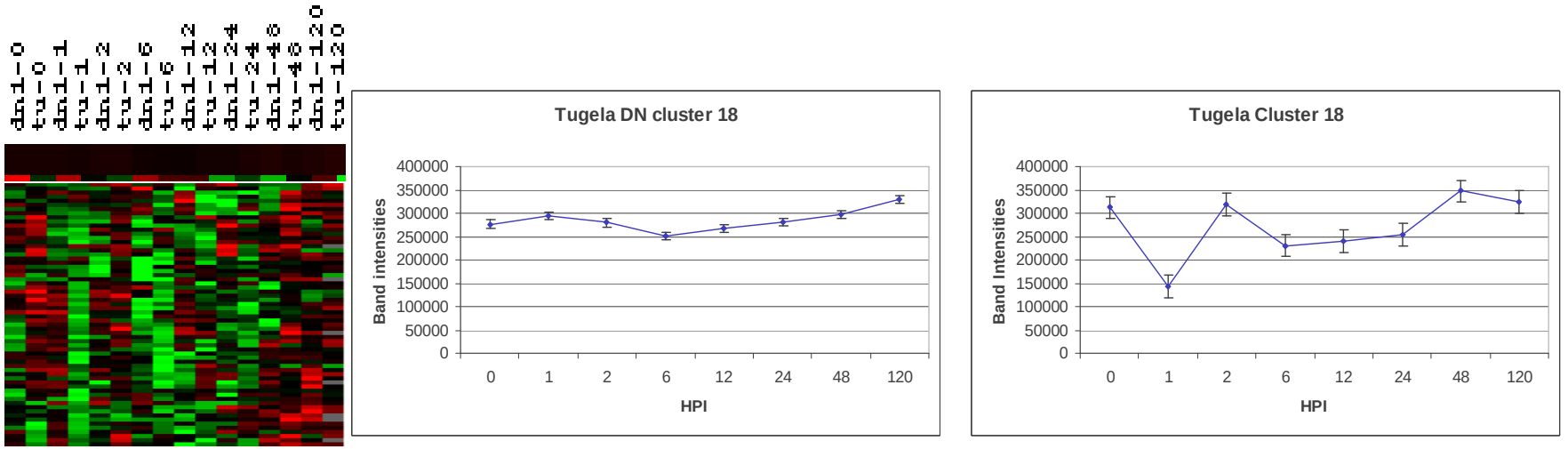


Figure 1 Enlargement of the hierarchical clusters in Figure 3.10. Panels P and Q Represent clusters 17 and 18, respectively. The panels are composed of an enlarged section of the respective clusters, followed by line graphs representing the general trends of expression in 'Tugela DN' and 'Tugela' in each cluster. Red = high expression and green = low expression.

APPENDIX C

Results – Supporting data

Table 1 Clone IDs of ESTs spotted on the cDNA microarray slides along with the Log2-fold change and net log*P* for significance.

