Profiling of gene expression in bread wheat (*Triticum aestivum* L.) line PI 137739 in response to Russian wheat aphid (*Diuraphis noxia* Mordvilko) feeding

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Declaration

I, the undersigned, hereby declare that the thesis submitted herewith for the degree Philosophiae Doctor to the University of Pretoria, contains my own independent work. This work has hitherto not been submitted for any degree at any other University faculty.

Lynelle Lacock
July 2003

The results presented in this thesis are original and were obtained from research carried out at the University of Pretoria and the Forestry and Agricultural Biotechnology Institute (FABI), Pretoria, under the supervision of Prof. Anna-Maria Botha-Oberholster.

The following articles have been published/submitted from the results of this study:

1. **Lynelle Lacock,** Chantal van Niekerk, Shilo Loots, Franco du Preez and Anna-Maria Botha. 2003. Functional and comparative analysis of expressed sequences from *Diuraphis noxia* infested wheat obtained utilizing the conserved Nucleotide Binding Site. African Journal of Biotechnology 2(4): 75 – 81.

2. **L. Lacock** and A-M Botha. 2002. Isolation and characterization of cDNA sequences from Russian wheat aphid induced wheat line PI 137739 cDNA libraries. Unpublished.
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3. **Lynelle Lacock** and Anna-Maria Botha. 2003. Suppression subtractive hybridization (SSH) employed to investigate gene expression after Russian wheat aphid infestation. Proceedings of the Tenth International Wheat Genetics Symposium, (1 – 6 September 2003, Paestum, Italy), 3: 1187 – 1189.

4. E. Swanepoel, **L. Lacock**, A.A. Myburg and A-M Botha. 2003. A leucine-rich homolog to *Aegilops tauschii* from bread wheat line PI 137739 obtained by suppression subtractive hybridization show linkage to Russian wheat aphid gene *Dn1*. Proceedings of the Tenth International Wheat Genetics Symposium, (1 – 6 September 2003, Paestum, Italy), 3: 1263 – 1265.

5. Botha A-M, **L. Lacock**, C. van Niekerk, M.T. Matsioloko, F.B du Preez, A.A. Myburg, K. Kunert and C.A. Cullis. 2003. Gene expression profiling during *Diuraphis noxia* infestation of *Triticum aestivum* cv. 'Tugela DN' using microarrays. Proceedings of the Tenth International Wheat Genetics Symposium, (1 – 6 September 2003, Paestum, Italy), 1: 334 – 338.

6. Botha A-M, **L. Lacock**, C. van Niekerk, M.T. Matsioloko, F.B. du Preez, K. Kunert and C.A. Cullis. 2003. Transcriptome changes in response to *Diuraphis noxia* feeding in *Triticum aestivum* utilizing a custom array. In progress.

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CONTENTS

List of Abbreviations

AA	*Triticum monococcum*
AABBDD	*Triticum aestivum*
Ae.	*Aegilops*
amp	Ampicillin
ANOVA	Analysis of variance
approx.	Approximately
ATP	Adenosine triphosphate
BB	*Aegilops speltoides*
BLAST	Basic local alignment search tool
bp	Base pairs
BSA	Bovine serum albumin
C-terminus	Carboxyl terminus
°C	Degree celsius
CC	Coiled-coil
cDNA	Complementary DNA
cfu	Colony forming units
chr.	Chromosome
cv.	Cultivar
dbEST	Data base EST
DD	*Aegilops tauschii*
DD	Differential display
dd	Double distilled
DEPC	Diethyl pyrocarbonate
DIG	Digoxigenin
DIMBOA	*2, 4-dihydroxy-7-methoxy-1, 4-benzoxazin-3-one*
Dn	*Diuraphis noxia*
DNA	Deoxyribonucleic acid
dNTP	Dinucleoside triphosphate

ed.	Editor
eds.	Editors
EDTA	Ethylenediamine tetra acetic acid
e.g.	For example
ER	Estrogen receptor
EST	Expressed sequence tag
et. al.	*Et alii* (and others)
etc.	Etcetera
EtOH	Ethanol
FHB	Fusarium head blight
f. sp.	Forma specialis
g	Gram(s)
HMW	High molecular weight
Hx	Hydroxamic acid
i.e.	It is
IPTG	Isopropylthio-β-D-galactoside
IWF	Intercellular washing fluids
JA	Jasmonic acid
kan	Kanamycin
kb	Kilo base pairs
LB	Luria Bertani
LOX	Lipoxygenase
LR	Leucine rich
LRR	Leucine rich repeat
LZ	Leucine zipper
M	Molar
MAS	Marker assisted selection
Min	Minutes
ml	Milliliter (s)
mm	Millimeter(s)
mM	Millimolar

mRNA	Messenger RNA
N-terminus	Amino terminus
NBS	Nucleotide binding site
NBT/BCIP	Nitroblue tetrazolium chloride and 5-bromo-4chloro-3-indolyl-phosphate
NCBI	National Centre for Biotechnology Information
NIL	Near isogenic line
nm	Nanometers
NZY	NZ amine yeast extract broth
OD	Optical density
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pg	Picogram(s)
PI	Plant introduction
pmol	Picomole(s)
PR	Pathogenesis related
QTL	Quantitative trait loci
R	Resistance
RACE	Rapid amplification of 5' and 3' cDNA ends
RAPD	Random amplified polymorphic DNA
RDA	Representational difference analysis
RFLP	Restriction fragment length polymorphism
RGA	Resistance gene analogue
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RWA	Russian wheat aphid
s	Seconds
SA	Salicylic acid
SCAR	Sequence characterised amplified region
SDS	Sodium dodecyl sulphate

SM	Sodium-magnesium sulphate buffer
SNP	Single nucleotide polymorphism
SP6	5'-ATT-CTA-TAG-TGT-CAC-CTA-AAT-3'
ss	Single-stranded
SSC	Sodium chloride-sodium citrate solution
SSH	Suppressive subtractive hybridisation
SSU	Small subunit
STS	Sequence-tagged-site
syn.	Synonym
T.	*Triticum*
T7	5'-TAA-TAC-GAC-TCA-CTA–TAG-GG-3'
TIR	Toll/Interleukin receptor
U	Units
µg	Microgram(s)
µl	Microliter(s)
µM	Micromolar
USA	United State of America
USDA	United states Department of Agriculture
USSR	Union of Soviet Socialist Republics
UTR	Untranslated region
UV	Ultraviolet
vol.	Volume
v/v	Volume per volume
w/v	Weight per volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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Preface

This thesis investigates the effect of Russian wheat aphid (RWA; *Diuraphis noxia*) infestation on the defence responses of the bread wheat line, PI 137739, on a molecular level. PI 137739 is known to contain the RWA resistance gene, *Dn*1. The study was conducted by utilising and combining a vast array of molecular biological techniques.

Chapter 1 introduces the reader to a summary of the resistance responses observed within infested plants. A detailed description of the Russian wheat aphid follows and the genes responsible for RWA resistance in wheat is discussed. A brief report of research performed on the bread wheat genome is given and the biochemical defence responses of plants against insect infestation are discussed. This is followed by a concise description of resistance (R) genes and resistance gene categories in plants. The last discussion concerns microarray technology, a molecular tool utilised during this study.

Chapter 2 aims at identifying genes involved in resistance against RWA infestation; specifically, genes containing the conserved nucleotide binding site-leucine rich repeat (NBS-LRR) motif. Genomic, as well as complementary DNA (cDNA), was utilised in order to compare functional gene expression in wheat infested with the RWA. This was executed by employing PCR-based methods, single-pass sequencing and basic local alignment search tool (BLAST) analyses.

Chapter 3 introduces suppression subtractive hybridisation (SSH) as a tool to further identify NBS-LRR or other resistance-related sequences in RWA infested wheat plants. SSH allows the comparative analysis of differential gene expression in RWA infested and uninfested wheat in order to identify resistance-related genes expressed in the infested, resistant wheat plants.

The effect of RWA infestation on wheat resistance responses was examined further in chapter 4 through microarray analysis. The aim was the introduction and establishment of the microarray technique and to test the feasibility of using microarrays for differential gene expression and regulation studies. Microarray slides were assembled in order to monitor the up- and down-regulation of genes at different time intervals – day 2, day 5 and day 8 – of RWA infestation. Clones isolated throughout this study were assembled on microarray slides and probed with control and RWA infested RNA. Differential gene regulation was assessed and further confirmed through Northern blot analyses, as well as quantitative real-time PCR.

The thesis concludes with a general summary of the results obtained in chapter 5 and future prospects are outlined.

Research Objectives

The outbreak of Russian wheat aphid infestations in wheat crops has mainly been combated through the use of chemical insecticides and breeding for aphid-resistant wheat cultivars. In order to aid marker assisted selection (MAS), it is imperative to possess a large amount of knowledge concerning the defence reactions within an infested plant. The research conducted during this study aimed at unravelling the resistance responses in bread wheat after infestation with Russian wheat aphids. The general defence responses following RWA infestation will be assessed and, thus, some of the genes involved in these responses will be identified. Further, it is suggested that the nucleotide binding site-leucine rich repeat (NBS-LRR) domain is involved in plant resistance mechanisms. Therefore, it is hypothesised that the NBS-LRR genes are involved in RWA resistance responses in wheat. This study aims at testing this hypothesis by utilising the following technical objectives:

Firstly, the isolation of NBS-LRR-containing gene regions in a resistant wheat line will be attempted. The feasibility of applying PCR-based approaches on genomic DNA in the process of identifying such NBS-LRR gene sequences will be tested (chapter 2).

Secondly, cDNA libraries will be constructed and screened for gene sequences that exhibit homology to GenBank NBS-LRR sequences. PCR-based approaches will be utilised during the screening procedures (chapter 2).

Thirdly, suppression subtractive hybridisation (SSH) will be employed in order to test the viability of obtaining resistance gene fragments, specifically NBS-LRR sequences, in a resistant wheat line following Russian wheat aphid infestation. Genes that are expressed in this line as a result of RWA infestation

will be highlighted and identified based on their sequence similarities to known GenBank sequences (chapter 3).

Fourthly, the clones identified during screening of wheat genomic DNA, cDNA and SSH will be classified into categories according to putative functions assigned to the sequences based on their homology to previously identified genes (chapter 2 and 3).

Lastly, the feasibility of employing microarray technology in order to investigate gene regulation in infested wheat plants will be tested. Material obtained throughout this study will be utilised as target material and regulation of the genes in RWA infested material will be determined at different time intervals. Microarray technology will be compared to Northern blot analyses, as well as to quantitative PCR analyses, as a means of determining the success of the microarray analysis (chapter 4).

This study further aims at determining linkage between the obtained resistance-related fragments and Russian wheat aphid infestation. The obtained fragments will be mapped on the wheat genome in order to indicate possible linkage to the *Dn*1 gene. Although the mapping data is not included as a part of this thesis, two fragments obtained during this study, namely a leucine rich-like protein (411 bp; GenBank accession # AF4446141.1) and a RGA2 fragment (368 bp; GenBank accession # AF326781), were analysed and mapped, confirming linkage to RWA resistance (see appendix 3).

Chapter 1

Literature review

1. INTRODUCTION

Nature provides the habitat where all plants, animals and other organisms continually interrelate. The features of this habitat result in unavoidable contact between all of these biological agents. Interactions between plants and insects, especially pests, lead to a vast array of responses within the relevant plant. Pests utilise the host plant's resources in two main ways: either by piercing the plant surface and sucking the nutritional substances or by chewing the plant tissue and ingesting nutrients. Aphids, together with whiteflies and mites, are piercing, phloem-feeding arthropods whose feeding behaviour results in little tissue damage (Walling, 2000).

Feeding by piercing insects involves the secretion of salivary substances as the stylet penetrates the host tissue. Two types of saliva have been identified. The first is sheath saliva composed of proteins, phospholipids and conjugated carbohydrates. The second type is a watery substance consisting of digestive enzymes (Miles, 1990). It is these substances, and not mechanical wounding alone (Srinivas *et al.*, 2001), that the attacked plant perceives as elicitors to which the plant responds by activating its signalling pathways and defence mechanisms (Walling, 2000). The signalling pathways that are activated are the salicylic acid (SA) pathway and the jasmonic acid (JA)/ethylene pathway (Van Kan *et al.*, 1995; Chao *et al.*, 1999). These pathways induce the synthesis of additional chitinases, β-1, 3-glucanases and peroxidases (Bronner *et al.*, 1991; Botha *et al.*, 1995; Van der Westhuizen and Pretorius, 1996; Broderick *et al.*, 1997; Van der Westhuizen *et al.*, 1998a, b). Higher levels of other pathogenesis related (PR) proteins, e.g. PR-2, PR-4 and PR-10, have also been observed after insect attack (Broderick *et al.*, 1997). Infestation of tomato plants with potato aphids (*Macrosiphum euphorbiae*) and green peach aphids (*Myzus persicae*) resulted in higher quantities of PR-1, as well as lipoxygenase (*LOX*; Fidantsef *et al.*, 1999). Hydroxamic acids (Hx) are another set of metabolites providing members of the Poaceae family with resistance to chewing and sap-sucking

insects (Niemeyer and Pérez, 1995). Tran and his colleagues (1997) found that the synthesis of certain proteinase inhibitors (potato proteinase inhibitors I and II) render wheat (*Triticum aestivum* L.) more resistant to aphid feeding. Aphid feeding further triggers the synthesis of volatile SA substances and lipids in several different plant genera, resulting in antibiotic and/or antixenosis effects on the aphid (Hildebrand *et al.*, 1993; Hardie *et al.*, 1994; Shulaev *et al.*, 1997).

Painter (1936, 1951) investigated several plant-pest interactions. His observations led to the conclusion that certain plant varieties are able to produce crops that are higher in yield and quality than other varieties when both were exposed to similar levels of insect infestation. According to the definition of plant resistance to insects certain plant cultivars carry genetically inherited traits enabling them to be less damaged when infested than other, susceptible cultivars (Smith, 1989). The plant's reaction to insect infestation was first categorised by Painter (1951). Painter differentiated between three defence mechanisms: non-preference, antibiosis and tolerance. During 1978, Kogan and Ortman proposed the term antixenosis as a replacement for non-preference.

Host plants exhibiting antibiosis display a negative effect on the biology of the invading pest (Smith, 1989; Kindler *et al.*, 1995). Painter (1951) explained that the infesting pest's growth and survival is hampered by such a host plant. This includes a decrease in growth rate of the insect (Farid *et al.*, 1998), a reduction in body weight, slower maturation rate, reduced fecundity when mature (Smith, 1989) and reduced longevity (Kindler *et al.*, 1995). The plant induces antibiotic effects due to the presence of allomones or toxic phytochemicals, or the absence of kariomones (Smith, 1989). The infested antibiotic plant may, further, lack sufficient nutrients essential for the insect's diet. The presence of lignin, silica and trichomes also instigate antibiotic effects on the insects (Smith, 1989). These factors in turn make the insects more vulnerable to attack by their natural enemies (Price, 1986). The infested plant, however, are also adversely affected when executing its antibiosis effects. Haile and his colleagues (1999)

found that an antibiotic cultivar, PI 137739, exhibited a reduction in the rate of photosynthesis after aphid infestation.

Antixenosis, as defined by Painter (1951), is described as the plant's influence on the insect's behavioural response towards the potential host plant. Such a plant executes its antixenosis effects through the presence of morphological or chemical agents that influence the insect's behaviour (Smith, 1989). Smith summarised several factors that may contribute to this resistance mechanism. These include thickened epidermal layers, wax layers on external surfaces, the presence of large numbers of trichomes (Bahlmann *et al.*, 2003) or specific phytochemicals that ward off the insects. The above-mentioned factors contribute to the fact that potential insect feeders are repelled and, thus, search for an alternative host (Smith, 1989; Kindler *et al.*, 1995).

Host plants displaying tolerance are distinguished from other plants in their ability to uphold their vigour, growth and yield despite being infested by insects (Coppel and Mertins, 1977). These plants may even be able to recover from the damage caused by the infesting insects (Smith, 1989). Tolerance of a plant is purely a result of the plant's characteristics and not due to plant-pest interaction (Smith, 1989). A reaction that has been observed in tolerant wheat after infestation is chlorosis (a loss in chlorophyll; Farid *et al.*, 1998). Reduced chlorosis in tolerant cultivars presumably occurs when RWA probe a leaf continually without successfully establishing a feeding site. This behaviour may be the result of physical barriers in the pathway of the stylet or a chemical barrier in the host's phloem (Farid *et al.*, 1998). A further observation by Haile and his colleagues (1999) led to the discovery that a tolerant line, PI 262660, altered its photosynthetic processes in order to render the plant more tolerant to Russian wheat aphid infestation.

2. THE RUSSIAN WHEAT APHID

2.1 Detection

Diuraphis noxia (Mordvilko) is commonly known as the Russian wheat aphid (RWA). The origin of the RWA is speculated to be the Caucasus in southern Russia (Robinson, 1992). The RWA is also native to Iran, Afghanistan and countries bordering the Mediterranean Sea (Walters *et al.*, 1979). Infestation by this aphid has resulted in yield losses to wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.) since 1900 (Grossheim, 1914). Spain reported minor occurrences of RWA infestations in 1945 (Robinson, 1992). The RWA spread from its native countries to Africa (Pakendorf, 1984; Torres, 1984) and then to South America and Mexico, where it was detected in 1980 (Gilchrist *et al.*, 1984; Zerene *et al.*, 1988). During 1986, the RWA spread towards the western USA and invaded south-western Canada (Stoetzel, 1987; Smith *et al.*, 1991).