Wheat clones (SSH) (van Niekerk and Botha, 2003)		
Sequence ID	**Log2FoldChange**	**NetLog*P***
Putative TNP-like transposable element [*Sorghum bicolour*]	0.040996052	0.146299454
Aegilops tauschii leucine-rich-like protein gene	0.103247471	0.371891062
Unknown protein	0.148718387	0.371628994
Wheat chloroplast ATP synthase CF-1 gene (M16843.1, e-value = 0)	0.106065242	0.326238798
Triticum aestivum chloroplast (AB042240.3, e-value = 0)	0.242273897	1.429580626
Unknown	0.045303961	0.101809568
Unknown	0.249410741	0.585781704
Unknown	0.246497169	1.037206847
Beta-1,4-endoglucanase 1 precurser [*Heterodera schachtii*]	0.282316322	0.467502971
Unknown	0.21466944	0.676734117
Unknown	0.02854643	0.070811972
Unknown	0.364226182	1.361602451
Triticum monococcum putative resistance protein (RGA-2) (AF326781, e-value = e-35)	0.502323164	3.826242227
Wheat chloroplast - ATP synthase (M16843.1, e-value = 0)	0.291690011	1.374355056
Unknown	0.105827507	0.29014216
Unknown	0.193432031	0.503924195
Putative TN-like transposable element [*Sorghum bicolour*]	0.206293783	0.451065081
Aegilops tauschii leucine-rich-like protein gene (AF947474.1, e-value = 1e-89)	-0.05834846	0.107513402
Unknown	0.1663231	0.353221114
Wheat chloroplast - ATP synthase (M16843.1, e-value = 0)	0.062619367	0.122615676
Wheat chloroplast ATP synthase CF-1 gene (M16843.1, e-value = 0)	0.158533075	0.524714987
Unknown	-0.012660781	0.025418899
Unknown	0.06114007	0.15038422
Unknown	0.157603454	0.469878114
Unknown	-0.06800017	0.140768298
Unknown	0.095002138	0.162798689
Wheat chloroplast - ATP synthase (M16843.1, e-value = 0)	-0.018776284	0.034520188
Unknown	0.532301553	1.282857438
Triticum monococcum putative resistance protein (RGA-2) (AF326781, e-value = e-105)/*Triticum*	0.62820682	2.482189652
Aegilops tauschii leucine-rich-like protein gene (AF947474.1, e-value = 9e-33)	0.124060574	0.231764955
Unknown protein	0.06907342	0.109932848
Unknown protein	0.012094301	0.026220291
Wheat chloroplast ATP synthase	0.378594668	2.566512173
Aegilops tauschii leucine-rich-like protein gene (AF947474.1, e-value = 2e-57)	0.12449775	0.209569888
Wheat mitochondrial small subunit rRNA gene (K01229.1 e-value = 0)	-0.041795694	0.188736023
Aegilops tauschii leucine-rich-like protein gene (AF947474.1, e-value = 2e-57)/*Triticum*	0.035094628	0.08380429
Aegilops tauschii leucine-rich-like protein gene (AF947474.1, e-value = 2e-44)	0.053272888	0.281490557
Unknown	0.135735308	0.392983817
Unknown	0.197112665	0.834971018
Unknown	0.236123865	0.498930514
Unnamed protein product [*Oryza sativa* (japonica cultivar group) e-value = 3e-14]	0.058782272	0.136078932
Unknown	0.062731988	0.165601611
Wheat chloroplast - ATP synthase (M16843.1, e-value = 0)	0.27352437	2.49044641
Wheat chloroplast - ATP synthase (M16843.1, e-value = 0)	-0.138749307	0.206302309
Aegilops crassa chloroplast genes – ATP synthase (AEGATPS2, e-value = 0)	0.153707078	0.485623962
Aegilops tauschii leucine-rich-like protein gene (AF947474.1, e-value = 3e-47)/Aegilops	-0.116372793	0.264150244
Wheat chloroplast - ATP synthase (M16843.1, e-value = 0)	0.158864146	0.695962433
Unknown	0.327922482	1.421861738
Wheat chloroplast - ATP synthase (M16843.1, e-value = 0)	0.228531544	1.22642995
Aegilops tauschii leucine-rich-like protein gene (AF947474.1, e-value = 1e-89)	0.044514636	0.097555413
Unknown	0.016570778	0.084282656
Not sequenced	0.205887787	0.313236501
Aegilops tauschii leucine-rich-like protein gene (AF947474.1, e-value = 9e-33)	-0.135286115	0.421241439
Unknown protein; protein id: At3g29760.1 [*Arabidopsis thaliana*]	-0.305938329	1.140860103
Unknown	-0.057499071	0.03079136
Unknown	0.551557513	1.615399917
Unknown	0.020140211	0.030165958
Beta nana Ty1-copia-like retrotransposon (AF48917.1 e-value = 4e-25)	0.028295894	0.063397278
Wheat chloroplast ATP syanthase CF-1 gene (M16843.1, e-value = 0)	0.1051727	0.213000727
Triticum aestivum chloroplast (AB042240.3, e-value = 0)	0.377731864	0.971760244
Wheat chloroplast - ATP synthase (M16843.1, e-value = 0)	0.375149669	0.97597103
Aegilops tauschii leucine-rich-like protein gene (AF947474.1, e-value = 2e-44)	-0.19417401	0.495537834
Wheat chloroplast - ATP synthase (M16843.1, e-value = 0)	0.283177752	1.935861907
Unknown	-0.05733526	0.123338105
Wheat chloroplast - ATP synthase (M16843.1, e-value = 0)	0.086640568	0.303295596
Aegilops tauschii leucine-rich-like protein gene (AF947474.1, e-value = 2e-44)	-0.18092888	0.79790412

APPENDIX C

Results – Supporting data

Table 1 (cont.)

Wheat mitochondrial small subunit	0.004740368	0.021142831
Aegilops tauschii leucine-rich-like protein gene (AF947474.1, e-value = 1e-89)	-0.032046123	0.051836079
Triticum monococcum putative resistance protein (RGA-2) (AF326781, e-value = 1e-36)	-0.055455427	0.146985886
Envelope protein [*Bovine immunodeficiency virus*]	-0.217331115	0.632657133
Unknown	-0.011231142	0.031025722
Unknown (e-value = >0.004)	0.308966555	0.824818184
Unknown	0.492292512	1.210085299
Unknown	0.086769836	0.141192063
Wheat chloroplast - ATP synthase (M16843.1, e-value = 0)	0.129770579	0.177571442
Unknown	0.242261562	0.604144666
Wheat chloroplast - ATP synthase (M16843.1, e-value = 0)	-0.011188844	0.011181443
Wheat chloroplast - ATP synthase (M16843.1, e-value = 0)	-0.052973438	0.049667716
Wheat chloroplast - ATP synthase (M16843.1, e-value = 0)	0.088068228	0.148816442
Unknown	0.21849785	0.479520049
Putative TNP-like transposable element [*Sorghum bicolour*]	0.201172672	0.321878641
Aegilops tauschii leucine-rich-like protein gene (AF947474.1, e-value = 1e-89)	0.050413401	0.086460201
Unknown	-0.128668451	0.125720996
Wheat chloroplast ATP syanthase CF-1 gene (M16843.1, e-value = 0)	0.304571156	0.412198645
Wheat chloroplast - ATP synthase (M16843.1, e-value = 0)	0.875457447	2.474465512
Putative TNP-like transposable element [*Sorghum bicolour*]	0.339622387	0.548637517
Beta-1,4-endoglucanase 1 precurser [*Heterodera schachtii*]	0.395500924	1.5098769
Unknown	0.493265144	1.635733672
Unknown	0.167337166	0.267579214
Unknown	0.254484353	0.333182994
Unknown	-0.186677717	0.205295916
Unknown	0.076285774	0.108545457
Triticum monococcum putative resistance protein (RGA-2) (AF326781, e-value = 4e-93)	0.493167396	3.032344665
Unknown	0.526125878	1.338408199
Hypothetical protein [*Nostoc punctiforme*]	0.234045234	0.514155443
Unknown	0.29949561	0.501548706
Wheat clones (SSH) (Lacock and Botha, 2003)		
Sequence ID	**Log2FoldChange**	**NetLog*P***
Not sequenced	0.780540179	7.737908921
Not sequenced	0.861213869	7.240683761
Oryza sativa T-DNA intergration target genomic sequence (U40814.1, e-value = 1e-09)	-0.95368986	3.986967579
Unknown	-0.391600306	3.379494072
*Cicer arietinum*Ty1-copia retrotransposon for putative reverse transcriptase (AJ535884.1)	0.538690469	3.066169252
Hordeum vulgare BARE-1 long terminal repeat (Z84562.1, e- value = 8e-19)	-0.471076359	2.191202418
Not sequenced	0.966802113	2.181668418
Hordeum vulgare BARE-1 long terminal repeat (Z84562.1, e- value = 1e-19)	-0.808891099	2.17127553