The RWA was recognised as a wheat pest in South Africa during 1978 (Walters *et al.*, 1979). Initially, only wheat in the eastern Free State was affected, but by late 1979 the aphids invaded the western Free State and Lesotho (Walters *et al.*, 1979). The result was severe disease outbreaks during 1970 to 1980 (Du Toit and Walters, 1982). Due to ignorance regarding the pest status of the RWA and infrequent applications of insecticides, the RWA became one of the most devastating pests infesting wheat in South Africa (Van der Westhuizen and Botha, 1993).

2.2 Description

Walters and his colleagues (1979) provided a thorough description of the Russian wheat aphid. It has a small (< 2mm in length), green, spindle-shaped body with especially short antennae. The RWA has a characteristic double tail above its cauda and an apparent absence of a prominent siphunculi.

Figure 1.1 A Russian wheat aphid during its aperous (wingless) life cycle. The body of the aphid is spindle-shaped and the apparent double tail is visible at the posterior of the body. Bar represents 0.2 mm. Photograph courtesy of Lieschen Bahlmann, University of Pretoria.

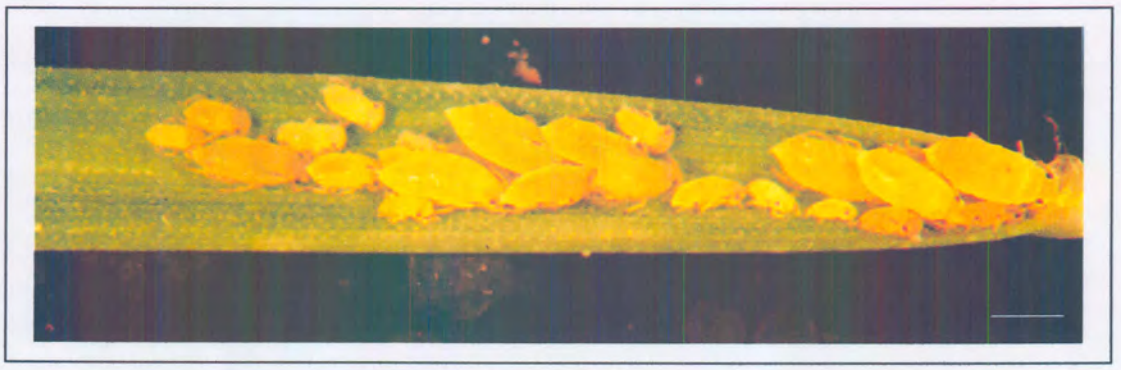

Figure 1.2 A wheat leaf infested with Russian wheat aphids. Adult, as well as juvenile aphids are present in their aperous form. Bar represents 1 mm. Photograph courtesy of Lieschen Bahlmann, University of Pretoria.

2.3 Life cycle

Russian wheat aphids mainly occur as female insects present either in a winged (alate) or a wingless (aperous) form (Walters *et al.*, 1979). The winged form distributes the pest to favourable areas as soon as the current field/area experiences stress or the growth stage of the plants no longer provides a suitable habitat. This distribution is aided by convection currents and wind, and the females start feeding as soon as it settles down on a new host plant (Walters *et al.*, 1979).

Male aphids are very seldom observed and, therefore, the RWA mainly reproduces through parthenogenesis (Robinson, 1992). The female aphids produce approximately four wingless, female nymphs per day when the conditions are favourable (Walters *et al.*, 1979). These nymphs produce offspring after two weeks. During adverse winter conditions, the Russian wheat aphid's reproductive cycle can include a sexual stage (Puterka *et al.*, 1993). Low winter temperatures do, however, restrict the reproduction of the aphids (Walters *et al.*, 1979). Sexual reproduction, known as holocyclic reproduction, enables the RWA to produce eggs during the over-wintering period (Puterka *et al.*, 1993).

2.4 Host species

During the warm seasons, when wheat fields are not available, the RWA needs alternative hosts in order to survive. In South Africa, the RWA exploits barley, rye, triticale and a variety of other grass species, such as rescue grass (*Bromus catharticus* Vahl), wild oats (*Avena fatua* L.) and volunteer wheat, to survive the warm seasons when wheat is unavailable (Kindler and Springer, 1989; Robinson, 1992; Prinsloo *et al.*, 1997).

2.5 Infestation

Russian wheat aphids establish themselves on their host plants throughout the plants' growth (Aalsberg *et al.*, 1989). Kriel and his colleagues (1984) observed that the aphids migrate upward on the plant as it grows. New emerging leaves are continually colonised, while the concentration of aphids on previously infested leaves increases (Walters *et al.*, 1979). Aalsberg and his co-workers (1989) found that the aphids prefer to colonise the flag leaves during anthesis and senescence. Since the flag and second leaves contain most of the carbohydrates necessary for grain development (Lupton, 1966), infestation of these leaves leads to considerable yield losses (Gray *et al.*, 1990).

2.6 Feeding behaviour

Russian wheat aphids possess tongue- and groove-connected stylets (Dreyer and Campbell, 1987) with which they probe their host plants in order to establish potential feeding sites. Probing occurs randomly on the host plant (Belefant-Miller *et al.*, 1994) and in the process causes the epidermal cell walls to break (Lopez-Abella *et al.*, 1988). Once the stylet has been inserted into the host plant tissue, it follows an intercellular path on the way to the host's phloem (Fouché *et al.*, 1984). The movement of the stylet is facilitated by salivary pectinase secreted by the aphid (Dreyer and Campbell, 1987). The pectinase is responsible for the depolymerisation of the pectin contained within the host tissue's middle lamellar layers (Dreyer and Campbell, 1987). The injected pectinase results in cell collapse and subsequent cell death (Al-Mouswi *et al.*, 1983). The saliva is, further, thought to contain cellulases, esterases, proteinases, amylases, polyphenoloxidases and phenolic compounds that could be destructive to the host's cells (Minks and Harrewijn, 1987; Robinson, 1992). Musser and his colleagues (2002) discovered the presence of glucose oxidase in the saliva of caterpillars (*Helicoverpa zea*). The glucose oxidase suppresses the functioning of jasmonic acid and, thus, inhibits the activation of signalling pathways (Musser *et al.*, 2002). As the stylet progresses between the plant cells, a lipoprotein sheath is secreted that surrounds and thus protects the stylet (Miles,

1990). Once the sieve elements of the phloem are reached, the phloem is pierced by the stylet and the aphid feeds on the sucrose-rich phloem (Dreyer and Campbell, 1987). Prolonged aphid feeding results in disruption and even disintegration of chloroplast and cell membranes (Fouché *et al.*, 1984).

2.7 Symptoms

The salivary enzymes injected into the plant during RWA feeding have dire consequences on its host. The action of these substances is responsible for the formation of longitudinal yellow, white and purple chlorotic streaks on the leaves of the infested plant (Du Toit, 1986). The leaf sheaths may also display an initial pink discoloration that turns white during prolonged infestation (Von Wechmar and Rybicki, 1981). These chlorotic symptoms can lead to necrosis and subsequent plant death (Robinson, 1992). The injected salivary substances further prevent leaf sheaths or leaf whorls from uncurling and the aphids are able to feed in these protected environments (Smith *et al.*, 1991). Heavily infested plants have a stunted, flattened appearance (Walters *et al.*, 1979; Elsidaig and Zwer, 1993). Spikes can become deformed (Elsidaig and Zwer, 1993), the heads entrapped in the rolled leaves (Ma *et al.*, 1998) and the ears become bent and turn white (Walters *et al.*, 1979). The total number of leaves produced by infested plants is also reduced (Burd and Burton, 1992). Damage caused to the flag leaves results in contorted heads that interfere with head extension and self-pollination (Smith *et al.*, 1991). These effects on the plant can result in yield losses ranging from 35% to 60% (Du Toit and Walters, 1982).

Since the RWA destroys the chloroplasts and cell membranes during feeding, the plant's photosynthetic ability is reduced (Fouché *et al.*, 1984). Feeding of the aphid further disrupts osmoregulatory processes (Riedell, 1989; Burd and Burton, 1992) and interferes with cold hardening (Thomas and Butts, 1990).

2.8 Control

Several basic strategies are employed worldwide in the combat against plant pests. These include proper cultural management, chemical and biological control, as well as the incorporation of resistance genes into the host plants. The implementation of proper cultural management in controlling Russian wheat aphid infestation involves careful planning concerning the choice of planting date, reducing the availability of volunteer hosts (Walters *et al.*, 1979) and considering the chances for aphid infestation from neighbouring fields (Robinson, 1992). Wheat grown under optimal environmental conditions, e.g. good fertilisation, moist soil and proper seedbed preparation, appear to be more resistant to RWA infestations (Walters *et al.*, 1979).

The use of biological agents such as predators and parasites of RWA has to date proven ineffective in the control of RWA, since the RWA's feeding behaviour impedes this approach. The enclosed environment of the rolled leaves protects the aphids from attack by other pests (Walters *et al.*, 1979). The introduction of a parasitoid (*Aphelinus varipes*) during 1993 to South African wheat crops was carried out in an effort to control RWA infestations (Prinsloo, 1998). Surveys two years later revealed that RWA were no longer present on *Bromus* species or volunteer wheat (Prinsloo, 1998). The Small Grain Institute (Bethlehem, South Africa) is also investigating the use of an entomopathogenic fungus to control the RWA (Tolmay *et al.*, 1999).

The routine application of chemical agents (Du Toit, 1989a) has been and is still being used in an effort to control the RWA. Insecticides have been applied in South Africa since the late 1970s, especially in the eastern Free State (Du Toit, 1987). The enclosed environment of the leaf sheaths and leaf whorls where the RWA feeds, however, decreases the efficiency of contact insecticides (Unger and Quisenberry, 1997). This necessitates the use of expensive systemic insecticides (Du Toit and Walters, 1982). Combinations of contact and systemic insecticides have also been applied in an effort to control the RWA (Walters *et*

al., 1979). These mixtures are, however, expensive and their use environmentally undesirable (Du Toit, 1986, 1989a; Tolmay *et al.*, 1999).

Fortunately, there is a more economical and environmentally safe approach towards the control of the RWA, namely the development of resistant wheat cultivars (Elsidaig and Zwer, 1993; Dong and Quick, 1995; Saidi and Quick, 1996). Several breeding programs are in progress in an attempt to develop such lines (Burd *et al.*, 1993). One such program in South Africa is the transfer of RWA resistance to commercially available cultivars at the Small Grain Institute, Bethlehem (Tolmay *et al.*, 1999).

2.9 Wheat lines resistant to the RWA

Resistance against the RWA has been identified in several wheat lines. The search for resistance in South Africa began during 1984 and was conducted on wheat lines from countries native to the RWA such as the former USSR, Iran, Israel, Lebanon, Jordan, Syria and Romania (Du Toit, 1987).

The wheat accession PI 137739 is a hard white, spring wheat originally found in Iran (Du Toit, 1987). It has been established that resistance is inherited through a single, dominant gene that is independently inherited (Du Toit, 1989b). This gene has been designated *Dn1* (Dong and Quick, 1995) and is located on the short arm of chromosome 7D (Marais and Du Toit, 1993; Schroeder *et al.*, 1994; Liu *et al.*, 2001). Resistance in PI 137739 has been ascribed to antibiosis (Du Toit, 1989b) and antixenosis (Du Toit, 1987).

PI 262660 is a hard white, winter wheat from Bulgaria and the gene expressing RWA resistance in this PI has been designated *Dn2* (Dong and Quick, 1995). Resistance to the RWA in *Dn2* is controlled by a single, dominant, independently inherited gene (Du Toit, 1989a). The resistance categories employed by PI 262660 are antibiosis, antixenosis (Du Toit, 1987) and tolerance to the RWA (Du Toit, 1989b).

Triticum taushcii, a progenitor species of bread wheat, also exhibits resistance against the RWA (Du Toit and Van Niekerk, 1985; Du Toit, 1987). Nkongolo and his co-workers (1991a) designated this accession as *dn3*. Resistance in *dn3* is thought to be the result of a recessive gene (Nkongolo *et al.*, 1991a).

Dn4 was designated to the PI 372129 wheat accession exhibiting resistance to the RWA (Dong and Quick, 1995). This wheat line originated in Russia (Quick, 1989) and resistance is controlled by a single, dominant, independently inherited gene (Du Toit, 1989a; Nkongolo *et al.*, 1991b). PI 372129 exhibits a high level of tolerance, and low levels of antixenosis and antibiosis (Quick, 1989). Liu and his colleagues (2001) mapped *Dn4* to the wheat chromosome 1DS.

The gene in wheat accession PI 294994 (originally from Bulgaria) has been designated as *Dn5* (Marais and Du Toit, 1993). Conflicting opinions exist regarding the mode of inheritance of resistance of this wheat line. Marais and Du Toit (1993) were of opinion that resistance is the result of a single dominant gene located on chromosome 7D. Elsidaig and Zwer (1993), however, found that resistance might be caused by one dominant and one recessive gene. Saidi and Quick (1996) proposed that two dominant genes are involved in resistance. The resistance categories employed by this wheat line are antibiosis and tolerance (Smith *et al.*, 1992). Liu and his colleagues (2001) identified the additional genes *Dn8* and *Dn9* when mapping *Dn5*. *Dn8* is located on wheat chromosome 7DS and *Dn9* is located on wheat chromosome 1DL. The mode of inheritance of these genes is unresolved.

A wheat line native to Iran (Quick, 1989), PI 243781, was designated as *Dn6* (Saidi and Quick, 1996). Resistance to the RWA is conferred by a single, dominant gene that is independently inherited (Saidi and Quick, 1996).

Russian wheat aphid resistance was also detected on the rye (*Secale cereale* L.) 1RS chromosome (Marais *et al.*, 1994). This dominant gene was transferred to the 1BL chromosome of the wheat line, "Gamtoos" and designated as *Dn7* (Marais *et al.*, 1994).

3. THE HOST: BREAD WHEAT (*Triticum aestivum* L.)

3.1 An introduction to *Triticum aestivum*

Bread wheat (*Triticum aestivum* L. em. Thell.) as it is known to man today is thought to have originated 10 000 years ago (Gill *et al.*, 1991). Kimber and Sears (1987) explained the origin of bread wheat – with an AABBDD genome composition – as follows: an ancestral, diploid wheat differentiated into other diploid forms, including a wild wheat containing the A genome. Today it is clear that this wheat is *T. monococcum*. The next phase in the development of hexaploid wheat commenced when a second diploid (BB) combined with the wild species and formed tetraploid wheat (AABB; *T. turgidum*). The source of the B genome, however, is still unknown (Devos and Gale, 1993). Sarkar and Stebbins (1956) speculated that *Aegilops speltoides* (syn. *T. speltoides*) is the donor of the entire B genome or a large portion of it. The final phase in the development of *T. aestivum* was the addition of the D genome. *Aegilops tauschii* (Coss.; syn. *Ae. squarossa* L.) was found to be the donor of the D genome (McFadden and Sears, 1944, 1946).

The combination of the A, B and D genomes to form *T. aestivum* has, evolutionary speaking, taken place recently (Devos and Gale, 1993). It is, therefore, surprising that wheat is separated from its ancestors in that reproduction between them cannot take place. Therefore, genetic variability in wheat may be limited (Talbert *et al.*, 1995). The fact that the three genomes are closely related enables this species to tolerate a deficient chromosome or even an extra chromosome (Jauhar and Chibbar, 1999). Genetic studies are, however, complicated by the large amount of information locked within these three genomes (Devos and Gale, 1993).

Each genome of bread wheat consists of seven chromosomes to form an allohexaploid (2n = 6x = 42; Bennett and Smith, 1976). The size of the entire genome is estimated to be 17 x 10^9 base pairs per chromosome or 18.1 picograms of DNA per nucleus (Devos and Gale, 1993; Moore *et al.*, 1993). Flavell and Smith (1976) investigated the sequence organisation of the nucleotides in each chromosome by using various renaturing techniques. These studies revealed that about 25% of the genome consist of non-repeated or unique sequences. The length of these sequences vary from 1 000 base pairs (15% of the genome) to several thousand base pairs (7% of the genome). The remaining non-repeated sequences are much longer. Thus, approximately 75% of the wheat genome consists of repeated DNA sequences (May and Appels, 1987). Ten percent of these repeated sequences consist of very long sequences or of long groups of short, identical repeated sequences. It is speculated that the percentage of coding genes contained within the wheat genome is relatively low (May and Appels, 1987).

Comprehending the complexity of the wheat genome is imperative in the search for resistance genes. Not only has the organisation of the wheat genome been investigated (Flavell and Smith, 1976; Kimber and Sears, 1987; May and Appels, 1987; Talbert *et al.*, 1995), but physical gene locations have also been identified and assembled into genetic maps (Gill *et al.*, 1991; Devos and Gale, 1993; Li *et al.*, 1999). Genetic maps of wheat are being utilised by plant breeders in order to deal with genes responsible for qualitative traits separately (Devos and Gale, 1993). Genetic maps further provide the means for gene tagging, which facilitates the selection for a specific trait (Gill *et al.*, 1991; Devos and Gale, 1993; Röder *et al.*, 1998; Li *et al.*, 1999; Börner *et al.*, 2000). Restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and sequence characterised amplified region (SCAR) markers are being utilised in map-based cloning programmes in order to identify biotic stress resistance genes (Myburg *et al.*, 1997; Feuillet and Keller, 1998; Venter and Botha, 2000). He and his co-workers (1992) and Dweikat and his colleagues

(1993), for example, have applied RAPD analysis together with denaturing gradient gel electrophoresis to differentiate between wheat cultivars. Wheat microsatellite maps are also being compiled by using restriction fragment length polymorphisms (RFLPs; Röder *et al.*, 1998; Börner *et al.*, 2000). Once genes of interest have been organised into such maps, they can serve as genetic markers for disease resistance (Spielmeyer *et al.*, 1998). The wheat genome is also being analysed by using sequence-tagged-site polymerase chain reaction (STS-PCR) markers as a means to identify genetic diversity (Chen *et al.*, 1994; Talbert *et al.*, 1995). Talbert and his colleagues (1995) showed that nine STS-PCR amplification products were polymorphic for 20 hexaploid wheat cultivars, all belonging to the same germplasm background. Chen and his co-workers (1994) further applied STS-PCRs to determine varying levels of diversity within and among different wheat accessions. Another molecular biology tool, quantitative trait loci (QTLs), is implemented in the search for disease resistance genes in wheat (Faris *et al.*, 1999). And once resistance genes have been identified, they can be characterised and cloned (Lagudah *et al.*, 1998).

3.2 Interaction between wheat and the RWA

The exact resistance mechanisms employed by wheat against the RWA are not yet known. When the feeding behaviour of RWAs on resistant and susceptible wheat is considered, two important aspects have been observed. Firstly, the aphids have difficulty in locating or accepting the phloem of a resistant host plant (Kindler *et al.*, 1992). As a result, the aphids continually probe these leaf surfaces, whereas probing on susceptible plants occurs less (Webster *et al.*, 1993b). The probing time of cereal aphids on a resistant plant is also twice as long as on a susceptible host (Dreyer and Campbell, 1987). Secondly, the duration of phloem ingestion by the aphid varies. Girma and his colleagues (1992) found that aphids feed for longer periods (at least 15 min) on susceptible wheat plants and ingestion can last up to 2 hours. Aphids can feed for up to 4 hours on susceptible barley (*Hordeum vulgare* L.; Webster *et al.*, 1993a).

The high probing frequency and short feeding period on resistant plants may be an indication that the plant produces substances that are unpalatable for the RWA (Belefant-Miller *et al.*, 1994). Phytoalexins are possible candidates resulting in unpleasant-tasting phloem (Mittler, 1988). The phloem of resistant plants could, further, lack adequate structural or chemical cues for phloem acceptance and, thus, feeding (Kindler *et al.*, 1992). The consequence is that the aphids engage in non-phloem feeding (Kindler *et al.*, 1992). Resistant wheat lines, further, inhibit the intercellular progress of the stylet due to the high depolymerisation rate of the pest's pectinase (Dreyer and Campbell, 1983).

The enzymatic plant defence responses have been shown to be triggered by plant wounding, elicitors, as well as pathogens (Bowles, 1990; Corcuera, 1993; Stinzi *et al.*, 1993). One of the initial responses following RWA infestation is the accumulation of salicylic acid (SA). SA may be involved in the signal transduction pathway responsible for the induction of several defence related proteins (Mohase and Van der Westhuizen, 2000). It has, in fact, been proved that the synthesis of pathogenesis related (PR) proteins are induced as a result of RWA infestation (Botha *et al.*, 1995, 1998; Van der Westhuizen and Pretorius, 1995, 1996; Van der Westhuizen *et al.*, 1998a, b; 2002). One such enzyme is β-1, 3-glucanase. β-1, 3-Glucanases accumulate in the apoplast of tissue of infested resistant wheat (Van der Westhuizen *et al.*, 1998a). This is indicative of its role in the plant's defence response. The subsequent increase in intracellular β-1, 3-glucanase activities were, further, found to spread systemically (Van der Westhuizen *et al.*, 1998a, 2002). Van der Westhuizen and his colleagues (1998a) speculated that β-1, 3-glucanase might be involved in callose formation at the necrotic lesions that form at the RWA's site of infestation. The function of the callose barrier is to limit the aphid's access to the host's food supply (Bowles, 1990; Bronner *et al.*, 1991).

A second protein thought to be involved in the plant's response to RWA infestation, is chitinase (Botha *et al.*, 1995, 1998; Van der Westhuizen and Pretorius, 1996; Van der Westhuizen *et al.*, 1998b). In studies conducted by Nagel (1995), she found a definite difference in chitinase induction between resistant and susceptible wheat. Resistant plants exhibited up to 300 times higher levels of endochitinases after RWA infestation (Nagel, 1995). Chitinase activity was, further, initiated at an earlier stage in resistant plants than in susceptible ones. A sudden increase in chitinase activity was observed between three and seven days after infestation (Botha *et al.*, 1998). This increase in activity occurred in the symplastic tissues, as well as in the intercellular washing fluids (IWF; Botha *et al.*, 1998), and could result from the plant's response to the chitinous elicitors produced by the RWA (Nagel, 1995).

Peroxidase is another probable candidate involved in plant resistance mechanisms. Bowles (1990) found that peroxidase is involved in defence-related occurrences in extra-cellular matrices of plant tissue. In wheat, peroxidase activity rapidly increases in infested, resistant plants and accumulates in the entire plant as a systemic event (Van der Westhuizen *et al.*, 1998b). The induction of peroxidase is closely related to the accumulation of SA in resistant wheat. SA accumulates 4 hours after RWA infestation and is followed by peroxidase accumulation within 12 hours (Mohase and Van der Westhuizen, 2002).

Plant proteinase inhibitors are also involved in plant defence reactions (Ryan, 1989) and have been shown to be effectual against insects (Xu *et al.*, 1993). Tran and his colleagues (1997), therefore, speculated that plant proteinase inhibitors are potential defence agents against cereal aphids.

A compound that has been investigated for its involvement in RWA resistance is hydroxamic acid (Hx; Mayoral *et al.*, 1996). Hydroxamix acids are present in wheat leaves, specifically in the mesophyll parenchyma and vascular bundles (Corcuera, 1990). Hx is a constituent of β-glucosides that is transformed to its corresponding glucones during plant damage (Virtanen and Heitla, 1960). The main glucone found in wheat is DIMBOA (*2, 4-dihydroxy-7-methoxy-1, 4-benzoxazin-3-one*; Niemeyer, 1988). DIMBOA concentrations in rye and wheat have been correlated to the levels of resistance against cereal aphids. However, conclusive evidence has not been found since there are no correlations between the levels of DIMBOA and the total time of RWA phloem feeding or growth rate (Mayoral *et al.*, 1996). Mayoral and his co-workers (1996) have, nevertheless, found that RWAs probe leaf surfaces less where the level of DIMBOA in artificial diets has been increased and, thus, fewer aphids could uphold phloem ingestion. Argondoña and his colleagues (1983) demonstrated a high level of greenbug (*Schizaphis graminum*) mortality on artificial diets containing DIMBOA. These facts lead to the assumption the Hx may also aid the wheat plant in its defence against the Russian wheat aphid.

4. PLANT RESISTANCE ON A MOLECULAR LEVEL

4.1 Interesting gene sequences linked to resistance

Investigations of plant resistance (R) genes have revealed many secrets that are locked within the sequences of these genes. One such secret is that R genes are enclosed in large groups consisting of similar sequences and characteristic domain organisation (Young, 2000). Therefore, R genes have been sorted into four main classes according to the amino acid motifs of the expressed proteins (Hammond-Kosack and Jones, 1997). The first distinct class encompasses the serine-threonine kinases that are involved in controlling certain signalling pathways during resistance responses (Martin *et al.*, 1993; Ritter and Dangl, 1996). The second class consists of transmembrane receptors bound to extracellular leucine rich (LR) domains (Dixon *et al.*, 1998). Receptor-like kinases make up the third class and exhibit characteristics of both the first and second class (Jones and Jones, 1997). The fourth and largest class of R genes are encoded by nucleotide binding site (NBS) and leucine rich repeat (LRR) sequences (Lagudah *et al.*, 1997; Rivkin *et al.*, 1999; Michelmore, 2000; Tao *et al.*, 2000; Halterman *et al.*, 2001). One percent of the *Arabidopsis* genome, for example, is thought to contain at least 200 different NBS-LRR genes (Ellis *et al.*, 2000; Sandhu and Gill, 2002).

The main structural characteristics of NBS-LRR sequences are a variable N-terminus of approx. 200 amino acids, a NBS domain of about 300 amino acids, followed by short tandem repeats of 10 to 40 LRRs at the C-terminus (Traut, 1994; Hammond-Kosack and Jones, 1997; Jones and Jones, 1997; Van der Biezen and Jones, 1998). The NBS domain can, further, be divided into two sub-classes namely the toll/interleukin receptor (TIR) class and the non-TIR class (Michelmore, 2000). The TIR domain exhibits great similarity to the *Toll* protein of *Drosophila* and *Interleukin* receptor-like sequences found in mammals (Hoffmann *et al.*, 1999; Qureshi *et al.*, 1999). The non-TIR class is characterised by a coiled coil (CC) or a leucine zipper (LZ) sequence (Lupas, 1996; Baker *et*

al., 1997). Lupas (1996) described the CC structures as a collection of helices, usually two to five, containing amino acid side chains with specific linking between the chains at their connection points. *Arabidopsis thaliana* has served as model system for investigating the NBS-LRR class of resistance domains and the TIR/non-TIR subclass (Meyers *et al.*, 2003). It has been discovered, however, that not all plant families contain the TIR subclass (Cannon *et al.*, 2002). TIR regions are not present in monocotyledonous plants and are absent from the genomes of the grass and palm families, as well as from certain Pinaceae genera, e.g. *Cryptomeria* (Cannon *et al.*, 2002).

Various opinions exist as to the function of the NBS-LRR and the TIR/non-TIR sequences and structural orientation. The main function of the nucleotide binding site region, especially the TIR region (Ellis and Jones, 1998), is thought to be signal transduction (Baker *et al.*, 1997; Meyers *et al.*, 1999; Van der Biezen *et al.*, 2000). Recent research by Ellis and his colleagues (1999) suggests the involvement of the TIR region in pathogen recognition. The LRR region, on the other hand, plays an important role in gene-for-gene (Staskawicz *et al.*, 1995) and protein-protein interactions (Kobe and Deisenhofer, 1994). One example of such protein-protein interaction is the binding of various ligands to the surface of the LRR structure (Braun *et al.*, 1991). In order to carry out this function, the LRR domain is thought to contain a β-sheet (Parniske *et al.*, 1997; Thomas *et al.*, 1997) consisting of changeable solvent-exposed residues (Van der Biezen and Jones, 1998).

Researchers have unravelled several resistance genes corresponding to the NBS-LRR structural design. Tomato plants subjected to several stress conditions, including mechanical wounding, expressed RNAs containing leucine rich aminopeptidases (Chao *et al.*, 1999). The tomato *Cf-2* gene, containing 38 LRRs, confers resistance to tomato leaf mould (Dixon *et al.*, 1996). Sugar beet is able to withstand the beet cyst nematode as a result of the HS^{pro-1} gene, which is composed of imperfect LRRs (Cai *et al.*, 1997). Lagudah and his colleagues

(1997) made a further interesting discovery concerning plant resistance genes. They isolated a gene family encoding a LRR, as well as a NBS region, at the *Cre3* cyst nematode resistance gene locus in wheat. In *Arabidopsis* the *RPS2* gene, containing NBSs and LRRs, confers resistance against certain *Pseudomonas syringae* strains (Tao *et al.*, 2000). Lawrence and his colleagues (1995) discovered that the *RPS2* gene is similar to the tobacco viral resistance gene, *N,* and the rust resistance gene in flax, *L6*. NBS sequences linked to disease resistance genes have also been cloned and amplified from soybean (Yu *et al.*, 1996), and wheat and barley (Seah *et al.*, 1998). Some plant resistance genes are, further, composed of LRR, as well as serine-threonine kinase-like domains. One such an example is the rice *Xa21* gene that confers resistance to *Xanthomonas oryzae* pv. *oryzae* race 6 (Song *et al.*, 1995). The wild tomato species, *Leucopiersocin peruvianum*, gene, *Mi*, has been found to exhibit nematode resistance, as well as resistance against the potato aphid (*Macrosiphum euphorbiae*), and has been designated as *Meu1* (Kaloshian *et al.*, 1995, 1997). It has been found that the *Mi-1.1* and *Mi-1.2* genes are constituents of the NBS-LRR resistance gene family (Milligan *et al.*, 1998) and Rossi and her colleagues (1998) concluded that *Mi* and *Meu-1* are the same gene, conferring resistance to both nematodes and aphids.

5. MICROARRAY TECHNOLOGY

5.1 The microarray principle

The concept behind microarrays and the implementation of this technique emerged during the late 1970s to late 1980s (Ekins and Chu, 1999). The principles on which microarrays are based have, however, been discovered several decades ago. Gillespie and Spiegelman (1965), for example, immobilised denatured DNA on nitrocellulose membranes and hybridised complementary RNA to these DNA strands. EM Southern (1975), in turn, transferred DNA from agarose gels to cellulose nitrate membranes. Southern hybridised the DNA to radio-labelled RNA and could, thus, detect DNA of specific sequences via autoradiography. "Dot-blotting" was the next technique to be developed (Kafatos *et al.*, 1979). Dot-blots involve the immobilisation of specific probes on a membrane that is hybridised against a labelled, target genomic DNA or cDNA population (Kafatos *et al.*, 1979). Microarrays are said to be second-generation dot-blots (Rockett and Dix, 2000) that underwent a process of miniaturisation and automation (Southern *et al.*, 1999). Two advances of microarray technology, however, are the preparation of microarrays on solid supports, and an increase in the number and density of spots that can be examined (Rockett and Dix, 2000).

The microarray principle is based on the concept of DNA sequence determination (Baringa, 1991; Hoheisel, 1997). Sequence determination is possible due to the characteristic binding of single stranded DNA and RNA to its single stranded complement (Chee *et al.*, 1996; Terryn *et al.*, 1999; Brazma *et al.*, 2000) through specific hydrogen bonds between complementary base pairs (Graves *et al.*, 1998). Therefore, the microarray hybridisation process takes place between single-stranded (ss) immobilised DNA or RNA of which the sequence is known and a DNA or RNA population of unknown sequence (Hoheisel, 1997). The immobilised DNA is present as high-density (Rockett and Dix, 1999; Van Hal *et al.*, 2000), microscopic spots (Graves, 1999) at specific

addresses on a solid support (Graves *et al.*, 1998; Gerhold *et al.*, 1999) and is referred to as the probes (Hoheisel and Vingron, 1998). The target, which is hybridised against the immobilised probes, consists of a sample of single stranded, labelled DNA or RNA in solution (Gerhold *et al.*, 1999). Labelling takes place through the incorporation of fluorescently labelled nucleic acids during reverse transcription of the target mRNA (Hoheisel and Vingron, 1998; Granjeaud *et al.*, 1999; Brazma *et al.*, 2000). Once hybridisation is complete, a fluorescent signal supplies information regarding the number of bound target molecules, their corresponding array position, as well as a measure of the level of expression of a specific gene (Hoheisel and Vingron, 1998; Lemieux *et al.*, 1998; Van Hal *et al.*, 2000).

Brazma and his co-workers (2000) explained how to interpret an acquired hybridisation pattern: A red fluorescent dye can be used to label RNA from a sample population (i.e. target 1) and green dye for RNA from a control population (i.e. target 2). Target 1 and target 2 are then hybridised against the immobilised probe DNA. The detection of a red signal indicates an abundance of sample (target 1) RNA. A green signal is an indication of abundant control (target 2) RNA. If the sample and control RNA binds equally to the probe, a yellow signal will be detected. A black spot is an indication that no binding took place (Brazma *et al.*, 2000).

5.2 Oligonucleotide microarrays

Microarrays can be categorised into two groups based on the probe sizes, the hybridisation and detection methods and the methods employed when assembling the arrays (Gerhold *et al.*, 1999). The first category employs a synthesis strategy (Schena *et al.*, 1998) and consists of probes ranging from 10 to 25 base pairs in length (Pease *et al.*, 1994; Thieffry, 1999; Rockett and Dix, 2000). These oligonucleotide chains are either synthesised fully and then spotted onto a glass slide (Marshall and Hodgson, 1998), or they are synthesised during a stepwise, *in situ* process to acquire a nucleic acid chain of the desired

length and sequence (Pease *et al.*, 1994; Schena *et al.*, 1998). *In situ* synthesis encompasses a series of enzymatic steps (Sapolsky and Lipshutz, 1996) that result in the formation of the oligonucleotide chains at specific addresses on the glass surface (Southern, 1996; Hoheisel, 1997). The glass surface, however, has to be treated chemically in order to ensure sufficient binding of the oligonucleotides. Oligoethylene (Cheung *et al.*, 1999), as well as a stable aliphatic polyether linker with a terminal hydroxyl group (Southern *et al.*, 1992) has been applied successfully for this purpose. During the extension of the oligonucleotides, labelled dideoxyribonucleoside triphosphates are incorporated into the oligonucleotide chain (Hacia, 1999). Each of the four dideoxyribonucleoside triphosphates is labelled with a different fluorescent dye (Nikiforov *et al.*, 1994). Hybridisation to the target sequence is the next step, after which analysis of the hybridisation pattern follows (Hoheisel, 1997).