Beta nana Ty1-copia-like retrotransposon (AF48917.1)	-0.818966105	1.941483197
Not sequenced	1.015234427	1.872016035
Beta nana Ty1-copia-like retrotransposon (AF48917.1)	-0.414904894	1.848813922
Oryza sativa T-DNA intergration factor (U40814.1, e-value = 2e-23)	-0.449690598	1.780630835
Not sequenced	-0.688737812	1.666318229
Cocos nucifera microsa (AJ458311.1, e-value = 3e-29)	0.855305696	1.665563063
Homo sapiens BAC clone (AC016773.8 e-value = 0.002)	-0.312107437	1.663820006
Oryza sativa T-DNA intergration factor (U40814.1, e-value = 3e-23)	0.371937356	1.558699268
Not sequenced	-0.242035278	1.457140391
Oryza sativa T-DNA intergration factor (U40814.1, e-value = 2e-23)	-0.455978815	1.439920798
Oryza sativa T-DNA intergration factor (U40814.1, e-value = 6e-23)	0.209389305	1.347873116
Anthoceros punctatus chloroplast (AB013664.1 e-value = 5e-22)	-0.335418209	1.332297661
Atropa belladonna partial mRNA 3'URT (AJ309392.1 e-value = 1e-19)	-0.810919069	1.327770732
Anthoceros punctatus chloroplast gene for photosystem I P700 apoprotein A1 (AB013664.1)	-0.271568414	1.212436153
Not sequenced	-0.843058801	1.168214077
Beta nana Ty1-copia-like retrotransposon (AJ489200.1, e-value = 4e-13)	-0.220396449	1.07597185
Hordeum vulgare BARE-1 long terminal repeat (Z84569.1, e- value = 4e-10)	-0.413197773	0.948394568
*Cicer arietinum*Ty1-copia retrotransposon for putative reverse transcriptase (AB086192.1)	-0.477510831	0.919152763
Unknown	-0.226198663	0.890738016
Hordeum vulgare BARE-1 long terminal repeat (Z84562.1, e- value = 9e-25)	-0.386128267	0.877713938
Not sequenced	-0.672875721	0.700953497
Not sequenced	-0.643557743	0.700568985
Human DNA sequence (AL121906.18, e-value = 0.081)	0.640119849	0.684349076
Oryza sativa T-DNA intergration factor (U40814.1, e-value = 3e-28)	-0.204335727	0.67029239
Not sequenced	0.33810053	0.655530276
Atropa belladonna partial mRNA 3'URT (AJ309392.1 e-value = 1e-16)	-0.172947963	0.639379389
Not sequenced	-0.436610125	0.634588458
Atropa belladonna partial mRNA 3'URT (AJ309392.1 e-value = 3e-17)	-0.154600444	0.605279361
Not sequenced	-0.667204774	0.603972121
Not sequenced	-0.186702366	0.59002135
Beta nana Ty1-copia-like retrotransposon for putative reverse transcriptase(AJ489202.1)	0.2247125	0.589170915
Atropa belladonna partial mRNA 3'URT (U30932.1, e-value = 7e-05)	-0.132589025	0.540077928
Beta nana Ty1-copia-like retrotransposon for putative reverse transcriptase(AJ489202.1)	-0.379544669	0.537625221

Table 1 (cont.)