Oligonucleotide microarrays have a diverse range of applications. A feature of this type of microarray commonly exploited is that of expression monitoring (Lockhart *et al.*, 1996). The mRNA levels that are present under certain conditions in a cell can be measured by hybridising the mRNA against specific oligonucleotides (Granjeaud *et al.*, 1999). These oligonucleotides represent the known sequence of the gene to be monitored (Granjeaud *et al.*, 1999). DNA chips bearing thousands of well-characterised genes are becoming available for use in the study of gene expression levels (Gerhold *et al.*, 1999).

Oligonucleotide microarrays, further, provide a rapid method for sequence determination (Southern, 1996). Since complementary nucleotides bind to each other, the sequence of a target oligonucleotide can be determined from its corresponding oligonucleotide probe (Southern, 1996; Hoheisel, 1997). Further implications of this feature are that mutations, as well as polymorphisms, can be detected (Gerhold *et al.*, 1999). Optimal hybridisation conditions can even lead to the detection of single nucleotide polymorphisms (SNPs; Gerhold *et al.*, 1999).

5.3 cDNA microarrays

The second category of microarrays involves a delivery strategy (Schena *et al.*, 1998). Generally, cDNA or polymerase chain reaction (PCR) products are used to assemble these microarrays (Schena *et al.*, 1995; Kozian and Kirschblaum, 1999) and are, therefore, known as DNA-fragment-based or cDNA microarrays (Richmond and Somerville, 2000). The length of these fragments vary from 500 to 2 000 base pairs for ESTs and cDNAs amplified by PCR (Lemieux *et al.*, 1998), to full-length cDNAs (Rockett and Dix, 2000). Small amounts of these fragments are delivered exogenously onto the solid supports (Schena *et al.*, 1998). The success of this technique largely depends on preparing pure RNA and, subsequently, pure cDNA (Lennon and Lehrach, 1991). When the cDNA fragments to be arrayed are identified these fragments are amplified by PCR in a 96-well microtiter plate (Schena *et al.*, 1995). cDNA microarrays are assembled by using either one of two distinct deposition techniques. The first is known as a contact (Rockett and Dix, 2000) or passive (Van Hal *et al.*, 2000) deposition method. This technique mainly makes use of a mechanical microspotter consisting of pins, tweezers or capillaries as utensils to deliver the DNA onto the glass slides (Lemieux *et al.*, 1998). The second technique is based on a non-contact (Rockett and Dix, 1999) or active (Van Hal *et al.*, 2000) principle. Here, ink-jet nozzles deliver the DNA onto the solid support (Lemieux *et al.*, 1998).

The cDNA or RNA to be hybridised with the immobilised probes, i.e. the target, is labelled with fluorescent tags through the incorporation of labelled oligonucleotides (Lee and Lee, 2000). The target population is generally labelled with two different fluorescent dyes (Schena *et al.*, 1995). This allows the comparison of expression levels of two differently treated RNA populations (Gerhold *et al.*, 1999). The immobilised cDNA is heat denatured before hybridisation with the single-stranded target population commences (Schena *et al.*, 1995). Thereafter, laser scanners are used to excite the fluorescent tags and the hybridisation images can be detected (Schena *et al.*, 1995).

cDNA microarrays are primarily tools for gene expression analysis (Schena *et al.*, 1998) and allow the comparison of expression levels of genes of two different cell or tissue types, or differently treated tissues (Jones and Fitzpatrick, 1999). Further, once the expression levels of a certain gene are determined the function of that gene can be established (Schena *et al.*, 1998). The advantage of measuring gene expression levels by using microarrays is that thousands of genes can be examined simultaneously on one glass slide (Rockett and Dix, 1999). Since ESTs can be used as probes during cDNA microarray analysis, expression levels of unknown genes can also be determined (Rockett and Dix, 1999). Thus, these genes can be characterised in order to determine their significance in a specific biological system (Rockett and Dix, 2000).

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Chapter 2

Functional sequence assessment in wheat (*Triticum aestivum*)

The data presented in Chapter 2 have been included in a journal publication entitled:

Functional and comparative analysis of expressed sequences from *Diuraphis noxia* infested wheat obtained utilizing the conserved Nucleotide Binding Site.

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1. ABSTRACT

Understanding the basic biological functions and interactions between plant proteins is imperative for investigations regarding plant reactions to and interactions with its environment. This comprehension is applicable when investigating important food crops such as wheat (*Triticum aestivum* L.) and its resistance mechanisms against pests and pathogens. A pest infesting wheat crops in South Africa is the Russian wheat aphid (RWA; *Diuraphis noxia* Mordvilko). Several resistance genes against the RWA have been identified (*Dn1* to *Dn9* and *Dnx*) and this study utilises the cultivar PI 137739 that contains the *Dn1* resistance gene. The strategy employed involved the utilisation of nucleotide binding site (NBS)-specific primers as a means to identify resistance-related fragments in RWA infested wheat. The nucleotide binding site-domain is a constituent of the NBS-LRR class of resistance genes in plants that is considered to be the largest class of resistance gene sequences and it has been shown that this domain is generally well-conserved between plants species. Identification of resistance-related fragments was attempted through the use of PCR-based methods with the NBS-specific primers, as well as DNA sequencing of both genomic and complementary DNA (cDNA). The gene fragments obtained were analysed using the BLASTN annotation and classified into groups according to their respective functions. The data obtained from the genomic DNA and cDNA approaches were analysed individually and collectively. According to the combined data the largest group of fragments identified consists of metabolic sequences (30%), 28% are related to protein synthesis, 10% to regulatory functions, only 4% is involved with structural functions, 18% are classified as miscellaneous and 10% of the fragments obtained are resistance-related. The resistance fragments exhibited homology to resistance gene analogues (RGAs), leucine-rich repeat regions, receptor-like kinases and a sequence homologous to a WIR pathogen resistance gene was identified.

2. INTRODUCTION

In nature, plants are continually under attack from various pests and pathogens. These attacks trigger constitutive or induced defence responses in plants that exhibit resistance to such attacks (Stotz *et al.*, 2000). The ceaseless battles between plants and their enemies necessitate the understanding and identification of resistance responses. One important enemy of bread wheat is the Russian wheat aphid (RWA; *Diuraphis noxia* Mordvilko). It is speculated that the RWA is native to southern Russia (Robinson, 1992), Iran, Afghanistan, and countries surrounding the Mediterranean Sea (Walters *et al.*, 1979). Since 1900, the RWA has infested wheat and barley crops in Spain (Robinson, 1992), Africa (Pakendorf, 1984), South America and Mexico (Gilchrist *et al.*, 1984), as well as Canada and North America (Stoetzel, 1987). The RWA has infested wheat crops in South Africa since 1978 (Walters *et al.*, 1979) and has caused considerable yield losses since then.

Bread wheat (*Triticum aestivum* L. em. Thell.) is one of the most important cereal crops in the world. It is an easily accessible source of food (Brettell and Murray, 1995) and nutrition (Vasil *et al.*, 1992), and wheat end products have a wide range of applications (Snape, 1998). Bread wheat as it is known to mankind today, has, in comparison with other plant species, an extremely large and complex genome (Devos and Gale, 1993). This hexaploid species (with a chromosome number of 42) has an AABBDD genome composition (Kimber and Sears, 1987). Various analytical studies conducted on the wheat genome revealed that 85% of the genes are present in only 10% of the chromosomal regions (Sandhu *et al.*, 2001). The extent of this large genome complicates the search for specific genes, especially resistance genes.

Today, molecular biology provides powerful tools for the investigation of complex genomes such as that of wheat. The synthesis of complementary DNA (cDNA), the polymerase chain reaction (PCR), sequencing and molecular cloning

are only some of these tools. A closer look at cDNA reveals that these molecules provide the gateway to unlocking the information contained within a specific gene (Alexander *et al.*, 1984). Since the analytical value of cDNA has been realised, various approaches have been developed for the synthesis and analysis of these informative molecules (Lapeyre and Amalric, 1985). During the synthesis of cDNA, for example, various factors have to be taken into consideration. One interesting consideration is the generation of hairpin loops during first strand synthesis at the 3' end of the mRNA: cDNA complex (Berger and Kimmel, 1987). Okayama and Berg (1982), for example, have developed S1-nuclease-mediated cleavage of the hairpin loop before synthesising second strand cDNA, where other scientists choose not to include such procedures (Land *et al.*, 1981). Access to the information enclosed within the cDNA has been made possible through the process of molecular cloning of such cDNA fragments (Gubler and Hoffman, 1983). An important aspect when cloning cDNA fragments is the vector to be used. Lapeyre and Amalric (1985) have used the λgt11 vector, where Ogihara and his colleagues (1998) successfully used pGEM-T vectors to clone wheat cDNA. Molecular cloning of genes, including disease resistance genes, has increased dramatically since it was first reported. A few examples of cloned resistance genes are *HM1* in maize (Johal and Briggs, 1992), *L6* in flax (Lawrence *et al.*, 1995), *Xa21* in rice (Song *et al.*, 1995), *N* in tobacco (Witham *et al.*, 1994), *Cf-2* in tomato (Dixon *et el.*, 1996), HS^{pro-1} in sugar beet (Cai *et al.*, 1997) and *RPS2* in *Arabidopsis* (Tao *et al.*, 2000).

A closer look at plant resistance genes has revealed some interesting information about the nucleotide composition of these genes. Many resistance (R) genes have been shown to be assembled in clusters (Jones *et al.*, 1993; Parniske *et al.*, 1997) and contain conserved nucleotide binding site (NBS) and leucine rich repeat (LRR) domains (Rivkin *et al.*, 1999; Halterman *et al.*, 2001). Generally, a low sequence homology is noticeable between NBS-LRRs, but some regions of these domains have been well conserved between plant species (Hammond-Kosack and Jones, 1997). It is these conserved areas that inspired

researchers to search for resistance gene analogues (RGAs) contained within plant genomes through PCR-based approaches (Leister *et al.*, 1996). These approaches utilise degenerate primers in order to amplify RGAs from genomes of diverse dicotyledonous and monocotyledonous plant species (Kanazin *et al.*, 1996; Leister *et al.*, 1996; Yu *et al.*, 1996). The process of targeting and amplifying specific gene regions through the utilisation of DNA markers has been termed chromosome landing (Tanksley *et al.*, 1995). Applying NBS-specific degenerate primers in the search for resistance gene analogues has been also been categorised as chromosome landing (Wang *et al.*, 2000).

Combining various molecular techniques serve as strategy in analysing certain scientific questions. Richard and his colleagues (1995), e.g., examined the differences between two repetitive DNA sequences using PCR and single-run partial sequencing. During this study, PCR-based strategies and DNA sequencing were employed in the search for gene fragments linked to RWA resistance in wheat. Specifically, the identification of gene fragments exhibiting homology to nucleotide binding site (NBS) sequences was attempted. This was achieved by using nucleotide binding site primers in order to amplify specific fragments. Complementary, as well as genomic DNA, was used as templates in order to compare the difference in the functional gene expression between Russian wheat aphid-infested and uninfested plants. The acquired fragments were PCR-amplified, cloned and sequenced in order to determine their possible relation to NBS sequences. Further, a general idea of the proportion of constitutive genes that are expressed during plant infestation, were also assessed. A hard spring wheat, PI 137739, that originated in Iran (Du Toit, 1987) was used as study material. PI 137739 is known to exhibit antibiotic and antixenotic resistance against the RWA and contains a single, dominant, independently inherited gene, *Dn*1 (Du Toit, 1989). Therefore, it was expected that sequences related to plant resistance fragments would be obtained.

3. MATERIALS AND METHODS

3.1 Wheat material

The wheat cultivar used during this study was PI 137739, which is known to contain the *Dn1* resistance gene (Du Toit, 1987). Wheat seed was obtained from the wheat germplasm source at the United States Department of Agriculture (USDA) Small Grains Repository, Aberdeen, Idaho, USA. The seed were planted in a sand: peatmoss (1:3; v/v) soil mixture, kept under consistent greenhouse conditions at 25 °C and watered daily. Infestation with Russian wheat aphids was performed at the second leaf-growth stage of the wheat plants; five aphids were scattered on each plant (Van der Westhuizen and Pretorius, 1996).

3.2 Primer design

The degenerate oligonucleotide primers used during this study were designed according to the amino acid sequences of two highly conserved nucleotide binding sites motifs of the tobacco *N* and *Arabidopsis RPS2* genes (Yu *et al.*, 1996). NB1 [5'-GGA-ATG-GG(AGCT)-GG(AGCT)-GT(AGCT)-GG(AGCT)-AA(AG)-AC-3'] and NB2 [5'-(CT)CT-AGT-TGT-(AG)A(CT)-(AGT)AT-(AGT)A(CT)-(C T)(CT)T-(AG)C-3'] primers were synthesised.

3.3 Genomic DNA analysis using NBS primers

Genomic DNA was extracted from 10-day old, uninfested wheat leaves using the monocot DNA isolation technique (Edwards *et al.*, 1991). The leaf tissue was homogenised in liquid nitrogen, 600 µl extraction buffer (200 mM Tris-HCl, 150 mM NaCl, 25 mM EDTA and 0.5% (w/v) SDS; pH 8.0) was added and mixed thoroughly. An incubation period of 30 minutes at 60 °C followed, with interruptions every 5 minutes when the tubes were inverted. 600 µl Chloroform/isoamylalcohol (24:1) was added, the solution was mixed

vigorously and subsequently centrifuged at 10 000 rpm for 15 minutes. The upper phase was removed and transferred to a sterile eppendorf tube. Two volumes of ice cold 95% (v/v) EtOH were added and the DNA was allowed to precipitate at -20 °C overnight. After precipitation, the DNA was scooped out and washed with 70% EtOH for 10 to 15 minutes. The wash step was repeated two more times, the extracted DNA resuspended in 200 µl double distilled (dd) H_20 and stored at -20 °C. DNA quantification was carried out on a Beckman DU® - 64 spectrophotometer at an absorbance of 260 nm.

The DNA was used in subsequent PCR reactions using the degenerate NB1 and NB2 primers that were designed according to the conserved NBS consensus region (Yu *et al.*, 1996). The PCR reaction consisted of 10 x PCR buffer (10 mM Tris-HCl, 50 mM KCl and 0.1% (v/v) Triton® X-100; pH 9.0), 25 mM $MgCl_2$, 200 µM of each dNTP, 10 pmol each of NB1 and NB2, and 1.25 U *Taq* DNA polymerase (Promega Corporation, USA) in a 25 µl reaction. A GeneAmp® PCR System 9700 (Applied Biosystems, USA) was used for all PCR reactions. The PCR reaction commenced at 94 °C for 5 minutes, was followed by 30 amplification cycles (1 minute at 94 °C, 1.5 minutes at 55 °C and 1 minute at 72 °C) and ended at 72 °C for 7 minutes. The PCR reactions were visualised on a 1% (w/v) low-melting agarose gel, the fragments were excised and cleaned using the Geneclean III Kit (Southern Cross Biotechnology, USA). Thereafter, the purified fragments were cloned into pGEM-T Easy Vectors (Promega Corporation, USA), containing SP6 (5'-TAT-TTA-GGT-GAC-ACT-ATA-G-3') and T7 (5'-TAA-TAC-GAC-TCA-CTA-TAG-GG-5') RNA polymerase promotors. The cloned products were transformed into *Escherichia coli* cells (JM 109 High Efficiency Competent Cells; $>10^8$ cfu/µg) and plated on LB plates containing ampicilin (2 µg/ml), IPTG (0.25 g/ml) and X-gal (20 µg/ml). Blue/white screening was used in order to assess transformation efficiency.

White colonies were used for further analyses. Positively transformed colonies were randomly selected and PCR-amplified on a GeneAmp® PCR System 9700 (Applied Biosystems, USA) using the colony PCR method (Gussow and Clackson, 1989). This PCR reaction consisted of 10 x PCR buffer (10 mM Tris-HCl, 50 mM KCl and 0.1% (v/v) Triton® X-100; pH 9.0), 2 mM of each dNTP, 25 mM $MgCl_2$ and 10 pmol each of SP6 and T7 primers and 0.5 U *Taq* DNA polymerase in a 5 µl reaction. Positively transformed colonies were selected and placed in the PCR reaction mixture. The PCR reaction involved 25 cycles of 30 seconds at 94 °C, 30 seconds at 50 °C and 1 minute at 72 °C, and a final step of 5 minutes at 72 °C. Once these PCR products were purified (Geneclean III Kit, Southern Cross Biotechnology, USA), they were sequenced and analysed. The sequencing reaction contained 250 ng purified DNA, 2 µl Terminator Ready Reaction Mix (BigDye Terminator Sequencing Reaction Kit, Perkin-Elmer) and 3.2 pmol SP6 or T7 primer in a 5 µl reaction. Sequencing was performed on an ABI 3100 Prism Automated Sequencer (Perkin-Elmer) and analysis of the obtained fragments was carried out using the Basic Local Alignment Search Tool (BLAST; Altschul *et al.*, 1990) at http://www.ncbi.nlm.nih.gov/BLAST/.