APPENDIX C

Results – Supporting data

Beta nana Ty1-copia-like retrotransposon for putative reverse transcriptase(AJ489202.1)	-0.123104808	0.532647044
Maize chloroplast DNA for 4.5S rRNA (X01365.1, e-value = 8e-08)	0.132546253	0.500051863
Beta nana Ty1-copia-like retrotransposon for putative reverse transcriptase(AJ489197.1)	0.208002615	0.441426601
Not sequenced	0.309391793	0.435554482
Not sequenced	-0.222873322	0.432008374
Homo sapiens BAC clone (AC125238.5, e-value = 0.64)	0.13275774	0.398815451
Not sequenced	-0.349438178	0.387939756
Nicotiana tabacum DNA topoisomerase (AY169238.1, e-value = 7e-05)	0.079523071	0.386265539
Oryza sativa T-DNA intergration factor (U40841.1, e-value = 2e-23)	-0.212688495	0.373065435
*Cicer arietinum*Ty1-copia retrotransposon for putative reverse transcriptase (AJ535884.1)	0.346439951	0.372422348
Beta nana Ty1-copia-like retrotransposon for putative reverse transcriptase(AJ489197.1)	-0.177153782	0.335719091
Not sequenced	-0.222795582	0.331637485
Hordeum vulgare BARE-1 long terminal repeat (Z84562.1, e- value = 2e-14)	-0.05530943	0.322225189
Not sequenced	-0.224690144	0.295062307
Not sequenced	-0.239384831	0.281556845
Unknown	-0.333196288	0.260110031
Not sequenced	0.363810262	0.256837266
Anthoceros punctatus chloroplast gene for photosystem I P700 apoprotein A1 (AB013664.1)	0.23304003	0.250959114
Not sequenced	0.26412877	0.235438737
Unknown	-0.181529677	0.234753835
Not sequenced	0.139652106	0.223858653
Zea mays serine hydroxymethyl-transferase mRNA (AF439728.1, e-value = 6e-52)	-0.072109819	0.216331508
Oryza sativa T-DNA intergration factor (U40841.1, e-value = 2e-23)	0.064226889	0.195055714
Not sequenced	-0.283964188	0.181482007
Not sequenced	-0.099855746	0.166458259
*Cicer arietinum*Ty1-copia retrotransposon for putative reverse transcriptase (AJ535884.1)	-0.090255203	0.165907057
Triticum aestivum chloroplast DNA (AB042240.3, e-value = 0)	0.114117828	0.161039155
Beta nana Ty1-copia-like retrotransposon for putative reverse transcriptase(AJ489202.1)	-0.04538102	0.156621168
Hordeum vulgare BARE-1 long terminal repeat (Z84562.1, e- value = 3e-14)	-0.141963961	0.138806002
Beta nana Ty1-copia-like retrotransposon for putative reverse transcriptase(AF489197.1)	0.050124961	0.134201782
Homo sapiens chromosome 3 clone (AC098647.2, e-value = 0.94)	0.139812853	0.126882114
Not sequenced	0.153536358	0.125201101
*Cicer arietinum*Ty1-copia retrotransposon for putative reverse transcriptase (AJ535884.1)	-0.069446648	0.110906213