3.4 Screening of a wheat genomic DNA library

A genomic, λ phage library constructed from the hexaploid wheat cultivar, Soleil, obtained from the John Innes Centre (Norwich, United Kingdom), was screened for gene fragments linked to Russian wheat aphid resistance. The library was constructed according to the Lambda FIX II Library Instruction Manual (Stratagene, USA). It consisted of approximately 2.4 million clones and was divided into five fractions. 1 µl of each of the five fractions of the genomic library was diluted in 9 µl SM buffer (100 mM NaCl, 10 mM $MgSO_4 \cdot 7H_2O$, 50 mM Tris-HCl, 0.1% (w/v) gelatine; pH 7.5), of which 1 µl was transformed into 200 µl XL1-Blue MRA (P2; Stratagene, USA) competent cells (OD_{600} = 0.5). Each transformed bacterial suspension was added to 6 ml top agar, plated on NZY plates (100 mM NaCl, 10 mM $MgSO_4$,

2% (w/v) Yeast Extract, 10% (w/v) casein hydrolysate, 15 g agar; pH 7.5) and incubated overnight at 37 °C.

Plaque lifts were performed subsequently (Sambrook *et al.*, 1989). The plates were incubated at 4 °C for two hours prior to plaque lifting in order to prevent agar from adhering to the nitrocellulose membranes. Transfer of the plaques onto the nictrocellulose membranes was carried out for 1 minute and then membranes were placed in denaturing solution for 5 minutes. The filters were transferred to a neutralising solution for 5 minutes and thereafter rinsed in 2 X SSC (3 M NaCl, 0.3 M sodium citrate; pH 7). The filters were air-dried and the DNA was fixed by exposure to UV light for 10 minutes at 0.15 Joules per second (Sambrook *et al.*, 1989).

The genomic library was screened using a fragment obtained during amplification of genomic DNA with NBS primers that displayed high homology to a putative resistance gene, RGA2 (Wicker *et al.*, 2001). Probe quantification was carried out and the fragment was labelled with digoxigenin-11-dUTP according to the manufacturer's instructions (DIG High Prime DNA Labelling and Detection Starter Kit I, Roche Molecular Biochemicals, Germany). 1 µg DNA was labelled overnight at 37 °C. Hybridisation of the labelled, denatured probe with the fixed genomic DNA on the nictrocellulose membranes was performed overnight at 50 °C. After the membranes were washed, probed with an antibody solution and equilibrated, they were incubated in 10 ml colour substrate [50 x stock solution; nitroblue tetrazolium chloride (18.75 mg/ml) and 5-bromo-4chloro-3-indolyl-phosphate (9.4 mg/ml); 2:1] and allowed to develop overnight in a dark container. The colour reaction was terminated by rinsing the membranes in 50 ml sterile double-distilled water (DIG High Prime DNA Labelling and Detection Starter Kit I, Roche Molecular Biochemicals, Germany).

3.5 Treatment of glassware, plastic ware and solutions

All glassware, plastic ware and solutions used during RNA isolation and first strand cDNA synthesis were treated with diethyl pyrocarbonate (DEPC) in order to remove all RNases. Glassware was treated overnight in 0.1% (v/v) DEPC, autoclaved for 20 minutes and baked at 200 °C overnight (Sambrook *et al.*, 1989). Mortars and pestles were rinsed with 0.25 M HCl and treated similarly as the glassware. The plastic ware and solutions were DEPC treated and autoclaved. Solutions containing Tris (2-Amino-2-(hydroxymethyl)-1, 3-propandiol) were not DEPC treated, but only autoclaved.

3.6 cDNA synthesis and cloning using the pGEM-T Easy Vector System

Total RNA was extracted from infested PI 137739 wheat plants using the second and third leaves, two to five days after infestation (Botha *et al.*, 1998). The RWAs were rinsed from the wheat leaves and the plant material was homogenised using liquid nitrogen. RNA was extracted according to the guanidine thiocyanate method of Chomczynski and Sacchi (1987). RNA extraction buffer (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% (w/v) N-laurocylsarcosine and 100 mM 2-mercaptoethanol) was added to the homogenised tissue in a 3:1 ratio. This mixture was placed at room temperature for 10 minutes and subsequently centrifuged at 10 000 rpm for 20 minutes. The supernatant was added to 50 µl 2 M NaOAc (pH 4) and 500 µl phenol:chloroform (1:1), and placed at room temperature for 10 minutes. After centrifugation at 10 000 rpm for 10 minutes, the supernatant was added to an equal volume of isopropanol. Total RNA was precipitated for 1 hour at -20 °C, pelleted at 13 000 rpm for 30 minutes and thereafter washed with 70% (v/v) EtOH. The RNA was subsequently air dried, resuspended in 20 µl DEPC-treated water and stored at -80 °C. RNA concentration was determined on a Beckman DU® - 64 spectrophotometer by reading the absorption at 260 nm. Protein contamination was determined by measuring the 260/280 nm ratios. RNA integrity was also

confirmed through visualising the RNA on a 2% (w/v) agarose gel containing ethidium bromide on UV light. The molecular mass standard used was lambda DNA restricted with Eco R1 and Hind III (Sambrook *et al.*, 1989).

mRNA was purified from total RNA using Oligo(dT)-cellulose columns supplied with the mRNA Purification Kit (Amersham Pharmacia Biotechnology USA). cDNA synthesis was carried out according to the manufacturer's instructions (cDNA Synthesis System, Roche Molecular Biochemicals, Germany). 1 μg mRNA was used as template and Pr 16 (5'-TTT-TGT-ACA-AGC-TT_{30} -3'; 200 pmol) was used as primer in order to synthesise the first strand cDNA. Thereafter, second strand cDNA was synthesised, the resulting cDNA was cloned into pGEM-T Easy Vectors, transformed into *E. coli* cells (JM 109; $>10^8$ cfu/μl) and blue/white screening was carried out in order to determine transformation efficiency. The white colonies were screened during subsequent PCR reactions and sequenced as described above.

3.7 cDNA synthesis and cloning using the GeneRacer System

Wheat plants were infested with Russian wheat aphids as described above. RNA was extracted from the infested plants using Chomczynski and Sacchi's (1987) RNA extraction method. Total RNA (2.5 μg) was used as template for cDNA synthesis. Single stranded cDNA was synthesised according to the GeneRacer Kit Instruction Manual (Invitrogen Corporation, The Netherlands). Second strand cDNA was generated by using the GeneRacer 5' (5'-CGA-CTG-GAG-CAC-GAG-GAC-ACT-GA-3') primer and the nucleotide binding site primer, NB1. The PCR reaction consisted of 100 ng first strand template, 10 pmol of each primer, 10 mM of each dNTP, 10 x reaction buffer (10 mM Tris-HCl, 50 mM KCl and 0.1% (v/v) Triton® X-100; pH 9.0), 25 mM $MgCl_2$ and 5 U Expand High Fidelity DNA polymerase (Roche Molecular Biochemicals, Germany) in a 50 μl reaction. The PCR reaction was assembled as follows: an initial step of 2 minutes at 94 °C was followed by the first 5 cycles (30 seconds at 94 °C and 1 minute at 72 °C); secondly 30

seconds at 94 °C, 30 seconds at 70 °C and 1 minute at 72 °C was repeated 5 times; lastly 30 seconds at 94 °C, 30 seconds at 68 °C and 1 minute at 72 °C was repeated 20 times. An extension at 72 °C for 10 minutes completed the PCR reaction.

A second PCR reaction was performed using the initial PCR reaction as template. This reaction contained 10 pmol each of the GeneRacer 5' nested primer [5'-GGA-CAC-TGA-CAT-GGA-CTG-AAG-GAG-TA-3'] and the NB2 primer [5'-(CT)CT-AGT-TGT-(AG)A(CT)-(AGT)AT-(AGT)A(CT)-(CT)(CT)T-(AG)C-3'], 1 µl primary PCR template, 10 x reaction buffer (10 mM Tris-HCl, 50 mM KCl and 0.1% (v/v) Triton® X-100; pH 9.0), 25 mM $MgCl_2$, 10 mM of each dNTP and 5 U Expand High Fidelity DNA polymerase (Roche Molecular Biochemicals, Germany) in a 50 µl reaction. The PCR reaction commenced at 94 °C for 1 minute, was followed by 20 cycles (94 °C for 30 seconds, 68 °C for 30 seconds and 72 °C for 1 minute) and was completed at 72 °C for 10 minutes.

The PCR products were separated on a 1% (w/v) low-melting agarose gel and visualised on UV light using ethidium bromide. The fragments were purified using the Geneclean III Kit (Southern Cross Biotechnology, USA).

The acquired blunt-ended fragments were cloned into the pCR® 4blunt-TOPO® vector (Zero Blunt TOPO PCR Cloning Kit for Sequencing, Invitrogen Corporation, The Netherlands). 2.5 ng/µl of the PCR product was used for each cloning reaction. Cloning was performed according to the manufacturer's instructions and the cloned products were transformed into One Shot Chemically Competent *E. coli* cells ($DH5\alpha^{TM}$-$T1^{R}$; $>10^9$ cfu/µl). The transformed cells were plated on LB plates containing IPTG (0.25 mg/ml), X-gal (20 µg/ml) and kanamycin (50 µg/ml). Blue/white screening was employed to access transformation efficiency and to select clones for subsequent sequencing and analyses.

4. RESULTS

4.1 Genomic DNA analysis using NBS primers

Transformation efficiencies of the cloned NBS PCR fragments from total genomic DNA ranged from 40 – 60%.

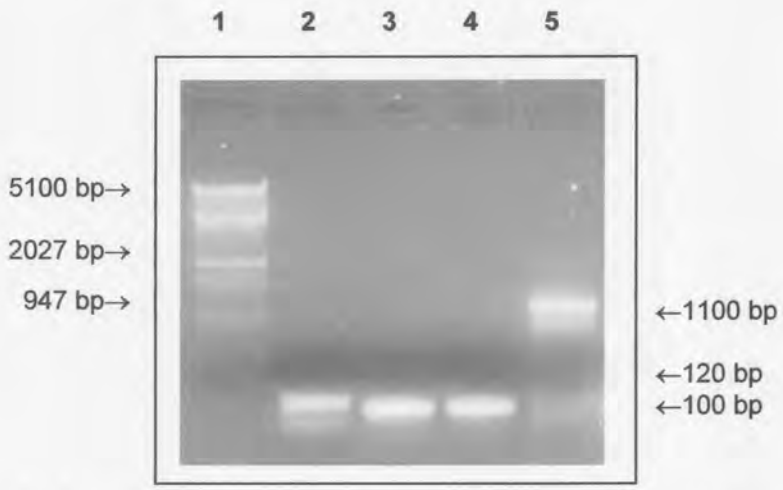

Figure 2.1 Fragments obtained after PCR of genomic DNA with NB1 and NB2 primers. Lane 1: molecular marker III (λ DNA restricted with Eco RI and Hind III); lanes 2 – 5: NBS fragments to be purified, cloned and sequenced.

After single-pass sequencing was performed, the vector sequences were removed and the sequences were analysed. The length of the sequences varied from 100 to 1100 base pairs. The attempt to isolate gene fragments from total genomic DNA belonging to the nucleotide binding site family yielded 10 fragments of which the data was used in this study. These fragments exhibited homology to 44 NCBI (National Centre for Biotechnology Information) data base ESTs (dbESTs). Of the 44 homologies, six exhibited correspondence to regions of a resistance gene analogue (RGA2; Fig. 2.2; Wicker *et al.*, 2001) and two exhibited correspondence to a leucine rich-like protein (Fig. 2.3; Anderson *et al.*, 2002). The remaining homologies consisted of 19% protein synthesis sequences, 34% metabolic sequences, 11% structural sequences, 7% regulatory sequences and 11% sequence classified as miscellaneous. Eighteen percent of the obtained sequences were related to resistance sequences (Table 2.1).

```
S:211       ttgtccctaggcaatcaataactagaacggtaatcattcttgcaattttatatcagggagaggcatgagctaaaatact 290
                      * *         *         * *          * *       *     *       *
Q:189162    ttgtccctaggcactcgataactagaccggtaatcactattgcaatttttatttgagggagtggcataagctaaca tact 189241


S:291       ttcattacttggatcatacgcacttatgattcgaactctagcaagcatccgcaactaccaaagatcattaaggtaaaac 368
              ***           *           *                                  *
Q:189242    ttctcttcttggatcatatgcacttatgattggaactctagcaagcatccgcaactactaaagatcattaaggtaaaac 189319


S:369       ccaaccagagcatt-aagtatcaagtcctcttta 401
                    *       *  *
Q:189320    ccaaccatagcattaaaatatcaagtcctcttta 189353
```

Figure 2.2 A sequence generated with the NBS primers (S) exhibiting 90% nucleotide homology to a resistance gene analogue (RGA2; Q) sequence when analysed using the BLASTN annotation. The length of the sequence = 190 bp, the score of the sequence = 222 bits, the e-value = 3e-55 and the proportion gaps = 1/192 (0%) (Wicker *et al.*, 2001). S = subject, Q = query, * represents single nucleotide polymorphisms (SNPs).

```
S:39        tctcacatcaactaggcgtatcaacgggctatggagatgcccattaatagatatcaatgtgagtgagtagggattgccat 118
                                    *                 *
Q:24296     tctcacatcaactaggcgtatcaat gggctatggagatgcccatcaatagatatcaatgtgagtgagtagggattgccat 24257


S:119       gcaacagatggactagagctataaatatatgaaagctcaacaaaagaaactaagtgggtgtgcatccaac188
                      *               * * *           *
Q:24258     gcaacagatgcactagagctataagtgtacgaaagctca--aaaagaaactaagtgggtgtgcatccaac 24208


S:189       ttgcttgctcatgaagacctagggcattttgaggaagcccatcgttggaatatacaagccaagttctata 258
                                          *             *
Q:24209     ttgcttgctcatgaagacctagggcatttt –aggaagcccattgttggaatatacaagccaagttctata 24157


S:259       atgaaaaatccccactagtatatgaaagtgataacatgagagactctctactatgaatatcatggtgctacatttgaagc 338
                  *                         * *  *  *    *         *       *              *
Q:24158     atgaaaagttcccactagtatatgaaagttac aaaataagaaactctctatcatgaagatcatggtgctac-tttgaagc 24001


S:339       acaagtgtggtaaaaggatagtaacattg 367
                      *
Q:24000     acaagtgtggaaaaaggatagtaacattg 23972
```

Figure 2.3 A sequence generated with the NBS primers (S) exhibiting 93% nucleotide homology to a leucine rich-like protein (Q) when using the BLASTN annotation. The length of the sequence = 328 bp, the score of the sequence = 456 bits, the e-value = e-125 and the proportion gaps = 4/329 (1%) (Anderson *et al.*, 2002). S = Subject, Q = Query, * represents single nucleotide polymorphisms (SNPs).

4.2 Screening of a wheat genomic library

Probe quantification of the nucleotide binding site fragment confirmed that DNA concentrations of 3 pg/µl to 1 ng/µl are sufficient for labelling with DIG-11-dUTP. The colour reaction following hybridisation was completed after 14 hours. However, screening a genomic library, especially one as large and complicated as that of wheat, requires an accurate and specific approach. The fraction sample that was screened during this study contained a very high amount of amplified clones (approximately 2.4 million clones). Although the NBS fragment could be labelled successfully, hybridisation of the library fraction was ineffective. In order to conduct a more thorough investigation of wheat genomic DNA it would be advisable to screen each sample of each fraction individually with the labelled fragment.

4.3 cDNA synthesis and cloning using the pGEM-T Easy Vector System

A cDNA library from RWA-infested wheat RNA was constructed and an average cDNA insert size of 1 kb was obtained. cDNA transformation into the pGEM-T Easy Vectors yielded a transformation efficiency of 40 – 50%. However, the amount of ESTs generated from the cloned cDNA fragments after single-run partial sequencing was low. Only nine cDNA ESTs were obtained, but they exhibited homology to a high number of NCBI dbESTs, namely 55. The homological data were used for further classification of the acquired sequences. 22% of the homologies displayed similarity to protein synthesis sequences, 16% to metabolic sequences, 22% to regulatory sequences and 40% exhibited homology to sequences with miscellaneous attributes (Table 2.1). Sequences that exhibit similarity to resistance gene analogues or other sequences thought to be involved in plant resistance could not be isolated from the constructed cDNA library.

4.4 cDNA synthesis and cloning using the GeneRacer System

Utilising the GeneRacer approach in synthesising and cloning cDNA yielded a higher percentage of significant information than the pGEM-T Easy Vector Sytem. Although the nucleotide binding site primers were utilised during the analysis of genomic, as well as complementary DNA, these primers seemed to be more informative when used as a gene-specific primer in the GeneRacer system. The transformation efficiency of the TOPO cloning system, however, was significantly lower than the efficiency of the pGEM-T Easy Vector System. Approximately 80 to 100 colonies per plate were obtained, with a transformation efficiency of 30 – 40%. The average length of the sequence obtained, however, ranged from 200 bp to 1 950 bp.