Atropa belladonna partial mRNA 3'URT (AJ30932.1, e-value = 7e-20)	-0.100493792	0.095142957
Not sequenced	0.081654494	0.092129855
Beta nana Ty1-copia-like retrotransposon for putative reverse transcriptase(AJ489197.1)	0.037519737	0.081714342
Unknown	-0.106876997	0.065724299
Oryza sativa T-DNA intergration factor (U40841.1, e-value = 1e-22)	0.024978414	0.06221645
*Cicer arietinum*Ty1-copia retrotransposon for putative reverse transcriptase (AJ535884.1)	-0.030458016	0.05874713
Not sequenced	-0.071932168	0.049621097
*Cicer arietinum*Ty1-copia retrotransposon for putative reverse transcriptase (AJ535884.1)	0.011425254	0.04262893
Not sequenced	-0.05535448	0.042066948
Homo sapiens chromosome 18 (AC015954.9, e-value = 0.02)	-0.027922187	0.038554614
Hordeum vulgare BARE-1 long terminal repeat (Z84562.1, e- value = 5e-09)	-0.020629152	0.032907049
Oryza sativa T-DNA intergration factor (U40841.1, e-value = 2e-23)	0.013468011	0.023429054
*Cicer arietinum*Ty1-copia retrotransposon for putative reverse transcriptase (AJ535884.1)	-0.010022476	0.023084828
Unknown	0.013827758	0.021035344
Homo sapiens BAC clone (AC019340, e-value = 0.044)	0.001831106	0.009035751
Oryza sativa T-DNA intergration factor (U40841.1, e-value = 7e-23)	-0.002672653	0.005118973
Denrobium chrysotoxum trnK, matK pseudogene, chloroplast genes (AF448862.1)	0.002720096	0.005078128
Not sequenced	-0.003523468	0.004006278
Not sequenced	-0.001115546	0.000889195
Oryza sativa T-DNA intergration factor (U40841.1, e-value = 6e-21)	6.1595E-05	9.994E-05
Flax and Banana clones (RDA) (Cullis Chris)		
Sequence ID	**Log2FoldChange**	**NetLog*P***
Allium grayi 5S ribosomal RNA gene (AF101256.1, e-value = 5e-32)	-0.17768198	0.542898282
Unknown	-0.37930738	0.564230265
Cloning vector pCMVTAG2b, complete sequence (e-value = 4e-21)	0.116983903	0.634123986
Unknown	0.142016493	0.149906414
Unknown	0.060646414	0.124678285
Unknown	-0.6457807	0.871253383
Unknown	-0.01855742	0.046504981
Unknown	-0.82226765	1.173782853
Linum usitatissimum clone 13 (AF074885, e-value = 1e-105)	0.579654972	0.569585728
Unknown	0.088182819	0.278384101
Linum usitatissimum LIS-1 insrtion sequence (AF104351, e-value = 0)	-0.11249723	0.534900609
Unknown	-0.071913	0.217254314
Unknown	0.428516238	0.543069578
Linum usitatissimum LIS-1 insrtion sequence (AJ131994.1)	0.058645708	0.19152195
Unknown	-0.35598755	0.548035168
Linum usitatissimum LIS-1 insrtion sequence (AJ131994.1, e-value = 0)	0.111438913	0.334335479
Unknown	-0.20256924	0.378669309
Unknown	0.259129186	1.785500303