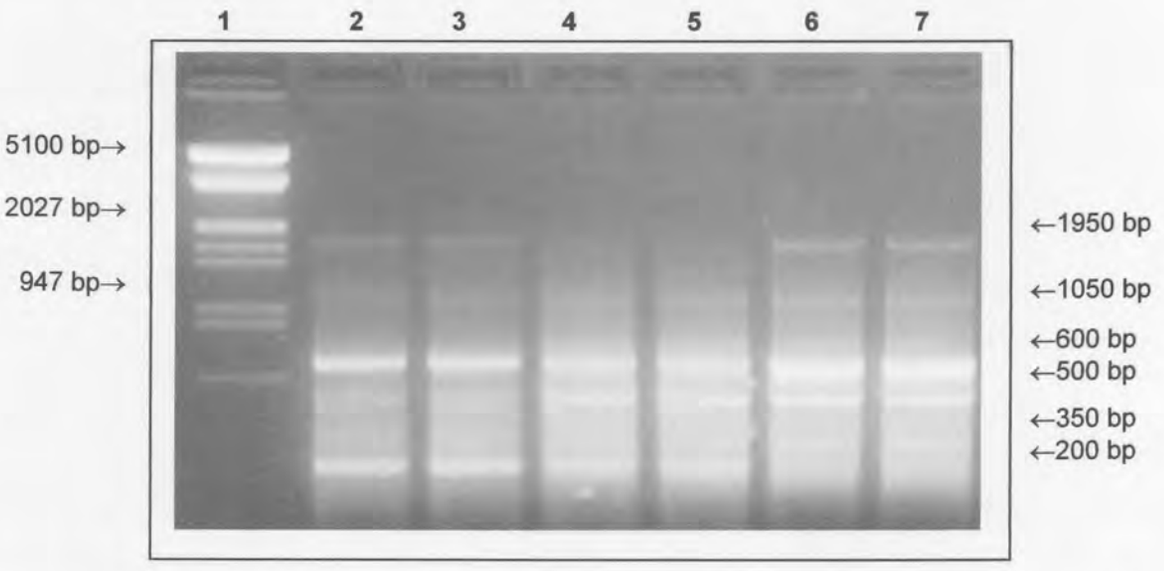

Figure 2.4 GeneRacer fragments obtained after secondary PCR separated on a 1% agarose gel. Lane 1: molecular marker III (λ DNA restricted with Eco RI and Hind III); lanes 2 – 7: samples of secondary GeneRacer PCR.

Although only ten EST fragments were obtained from the GeneRacer procedure, these fragments exhibited homology to 58 NCBI dbESTs. Sequences displaying homology to protein synthesis sequences (40%), metabolic sequences (42%) and miscellaneous sequences (6%) were obtained. Two sequences

displayed homology to a leucine rich repeat (LRR19; Feuillet *et al.*, 2001), three sequences displayed similarity to the WIR pathogen resistance gene (Franck and Dudler, 1995) and two sequences were homologous to a receptor-like kinase fragment (Feuillet *et al.*, 2001). The sequence homological to the WIR pathogen resistance gene was also translated into its amino acid configuration and confirmed its similarity to this pathogen resistance gene (Fig. 2.5). The sequences related to plant resistance genes, constituted 12% of the total amount of fragments obtained (Table 2.1).

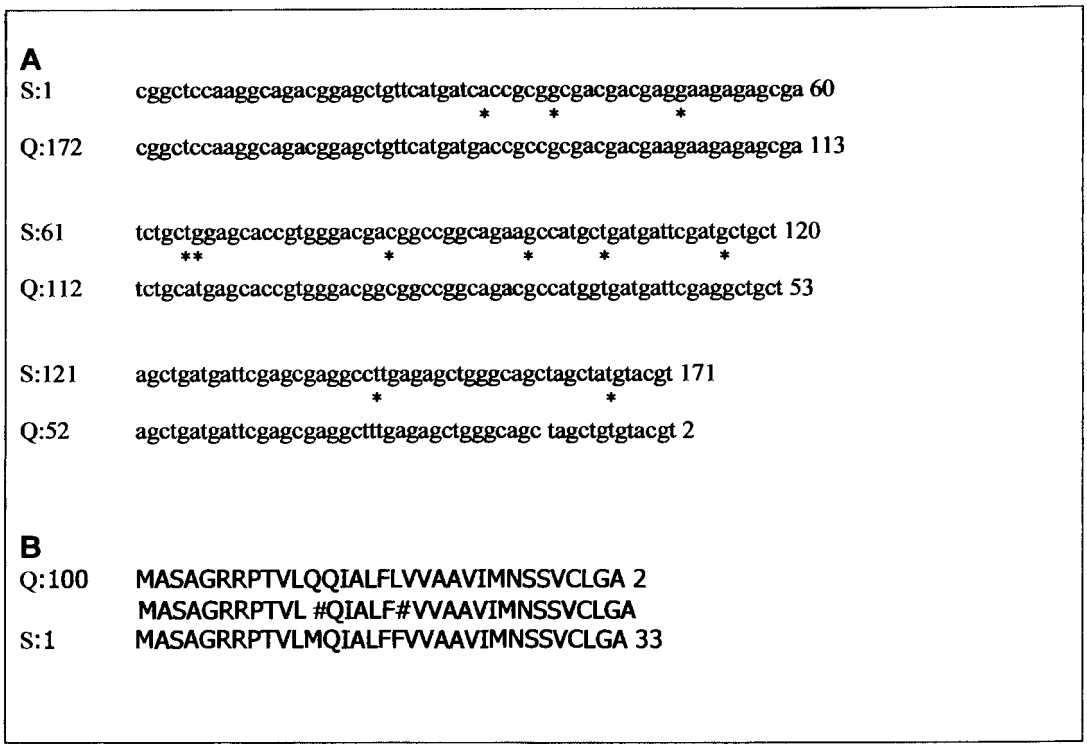

Figure 2.5 A sequence obtained from a GeneRacer fragment (S) exhibiting 93% homology to the WIR pathogen resistance gene (Q; Franck and Dudler, 1995). **A** represents the nucleotide homology obtained using the BLASTN annotation and **B** represents the amino acid homology using the BLASTX annotation. The length of the nucleotide sequence = 171 bp, the score of the sequence = 252 bits and the e-value = 2e-64. The score of the amino acid annotation = 60.8 bits and exhibits 93% homology. S = subject, Q = query, * represent single nucleotide polymorphisms (SNPs), # represents amino acid differences.

4.5 Sequence analysis

All the sequences obtained during the present study were compared to the sequences of previously identified dbESTs in order to determine their putative identities and functions using the BLASTN (nucleotide homology) algorithm (Altschul *et al.*, 1990). Putative functions were assigned to the obtained EST fragments according to their nucleotide homologies, where sequences with a maximum probability threshold of less than 10^{-3} were considered for further data analysis (Table 2.1). Several sequences exhibiting E-values larger than 10^{-3} were also listed for completeness, but not included in the data analysis. The obtained fragments were classified into functional groups and the data presented as a distribution of the total amount of ESTs evaluated (Fig. 2.6).

The protein synthesis sequences encompass the mitochondrial and chloroplast ribosomal RNA sequences, the S7 and S13 ribosomal fragments, the actin gene sequences, the partial mRNA 3' UTR (untranslated region) sequences and the high molecular weight (HMW) gene sequences. The metabolic ratio is represented by the Rubisco small subunit sequences, chloroplast genes, aldehyde dehydrogenases, serine hydroxymethyl transferases and ATP synthase β subunit sequences. The regulatory sequences consist of the T-DNA integration factors, chromosome condensation factors and topoisomerases. The structural ratio is represented by the tonoplast DNA and the noduline like-like protein sequences. A fifth class was included namely miscellaneous sequences (long terminal repeats, retrotransposon-related sequences and microsatellite fragments). The resistance fraction contains sequences coding for a resistance gene analogue (RGA2; Wicker *et al.*, 2001), receptor-like kinases (Feuillet *et al.*, 2001), leucine-rich repeats (LRR19; Feuillet *et al.*, 2001) and the WIR pathogen resistance gene (Franck and Dudler *et al.*, 1995).

Table 2.1 Data of sequence analyses displaying the organisms and proteins to which homology was obtained, the GenBank accession numbers of the sequences to which homology was obtained in brackets, the method employed in generating the gene fragments and the average expectancy (E) value. Values in the accession number column indicate the accession numbers of the sequences from this study submitted into GenBank.

Accession numbers[a]	BLASTN annotations	Number of homologous ESTs cDNA[b]	NBS[c]	GR[d]	Average E-value cDNA[b]	NBS[c]	GR[d]
CA407987	Mitochondrial 26S rRNA: *Triticum aestivum* (Z11889)	2		6	0.0		0.0
CA407988	*Zea mays* (K01868)	1		4	1.0		0.0
CA407993	*Beta vulgaris* (AP000397)	2		2	1.0*		3e-23
	Arabidopsis thaliana (Y08501)	1		2	0.0		e-116
	Mitochondrial DNA: *Secale cereale* (Z14059)			2			4e-13
	Mitochondrial 18S rRNA: *Zea mays* (X00794)			4			0.0
	Mitochondrial 23S rRNA: *Beta lupini* (X87283)	1			2e-87		
	Mitochondrial 16S rRNA: *Beta japonicum* (Z35330)	2			2e-87		
	SSU[e] rRNA: *Drimys winteri* (AF197162)			1			3e-04
	Tetracentron sinense (AF193998)			1			3e-04
	Grevillea robusta (AF193995)			1			3e-04
	Trochodendron aralioides (AF161092)			1			3e-04
	Nelumbo nucifera (AF193983)			1			$1e^{-3}$
	Platanus occidentalis (AF161090)			1			0.085*
	S7 ribosomal fragment: *Triticum aestivum* (X67242)			1			$1e^{-3}$
	S13 ribosomal fragment: *Triticum aestivum* (Y00520)			1			$5e^{-3}$
CA407992	Chloroplast DNA: *Triticum aestivum* (AB042240)	2	2		e-164	0.0	
	Oryza sativa (X15901)	2			7e-13		
	Zea mays (X86563)	1	2		4e-17	0.0	
	Hordeum vulgare (X00408)		1			0.0	
	Chloroplast DNA for 4.5S rRNA: *Zea mays* (X01365.1)	1			8e-08		
	Chloroplast gene for P700 protein: *Anthoceros* (AB013664.1)	2	1		2e-24	4e-16	
	Chloroplast genes: *Dendrobium chrysotoxum* (AF448862.1)	1			2e-14		
CA407986	BARE-1 long terminal repeat: *Hordeum vulgare* (Z84569)	6	1	2	8e-19	9e-25	0.007*
	Actin gene: *Triticum monococccum* (AF326781)		5	2		8e-31	4e-19
	T-DNA integration factor: *Oryza sativa* (U40814.1)	11	1		6e-21	3e-28	
	Microsatellite DNA: *Cocos nucifera* (AJ458311.1)	1			3e-29		
	Tonoplast intrinsic proteins: *Hordeum vulgare* (AF254799)		3			6e-50	
	DNA topoisomerase II: *Nicotiana tabacum* (AY169238.1)	1			7e-05		
	HMW[f] glutenin gene: *Aegilops tauschii* (AF497474)		2			e-116	

Accession numbers[a]	BLASTN annotations	Number of homologous ESTs cDNA[b]	NBS[c]	GR[d]	Average E-value cDNA[b]	NBS[c]	GR[d]
	Noduline like-like protein: *Triticum monococcum* (AF326781)		2			2e-43	
	Chr.[g] condensation factor: *Triticum monococcum* (AF326781)		2			2e-43	
CA407990	mRNA for SSU, Rubisco: *Triticum aestivum* (M37328)		3	4		3e-05	5e-34
	Secale cereale (AJ131738)			1			6e-58
	Hordeum vulgare (U43493)			1			2e-42
	Triticum timopheevii (AB020955)			1			2e-11
	Triticum urartu (AB020954)			1			2e-11
	Oryza sativa (AF052305)			6			1e-09
	Avena strigosa (AF097360)			1			3e-10
	Avena maroccana (AF104250)			1			3e-10
	Aegilops squarrosa (AB020938)			1			5e-09
	Aegilops bicornis (AB020936)			1			5e-09
	Aegilops sharonensis (AB020935)			1			5e-09
	Aegilops longissima (AB020933)			2			5e-09
	Partial mRNA 3'UTR: *Atropa belladonna* (AJ309392.1)	4	1		7e-20	7e-05	
CA407991	ATP synthase β subunit: *Clinostigma savoryanum* (AF449171)		1			e-168	
	Elaeis oleifera (AY012452)		1			e-168	
	Cyphophoenis nucele (AY012445)		1			e-168	
	Howea belmoreana (AY012435)		1			e-168	
	Phoenix canariensis (AF209652)		1			e-166	
	Linospadix longicruris (AF449172)		1			e-166	
	Aldehyde dehydrogenase: *Zea mays* (AF348415)			1			5e-19
	Microsatellite fragment: *Oryza sativa* (AY021654.1)			2			3e-14
	Ty1-copia retrotransposon: *Cicer arietinum* (AJ535884.1)	6	2		9e-49	5e-28	
	Beta nana (AJ489200.1)	9	2		9e-20	8e-20	
	Serine hydroxymethyl transferase: *Zea mays* (AF439728.1)	1			6e-52		
BU808664,5,6	RGA2[h]: *Triticum monococcum* (AF326781)		6			7e-13	
BU808668	Receptor-like kinase: *Triticum aestivum* (AF325196)			2			3e-07
BU808662,3	LRR[i]19: *Triticum aestivum* (AF325196)			2			6e-07
BU808661	WIR pathogen R[j] gene: *Triticum aestivum* (X87686)			3			6e-65
BU808667	Leucine rich-like protein: *Aegilops tauschii* (AF497474.1)		2			e-125	

a. Accession numbers of sequences submitted from this study (Lacock and Botha, 2002)
b. ESTs produced through PCR amplification of cDNA with NBS primers
c. Fragments produced through PCR amplification of genomic DNA with NBS primers
d. ESTs produced employing the GeneRacer cDNA synthesis system
e. Small subunit
f. High molecular weight
g. Chromosome
h. Resistance gene analogue 2
i. Leucine rich repeat
j. Resistance
* Sequences with E-value > 10^{-3}

A comparative analysis between DNA fragments and cDNA ESTs is indicative of the diverse reactions taking place in infested and uninfested plant tissue. The amount of representation of the different functional classifications between uninfested and infested treatments was determined, where the first value given in brackets (as discussed below) represents the cDNA library-obtained value and the second value the GeneRacer EST value (Fig. 2.6).

The most noticeable difference between the two types of treatments is the quantity of proteins synthesised. Infested plants synthesise a considerable larger amount of proteins, namely 32% (22% and 42%) than unstressed plants (19%). Metabolic activities in stressed (30%; 16% and 43%) and unstressed tissue (34%) appear to be comparable. It is noticeable that plants seem to suppress all structural activities during pest infestation. Structural activities in uninfested plants amount to 11%. Sequences related to regulatory activities in stressed plant tissue amounted to 11% (22% and 0%), where uninfested plants exhibit a rate of 7 % regulation. Sequences not linked to the above mentioned classifications amount to 11% in uninfested plant tissue and 21% (40% and 3%) in infested wheat plants. As expected, sequences linked to plant resistance genes are expressed during RWA infestation and accounts for 6% (0% and 12%) of the ESTs obtained. Gene fragments related to plant resistance (18%) were also isolated from non-infested plant material. This is expected since the wheat line used, PI 137739, is known to exhibit resistance against the RWA and the resistance fragments can, thus, be isolated from this DNA.

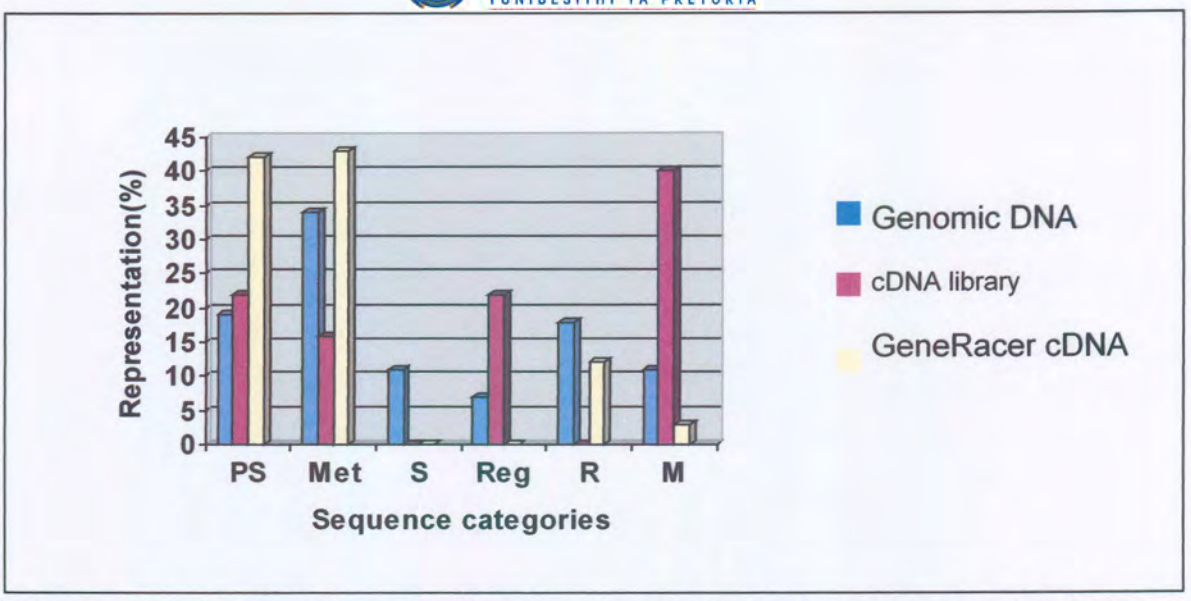

Figure 2.6 Comparative, functional analyses of the gene fragments obtained from genomic DNA, cDNA packaged in pGEM-T Easy Vectors and cDNA generated with the GeneRacer system. PS = protein synthesis; Met = metabolic; S = structural; Reg = regulatory; R = resistance; M = miscellaneous.