APPENDIX C

Table 1 (cont.) Results – Supporting data

Linum usitatissimum LIS-1 insrtion sequence (AJ131994.1, e-value = 0)	0.179011875	0.696415286
Gossipium nirsutum clone D1F08 unknown chloroplast sequence	0.524604725	3.446214638
Unknown	0.151473179	0.146449444
Linum usitatissimum LIS-1 insrtion sequence (AJ131994.1, e-value = 0)	0.005852952	0.012245197
Gossipium nirsutum clone D1F08 unknown chloroplast sequence	0.547345678	3.467313523
Unknown	-0.3026328	0.219766704
Linum usitatissimum LIS-1 insrtion sequence (AJ131994.1)	0.230301458	1.406532124
Unknown	-0.15198015	0.145523367
Unknown	-0.5590012	1.324353712
Unknown	0.16778236	0.451439359
Unknown	-0.69463165	1.367466859
Unknown	-0.73099492	1.026630795
Unknown	-0.3817393	0.93361799
Unknown	-0.55045942	0.689917301
APC/C ubiquitin-protein ligase (cell cycle regulation)	0.034363126	0.048707377
Unknown	0.010447854	0.019131142
Physcomitrella patens mRNA for calmodulin(X90560.1, e-value = 2e-07)	0.267643914	0.489638056
Unknown	0.02532663	0.028296174
Unknown	-0.22550356	0.631048559
Unknown	0.836927028	1.161517864
Unknown	-0.17032777	0.225894612
Unknown	-0.25904172	0.226395155
Unknown	-0.19370031	0.23924183
Misgurnus anguillicaudatus DNA, microsatellite Mac2 (AB060172.1)	-0.78745854	1.926920625
Cloning vector pCRSCRIPT Cam, complete sequence (U46018.1)	-0.24370939	1.310981089
Uncultured bacterium partial 16S rRNA gene, clone B5.2 ((AJ517906.1)	0.644605512	1.019552745
Unknown	0.10023002	0.067836186
Unknown	0.78984632	1.272856249
Unknown	-0.2559091	0.313773512
Linum usitatissimum LIS-1 insrtion sequence (AF104351, e-value = 0)	0.055778438	0.204894799
Xenopus laevis mRNA for KREMEN, complete cds (e-value = 9e-24)	0.052099797	0.140461489
Unknown	0.722820227	0.432006597
Misgurnus anguillicaudatus DNA, microsatellite Mac2 (AB060172.1)	-0.31755812	2.698674358
Linum usitatissimum regulatory protein (AF074883, e-value = 1e-121)	0.073062612	0.248876761
Cloning vector pCMVTAG4a, complete sequence (AF073000.1)	-0.1498283	0.690153311
Linum usitatissimum LIS-1 insrtion sequence (AF104351, e-value = 0)	0.512780036	1.507335061
Unknown	0.215874016	0.904899772
Linum usitatissimum clone 5-2 (AF074884.1, e-value = 1e-177)	0.053396268	0.294510415
Linum usitatissimum LIS-1 insrtion sequence in genotrophs induced by	0.197948885	0.58463936
Unknown	-0.01984079	0.055071627
Unknown	0.055435616	0.104952274
Uncultured bacterium partial 16S rRNA gene (AF327894, e-value = 4e-24)	0.093959752	0.475514808
Hordeum californicum clone HCAL016 5S ribosomal RNA gene	1.356140377	1.365613283
Unknown	0.104769223	0.07729084
Linum usitatissimum LIS-1 insrtion sequence (AF104351, e-value = 0)	0.148208436	0.568691632
Linum usitatissimum LIS-1 insrtion sequence (AF104351, e-value = 0)	-0.0787657	0.30729176
Unknown	0.001307404	0.00389972
Unknown	0.159221917	0.185048682
Unknown	-0.15575562	0.105013113
Oryza sativa chromosome 10 BAC OSJNBb0005J14genomic sequence	0.057981494	0.284053338
Unknown	0.262658142	0.225440765
Unknown	-0.77894684	0.953577858
Unknown	0.654383306	4.42370322
Linum usitatissimum LIS-1 insrtion sequence (AF104351, e-value = 0)	0.097462806	0.258028904
Unknown	0.470446417	0.449602881
Cloning vector pCMVTAC5c, complete sequence (AF073000.1)	0.161376174	0.134782801
Unknown	0.185057046	2.332585053
Cloning vector pCMVTAC2a, complete sequence (AF073000.1)	0.253401851	1.129831709
Cloning vector pCRSCRIPT Cam, complete sequence (e-value = 4e-26)	-0.09447968	0.185974167
Cloning vector pCMVTAC2a, complete sequence (AF073000.1)	0.055176119	0.18484116
Unknown	0.078391636	0.370851359
Gossipium nirsutum clone D1F08 unknown chloroplast sequence	0.549904679	3.193121512
Unknown	0.127251402	0.445526821
Cloning vector pCMVTAC2a, complete sequence (e-value = 5e-26)	0.179573628	0.456617258
Branchiostoma belcheri Amphi-nCalponin mRNA for calponin	-0.15380583	0.279616656
Cloning vector pCMVTAC4a, complete sequence (AF073000.1)	0.505739043	2.172996032
Uncultured bacterium partial 16S rRNA gene (clone group A54n) (X91477)	0.004388723	0.015313348
Uncultured bacterium partial 16S rRNA gene (clone group A54n) (X91477)	0.087457909	0.397600923
Cloning vector pCRSCRIPT Cam, complete sequence (U46018.1)	0.006203718	0.011763491