The proportion of non-resistance related fragments obtained during this study when taking all gene fragments – isolated from genomic as well as cDNA – into account amounts to 90%, while the sequences involved in plant resistance constitutes 10% of the analysed sequences (Fig. 2.7).

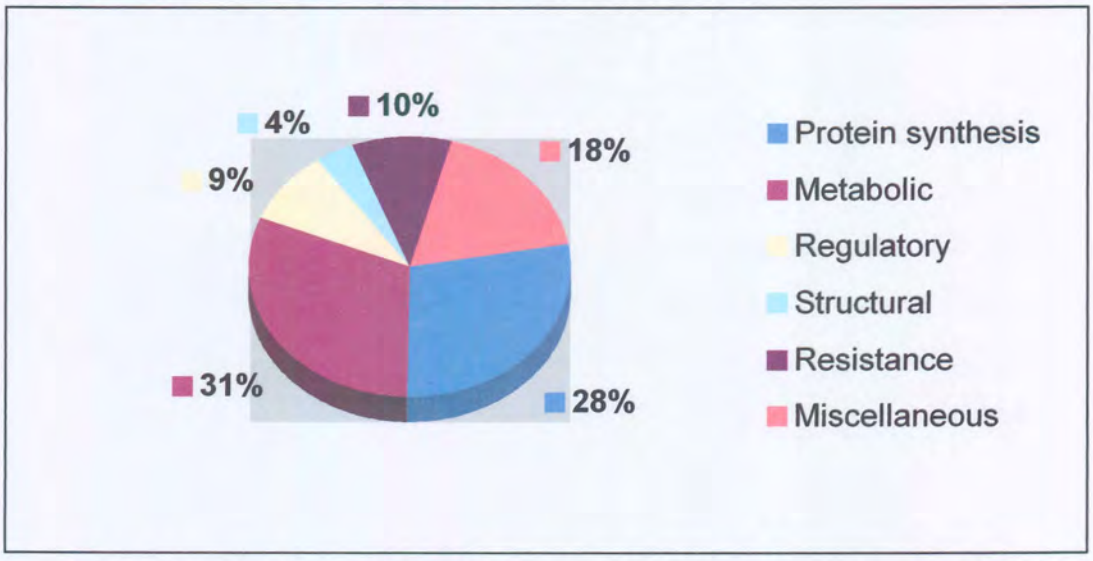

Figure 2.7 The proportional distribution of the analysed sequences presented as functional groups. Protein synthesis sequences constitutes 28%, the metabolic sequences 31%, the regulatory sequences 9%, the structural sequences 4%, miscellaneous sequences 18% and the resistance sequences 10% of the acquired sequences.

5. DISCUSSION AND CONCLUSION

The characterisation of resistance genes in various plant species is becoming an increased priority worldwide. The identification of resistance gene clusters (Pangstruga *et al.*, 1998) highlighted the importance of the NBS-LRR gene family in plant resistance (Yu *et al.*, 1996; Seah *et al.*, 1998; Tao *et al.*, 2000). The existence of these conserved domains has initiated the possibility of cloning other resistance genes utilising a PCR-based approach (Leister *et al.*, 1996). This study focused on the utilisation of NBS-specific primers in order to identify RWA resistance fragments in wheat.

We utilised two different templates, namely DNA and complementary DNA (cDNA) in the search for resistance gene fragments, and diverse results were obtained. Since the wheat genome is large, complex and thus difficult to investigate, the amount of sequences obtained from analysing total genomic DNA with the NBS-specific approach (10 ESTs with homology to 44 BLAST ESTs), were more than expected. Further analysis of the genomic DNA library with the DIG High Prime DNA Labelling and Detection System, however, yielded disappointing results even though the probe labelling was effective. The size and complexity of the genome (Moore *et al.*, 1993) impaired the screening of the genomic library. The aim of synthesising cDNA during this study was to eliminate non-expressed sequences and in the process ease the isolation of the gene(s) of interest. Although the synthesis and cloning of cDNA into the pGEM-T Easy Vector System was successful, screening of the cDNA library for resistance sequences with NBS-specific primers proved to be ineffective. Sequences involved with protein synthesis and metabolic activities, as well as miscellaneous sequences, were identified, but not sequences related to plant resistance. When a more targeted method for the analysis of cDNA was employed, namely the GeneRacer System, seven resistance-specific sequences were obtained.

An unusually high proportion of protein synthesis was detected in Russian wheat aphid infested plants (an average of 32%) when compared to the proportion in uninfested cells (19%). During a study conducted by Kruger and his colleagues (2002) on wheat after Fusarium head blight (FHB; *Fusarium graminearum*) infestation, the proportion of protein synthesis-related ESTs obtained was only 6%. The high amount of protein synthesis can be expected if it includes the synthesis of proteins linked to plant defence and resistance responses, e.g. pathogenesis-related (PR; Danhash *et al.*, 1993) proteins.

The average rate of metabolic activity within plants is reflected by the proportion of metabolic sequences obtained. Stressed tissue exhibited a slightly lower rate of metabolic activity (30%) than unstressed tissue (34%). Monocotyledonous species appear to maintain relatively low metabolic rates when compared to dicotyledonous species (Lee *et al.*, 1998). Lee and his colleagues (1998) compared the amount of metabolic activity of oilseed rape (*Brassica napus*) with that of maize (*Zea mays*) and rice (*Oryza sativa*). Oilseed rape exhibited a proportion of 62.2% metabolic activity, whereas that of maize and rice was much less, 26.3% and 6.8% respectively (Lee *et al.*, 1998). The proportion of sequences linked to metabolic activity in infested wheat tissue (29%) obtained during this study is comparable with that of maize (26.3%). Kruger and his colleagues (2002) found that only 18% of the expressed genes in wheat infected with FHB encoded genes for metabolic functions.

Average cell regulation (9%) takes place at a much lower rate than that of protein synthesis and metabolism. The difference in cell regulation between stressed (11%) and unstressed plants (7%) is negligible. The percentage regulatory activity in unstressed tissue is comparable to the findings of Kruger and his colleagues (2002). Genes expressed for cell regulation in FHB-infected wheat consisted of only 7% of the total amount of ESTs investigated.

Our study failed to isolate gene fragments related to structural functions from infested plant material. Uninfested material, however, exhibited structural gene expression of 11%. It appears that the amount of structural genes expressed during defence responses should not differ significantly between uninfested and infested plant material. Kruger and his colleagues (2002) found that 12% of genes expressed in wheat after infection with FHB were related to structural functions.

It is interesting to observe that all the resistance sequences obtained from genomic DNA exhibited homology to resistance gene analogues (RGAs). Further, these sequences are homologous to RGAs identified in *Triticum monococcum*, the donor of the A genome of wheat (Kimber and Sears, 1987). RGAs have been identified by several researchers when applying PCR-based methods in order to isolate NBS genes (Leister *et al.*, 1996, 1999; Mago *et al.*, 1999; Wang *et al.*, 2000). This includes genes from tomato (Ohmori *et al.*, 1998), soybean (Graham *et al.*, 2000), wheat and barley (Seah *et al.*, 1998), *Arabidopsis* (Bent *et al.*, 1994) and maize (Collins *et al.*, 1998). It is important to mention that most of these RGAs have been linked with known resistance gene sequences (Wang *et al.*, 2000).

Most importantly, our study revealed that 10% of the obtained gene fragments isolated from genomic, as well as cDNA, were related to resistance sequences. Of these, 27% corresponded to leucine rich repeats, but homology to nucleotide binding site sequences was not found. The LRR fragments identified in this study all exhibited homology to leucine rich repeats identified in *Aegilops tauschii*, which is thought to be the donor of the D genome of bread wheat (McFadden and Sears, 1944). The remaining resistance fragments exhibited homology to the WIR pathogen resistance gene (20%; Franck and Dudler, 1995), resistance gene analogues (40%; Wicker *et al.*, 2001) and receptor-like kinases (13%). Receptor-like kinases have been classified as part

of the serine-threonine class of resistance proteins (Martin *et al.*, 1993; Ritter and Dangl, 1996).

This study pointed out the difficulty of isolating and characterising genes or gene fragments involved in plant resistance responses. We have, however, indicated the feasibility of isolating some resistance gene fragments contained within NBS-LRR domains from wheat using a PCR-based approach. The procedures utilised were complicated by the presence of excess genes that could possibly contain NBS regions, but that are not related to resistance (Yu *et al.*, 1996). Hulbert (1998) speculated that these could be relics of previously functional resistance genes. However, the use of a target specific approach proved useful since a larger than expected percentage of ESTs obtained were related to leucine rich repeats, nucleotide binding sites or resistance gene analogues.

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Chapter 3

SSH employed to investigate gene expression after Russian wheat aphid infestation

The data presented in Chapter 3 have been included in two journal publications entitled:

1. Suppression subtractive hybridisation (SSH) employed to investigate gene expression after Russian wheat aphid infestation.

Lynelle Lacock and Anna-Maria Botha

Proceedings of the Tenth International Wheat Genetics Symposium, (1 – 6 September, Pasetum, Italy), 3: 1187 – 1189.

2. A leucine-rich homolog to *Aegilops tauschii* from bread wheat line PI 137739 obtained by suppression subtractive hybridisation show linkage to Russian wheat aphid resistance gene *Dn1*.

E. Swanepoel, **L. Lacock,** A.A. Myburg and A-M Botha

Proceedings of the Tenth International Wheat Genetics Symposium, (1 – 6 September, Pasetum, Italy), 3: 1263 – 1265.

1. ABSTRACT

The Russian wheat aphid (RWA; *Diuraphis noxia* Mordvilko) is a major pest of wheat, barley and other triticale in South Africa. Infestation by this aphid results in altered gene and, ultimately, protein expression patterns. Several approaches have been developed as a means to identify differentially expressed genes, including suppression subtractive hybridisation (SSH). SSH involves the selective amplification of target cDNA while simultaneously suppressing the amplification of non-target cDNA. During this study, SSH was employed to isolate gene sequences that are involved in wheat resistance against RWA infestation. Therefore, mRNA from RWA-infested wheat plants (PI 137739; *Dn*1) and mRNA from uninfested plants were compared with each other in an effort to isolate sequences related to RWA resistance. The fragments obtained ranged from 150 to 1000 base pairs. Gene fragments related to several resistance gene families were identified and constituted 18% of the isolated SSH fragments. This includes a leucine rich-like fragment (e–149; 377 bp) and leucine zipper-nucleotide binding site-leucine rich repeats (LZ-NBS-LRR; e–131; 440 bp) homologous to *Aegilops tauschii*. The expression of these fragments during plant infestation was confirmed through Northern blot hybridisations, as well as through quantitative real-time PCR. Hybridisations and quantification were performed using uninfested wheat RNA and RNA extracted at day two, three, four and five after infestation. On average, the resistance fragments exhibited increased expression after two days of infestation (approx. 2 – 11 fold), less expression during day three (approx. 2 – 9 fold down-regulation) and gradual up-regulation of the gene expression during day four (approx. 8 – 350 fold) and day five (approx. 890 – 10 200 fold). Thus, differential expression of these resistance-related fragments was undoubtedly observed after RWA infestation.

2. INTRODUCTION

Living organisms are assembled of a vast amount of biological processes and tissue types that form a balanced equilibrium. Each of these biological processes and developmental stages are regulated by specific patterns of gene expression (Diatchenko *et al.*, 1996). These specifically expressed genes are known to be differentially expressed (Birch *et al.*, 2000). Several approaches have been developed in the quest to identify differentially expressed genes in plants subjected to specific treatments as a means to identify the genes involved in the plant's response to such treatment.

One of the earliest methods employed in this quest is differential display (DD; Liang and Pardee, 1992). This involves the synthesis of cDNA from differing sets of RNA using an oligo-dT 3' primer (the 3' amplimer). The cDNA is then amplified with the polymerase chain reaction (PCR) using the 3' amplimer and a 5' amplimer (Sompayrac *et al.*, 1995). The obtained fragments are visualised on a sequencing gel from which they are excised and further amplified (Liang and Pardee, 1992). DD, however, only detects differences at the 3' ends of cDNA and not the 5' end (Von Stein *et al.*, 1997) and differences obtained can often not be reproduced through Northern blot hybridisations (Sompayrac *et al.*, 1995).

Representational difference analysis (RDA) was developed by combining subtraction with hybridisation in order to isolate differences between two genomic DNA populations (Lisitsyn *et al.*, 1993). Genomic, as well as cDNA RDA, is based on restriction of the DNA and the target sequence is enriched through subtractive hybridisation from this simplified representation of the genomic DNA or cDNA (Hubank and Schatz, 1994). The disadvantage of RDA is that several cycles of hybridisation is required due to the high amount of differing individual mRNA transcripts present in the mRNA populations (Von Stein *et al.*, 1997).

The sensitivity of subtraction methods, however, increases drastically when PCR is employed as an additional tool in differential gene expression

analyses (Hara *et al.*, 1991; Balzer and Baumlein, 1994; Hubank and Schatz, 1994). A new discovery involving PCR changed the approach towards isolating rare gene transcripts completely. Long inverted terminal repeats at the ends of DNA fragments form stem-loop configurations after each denaturation and primer annealing PCR cycle (Diatchneko *et al.*, 1999). These structures selectively suppress the exponential amplification of sequences that are present in equal abundance, but cDNAs differing in abundance are exponentially amplified (Siebert *et al.*, 1995; Von Stein *et al.*, 1997). This discovery led to the development of suppression subtractive hybridisation (SSH; Diatchenko *et al.*, 1996). The principle behind SSH, therefore, is the selective amplification of target cDNA while simultaneously suppressing the amplification of non-target cDNA (Diatchenko *et al.*, 1996). SSH utilises two cDNA populations: the tester population, containing the target sequence, and the driver population, with which the tester is compared, but without the target sequence (Diatchenko *et al.*, 1996; Gurskaya *et al.*, 1996). Birch and his colleagues (2000) briefly describe the SSH process as follows: Two similar tester sub-populations are each ligated to a unique adapter. Each tester sub-population is individually hybridised with excess driver cDNA. During this step, complementary tester and driver sequences form double strands, but the target sequences remain single-stranded. A second hybridisation follows where the two tester sub-populations are combined and added to freshly denatured driver cDNA. The additional denatured driver cDNA causes the single-stranded tester population to be enriched even more (Gurskaya *et al.*, 1996). Subsequently, two rounds of PCR are performed by using primers complementary to the adapter sequences. During the primary PCR the adapter ends are filled in; during secondary PCR the fragments possessing both adapters are amplified, since amplification of fragments containing only one adapter is suppressed by their stem-loop configurations (Diatchenko *et al.*, 1999).

Suppression subtractive hybridisation has a wide range of applications in medical, as well as botanical research. Hara and his colleagues (1991) employed SSH in studying undifferentiated human carcinoma cells during embryonic development. Diatchenko and his colleagues (1996) screened a

human Y chromosome cosmid library using SSH. They used subtracted testis-specific cDNA and were able to identify sequences specific to the Y chromosome (Diatchenko *et al.*, 1996). During further studies, more differentially expressed genes were identified in testis and ovary tissue when a subtracted testis cDNA library was screened (Jin *et al.*, 1997). SSH was employed by Kuang and his colleagues (1998) in studying gene expression in estrogen receptor (ER)-positive and -negative cell lines. They were able to identify genes that are expressed in hormone-responsive ER positive cells but not in, or minimally, in hormone-unresponsive ER negative cells (Kuang *et al.*, 1998).

Reports of SSH being applied in botanical research are more recent. Low temperature-induced genes were isolated from cold-treated winter barley (*Hordeum vulgare* L. cv. Dongbori) by utilising SSH (Bahn *et al.*, 2001). Zang and his colleagues (2002) investigated a cDNA library of *Dunaliella salina* that was exposed to hyperosmotic stress. The subtracted libraries were screened using SSH and several differentially expressed genes were identified (Zang *et al.*, 2002). SSH was, further, utilised by Kloos and his colleagues (2002) in order to isolate cDNA transcripts for taproot-expressed genes in sugar beet.

During the present study, SSH was employed in an attempt to isolate gene sequences that are involved in wheat resistance responses against insect infestation, specifically the Russian wheat aphid (RWA). The wheat line PI 137739, which is known to exhibit a certain level of resistance against the RWA and contains the *Dn*1 resistance gene (Du Toit, 1989), was used as the RNA source. Tester cDNA was prepared from wheat leaf material infested with Russian wheat aphids, whereas driver cDNA was prepared from uninfested tissue. These two types of cDNA were used to isolate sequences that are present only in the infested cDNA population. The SSH fragments were then cloned and their sequences determined in order to compare them with putative resistance gene fragments. The level of induction of the resistance-related fragments was assessed through Northern blot analyses, as well as quantitative PCR reactions.

3. MATERIALS AND METHODS

3.1 Wheat material

The wheat cultivar used for SSH analysis was PI 137739, which is known to contain the *Dn1* resistance gene (Du Toit, 1989). Wheat seed was obtained from the wheat germplasm source at the United States Department of Agriculture (USDA) Small Grains Repository, Aberdeen, Idaho, USA. The seed were planted in a sand: peatmoss (1:3; v/v) soil mixture and kept under consistent greenhouse conditions at 25 °C. RWA infestation of the plants was performed at the second leaf-growth stage of the plants and five aphids were scattered on each plant (Van der Westhuizen and Pretorius, 1996).

3.2 Treatment of glassware, plastic ware and solutions

All glassware, plastic ware and solutions used during RNA isolation and first strand cDNA synthesis were treated with diethyl pyrocarbonate (DEPC) in order to remove all RNases. Glassware was treated overnight in 0.1% (v/v) DEPC, autoclaved for 20 minutes and baked at 200 °C overnight (Sambrook *et al.*, 1989). Mortars and pestles were rinsed with 0.25 M HCl and treated similarly as the glassware. The plastic ware and solutions were DEPC treated and autoclaved. Solutions containing Tris (2-Amino-2-(hydroxymethyl)-1, 3-propandiol) were not DEPC treated, but only autoclaved.

3.3 RNA isolation and mRNA purification

Wheat plants were infested with Russian wheat aphids after the plants had reached the third-leaf stage. RNA was isolated from the second and third leaves of the infested plants, two to five days after infestation (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 homogenised using liquid nitrogen and RNA extracted according to the guanidine thiocyanate method of Chomczynski and Sacchi (1987). RNA

extraction buffer (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% (w/v) N-laurocylsarcosine and 100 mM 2-mercaptoethanol) was added to the homogenised tissue in a 3:1 ratio. This mixture was placed at room temperature for 10 minutes and subsequently centrifuged at 10 000 rpm for 20 minutes. The supernatant was added to 50 µl 2 M NaOAc (pH 4) and 500 µl phenol:chloroform (1:1), and placed at room temperature for 10 minutes. After centrifugation at 10 000 rpm for 10 minutes, the supernatant was added to an equal volume of isopropanol. The RNA was precipitated for 1 hour at -20 °C, pelleted at 13 000 rpm for 30 minutes and thereafter washed with 70% (v/v) EtOH. The RNA was subsequently air dried and resuspended in 20 µl DEPC-treated water. RNA concentration was determined on a Beckman DU® - 64 spectrophotometer by reading the absorbance at 260 nm. mRNA was purified from total RNA using Oligo(dT)-cellulose columns supplied with the mRNA Purification Kit (Amersham Pharmacia Biotechnology, USA).

3.4 cDNA synthesis

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

3.5 Suppression subtractive hybridisation

3.5.1 Driver preparation

The driver cDNA sample was subsequently restricted with 15 U *Rsa*I for 5 hours at 37 °C. The driver cDNA was cleaned once again using the MinElute Reaction Cleanup Kit (Qiagen, USA) and eluted with 30 µl dd H_2O.

3.5.2 Tester preparation

Tester cDNA was manipulated by restricting 1.8 µg of cDNA with 15 U *Rsa*I restriction enzyme for 5 hours at 37 °C. The cDNA was cleaned with the MinElute Reaction Cleanup Kit (Qiagen, USA) and eluted in 30 µl dd H_2O. A 1:6 dilution of the restricted tester cDNA was prepared and 100 ng of this dilution was used for subsequent ligation reactions.

3.5.3 Adapter ligation

Two ligation reactions were performed (CLONTECH PCR-Select, cDNA Subtraction Kit User Manual, CLONTECH Laboratories Incorporated, USA). Each reaction contained 100 ng template, 3 µl H_2O, 2 µl 5 x ligation buffer (CLONTECH PCR-Select, CLONTECH Laboratories Incorporated, USA), 0.5 U T4 DNA ligase and 10 pmol of either adapter I (5'-GTA-ATA-CGA-CTC-ACT-ATA-GGG-CTC-GAG-CGG-CCG-CCC-GGG-CAG-GT-3'; 3'-CCC-GTC-CA-5') or adapter II (5'-TGT-AGC-GTG-AAG-ACG-ACA-GAA-AGG-GCG-TGG-TGC-GGA-GGG-CGGT-3'; 3'-GCC-TCC-CGC-CA-5') in a 10 µl reaction. The reaction was placed at 4 °C overnight, terminated by adding 0.2 M EDTA and the ligase inactivated at 70 °C for 5 minutes.

In order to confirm the ligation of adapters I and II to the tester cDNA, ligation test PCRs were performed (GeneAmp® PCR System 9700, Applied Biosystems, USA). The tester cDNA ligated to adapter I (A1) and II (A2) was used as template – diluted 1:200 – in separate reactions. The PCR reactions consisted of 2.5 µl 10 x PCR buffer (10 mM Tris-HCl, 50 mM KCl and 0.1% (v/v) Triton® X-100; pH 9.0) with 2.5 mM $MgCl_2$, 10 mM of each dNTP, 10 pmol of each PN1 (5'-TCG-AGC-GGC-CGC-CCG-GGC-AGG-T-3') and PN2 (5'-AGG-GCG-TGG-TGC-GGA-GGG-CGG-T-3'), 1 U Taq DNA polymerase and 0.1 ng template in a 25 µl reaction. The PCR reaction commenced at 94 °C for 30 seconds and was followed by 20 cycles consisting of 94 °C for 10 seconds, 55 °C for 30 seconds and 72 °C for 2.5 minutes, and concluded for 7 minutes at 72 °C. Ligation of the adapters was confirmed visually using a 1% (w/v) low-melting agarose gel containing ethidium bromide on UV light.

3.5.4 Primary hybridisation

Prior to the primary hybridisation, the two adapter-ligated tester samples were further diluted: 1) adapter-I (A1a) and -II (A2a) to a 1:5 ratio, and 2) adapter-I (A1b) to a 1:3 and adapter-II (A2b) to a 1:1 ratio. 1.5 µl (~20 ng) of each of the diluted adapter samples was added individually to 600 ng *Rsa*I-digested driver cDNA and 1.0 µl 4 x hybridisation buffer (50 mM Hepes, 0.5 M NaCl, 0.02 mM EDTA, 0.2% (w/v) PEG 8000; pH 8). These samples were heat denatured in a GeneAmp® PCR System 9700 (Applied Biosystems, USA) at 98 °C for 1.5 minutes and subsequently incubated at 68 °C for 6 hours.

3.5.5 Secondary hybridisation

Prior to secondary hybridisation 150 ng driver cDNA was added to 2 µl 4 x hybridisation buffer (50 mM Hepes, 0.5 M NaCl, 0.02 mM EDTA, 0.2% (w/v) PEG 8000; pH 8) and 4 µl ddH_2O. The driver was heat denatured at 98 °C for 1.5 minutes. The primary hybridisation samples containing the 1:5 diluted adapter fractions (A1a and A2a), were combined, as were the adapter fractions containing the 1:3 and 1:1 (A1b and A2b) diluted samples. 4 µl Denatured driver was immediately added to each of these two primary reactions. Hybridisation was performed at 68 °C overnight and thereafter 100 µl dilution buffer (20 mM Hepes, 50 mM NaCl and 0.2 mM EDTA; pH 8.3) was added to the mixture and incubated for a further 7 minutes at 68 °C.

3.5.6 Primary PCR amplification

Primary PCR amplifications were carried out using the respective subtracted, secondary hybridisation templates, as well as a combined sample of the unsubtracted, adapter-ligated tester samples. The PCR reactions consisted of 2.5 µl 10 x PCR buffer (10 mM Tris-HCl, pH 9.0, 50 mM KCl and 0.1% (v/v) Triton® X-100; pH 9.0), 2 mM $MgCl_2$, 10 mM of each dNTP, 5 pmol P1 primer (5'-GTA-ATA-CGA-CTC-ACT-ATA-GGG-C-3'; Diatchenko *et al.*, 1996), 1 U Taq DNA polymerase and 50 ng template in a 25 µl reaction.

Amplification commenced at 94 °C for 30 seconds, followed by 27 cycles (30 seconds at 94 °C, 30 seconds at 66 °C and 2 minutes at 72 °C) and ended with an extension period of 5 minutes at 72 °C.

3.5.7 Secondary PCR amplification

The primary PCR reaction was diluted 1:10 and PCR was carried out using diluted, as well as undiluted primary PCR templates. The secondary PCR reactions were performed with nested primers PN1 (5'-TCG-AGC-GGC-CGC-CCG-GGC-AGG-T-3') and PN2 (5'-AGG-GCG-TGG-TGC-GGA-GGG-CGG-T-3'). The PCR reactions were assembled similarly to the primary reactions and 0.5 pmol of each primer was used. PCR was performed for 27 cycles (94 °C for 30 seconds, 68 °C for 30 seconds and 72 °C for 1.5 minutes) with a final extension of 5 minutes at 72 °C. The SSH products were visualised on a 1% (w/v) low-melting agarose gel containing ethidium bromide on UV light.

3.6 Cloning and sequencing

The secondary SSH fragments were excised from the gel and purified using the Geneclean III Kit (Southern Cross Biotechnology, USA). The fragments were cloned into pGEM-T Easy Vectors (Promega Corporation, USA), colony PCR was performed on the positively cloned fragments (Gussow and Clackson, 1989) and used as template in subsequent sequencing reactions. Sequencing (BigDye Terminator Sequencing Reaction Kit, Perkin-Elmer) was performed 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 on an ABI 3100 Prism Automated Sequencer (Perkin-Elmer).

3.7 Functional annotation

The sequenced SSH fragments were analysed using the nucleotide BLASTN annotation of the Basic Local Alignment Search Tool (BLAST; Altschul *et al.*, 1990) at http://www.ncbi.nlm.nih.gov/BLAST/. Three

expressed sequence tags were identified that exhibited homology to a LZ-NBS-LRR (leucine zipper-nucleotide biding site-leucine rich repeat) gene, one fragment was homologous to a leucine rich-like protein and one fragment was homologous to a wheat ubiquitin carrier protein. These fragments were further characterised using Southern and Northern blot hybridisations, and quantitative PCR. The clone identities and the Genbank accession numbers assigned to the LZ-NBS-LRR fragments were L-SSH G (ABO00040; CA798960), L-SSH H (ABO00037; CA798957), L-SSH J (ABO00041; CA798961); the leucine rich-like fragment was L-SSH L (ABO00042; CA798962) and the ubiquitin carrier protein fragment was L-SSH E (ABO00039; CA798959).

3.8 Genomic DNA extraction

Genomic DNA was extracted from the PI 137739 cultivar, *Triticum urartu* and *Aegilops speltoides* using 10-day old, uninfested wheat with the monocot DNA isolation technique (Edwards *et al.*, 1991). The leaf tissue was homogenised in liquid nitrogen, 600 µl extraction buffer (200 mM Tris-HCl, 150 mM NaCl, 25 mM EDTA and 0.5% (w/v) SDS; pH 8) was added and mixed thoroughly. An incubation period of 30 minutes at 60 °C followed, with interruptions every 5 minutes when the tubes were inverted. 600 µl Chloroform/isoamylalcohol (24:1) was added, the solution was mixed vigorously and subsequently centrifuged at 10 000 rpm for 15 minutes. The upper phase was removed and transferred to a sterile eppendorf tube. Two volumes of ice cold 95% (v/v) EtOH were added and the DNA was precipitated at -20 °C overnight. The DNA was scooped out and washed with 70% (v/v) EtOH for 10 minutes. The wash step was repeated two more times. The extracted DNA was resuspended in 200 µl dd H_2O and stored at -20 °C. DNA quantification was carried out on a Beckman DU® - 64 spectrophotometer at an absorbance of 260 nm.

3.9 Southern blot analysis

Southern blot analyses were performed using the PI 137739 (AABBDD), *Triticum urartu* (AA; Kimber and Sears, 1987) and *Aegilops speltoides* (BB; Sarkar and Stebbins, 1956) genomic DNA. 200 ng of each sample was spotted on five nylon membranes (Roche Diagnostic Corporation, Germany) – four membranes for each SSH clone (L-SSH G, L-SSH H, L-SSH J and L-SSH L) to be characterised, as well as one for the control fragment (L-SSH E) – and UV cross-linked at 0.15 Joules per second (Sambrook *et al.*, 1989).

The hybridisation probes were prepared from the one leucine rich-like clone (L-SSH L), the three LZ-NBS-LRR clones (L-SSH G, L-SSH H and L-SSH J) and the ubiquitin carrier protein (L-SSH E) identified during sequence analysis of the acquired SSH fragments. 50 ng of each fragment was labelled using the *Gene Images* Random Prime Labelling Module (Amersham Pharmacia Biotechnology, USA).

Pre-hybridisation of the membrane-bound genomic DNA was performed at 60 °C using hybridisation buffer [5 x SSC (75 mM NaOAc and 0.75 M NaCl), 0.1% (w/v) SDS, 5% (v/v) Denhardt's solution] and a 20-fold dilution of the Liquid Block (*Gene Images* Random Prime Labelling Module, Amersham Pharmacia Biotechnology, USA) for 3.5 hours. 15 µl of each probe was heat denatured for 5 minutes and added to the respective pre-hybridised membranes. Hybridisation continued at 65 °C overnight in a HB-1D Hybridiser (TECHNE, Cambridge, United Kingdom). Two stringency washes followed hybridisation: the first wash [1 x SSC (15 mM NaOAc and 0.15 M NaCl) and 0.1% (w/v) SDS], as well as the second wash [0.1 x SSC (1.5 mM NAOAc and 15 mM NaCl) and 0.1% (w/v) SDS] was performed at 65 °C for 15 minutes each. Thereafter, the blots were incubated in buffer A (100 mM Tris-HCl and 300 mM NaCl) plus a 10-fold dilution of the Liquid Block (*Gene Images* CDP-*Star*™ Detection Module, Amersham Pharmacia Biotechnology, USA) for 1 hour at room temperature. The next incubation took place in buffer A (100 mM Tris-HCl and 300 mM NaCl) containing 0.5%

(w/v) BSA and a 5000-fold dilution anti-fluorescein-AP conjugate for 1 hour. Finally, three wash steps [buffer A (100 mM Tris-HCl and 300 mM NaCl) and 0.3 % (v/v) Tween] for 10 minutes each followed.

CDP-*Star*™ detection reagent (500 µl per blot) was pipetted onto the membranes, removed after 5 minutes and the membranes were wrapped in Saran Wrap. The membranes were exposed to X-ray film (HyperFlim for fluorescence, Amersham Pharmacia Biotehcnology, USA) for 30 minutes after which the films were developed (Sambrook *et al.*, 1989) and visually evaluated.

3.10 Northern blot analysis

Northern blot analyses were performed using total RNA extracted from uninfested wheat leaves, as well as RNA isolated at day 2, day 3, day 4 and day 5 after infestation with RWAs. The RNA was spotted onto nylon membranes (Roche Diagnostic Corporation, Germany) – each spot consisting of 200 ng RNA. Five membranes were prepared: one membrane for each of the SSH clones and one membrane for the control fragment. The membranes were allowed to air dry and the RNA was UV-cross linked to the membranes at 0.15 Joules per second (Sambrook *et al*, 1989). The procedures followed for the Southern blot hybridisations were applied during the subsequent Northern blot hybridisations.

3.11 Quantitative PCR analyses

Primers were designed in order to amplify each of the SSH fragments characterised during Northern and Southern blot hybridisations, as well as primers specific for ribulose-1, 5-bisphosphate (Rubisco) used during the $MgCl_2$ titration (Table 3.1). First strand cDNA was synthesised from uninfested PI 137739, as well as from day 2, 3, 4, and 5 RWA-infested total RNA using AMV Reverse Transcriptase (Promega Corporation, USA). The concentrations of the first strand cDNA samples were determined on a Beckman DU® - 64 spectrophotometer at an absorbance of 260 nm.

Table 3.1 Primer sequences of the SSH fragments and Rubisco control fragment used for quantitative PCR analysis.

SSH primer	Primer sequence
Rubisco f	5'-ATT-GTC-TCC-GTG-GTG-GAC-TC-3'
Rubisco r	5'-TAA-TTT-CAC-CCG-TCT-CAG-CC-3'
SSH Ef	5'-CAT-CCA-CCA-GTC-AAG-GGT-TC-3'
SSH Er	5'-CTT-CTC-CTC-TAC-CCG-AAC-CC-3'
SSH Gf	5'-TGG-GAT-ATT-CAC-GTG-ATC-CA-3'
SSH Gr	5'-CTT-CAA-AGA-GTG-CCC-CAA-AG-3'
SSH Hf	5'-TTT-TGG-TGA-TCA-ACT-TGC-GA-3'
SSH Hr	5'-AAG-AGT-GCC-CCA-AAG-GTT-CT-3'
SSH Jf	5'-GTA-CCG-CGA-GCT-TTG-CTA-TT-3'
SSH Jr	5'-TCA-AGA-TGA-AAA-CGT-GTG-CC-3'
SSH Lf	5'-CAC-AGG-ATC-ATG-CAT-TAC-GG-3'
SSH Lr	5'-GGT-ACG-TTA-TTT-GCC-CGA-GA-3'

f forward primer
r reverse primer

A $MgCl_2$ titration was performed using the Rubisco primers in order to determine the optimum $MgCl_2$ concentration (1 mM, 3 mM or 5 mM) for subsequent PCR reactions (Fig 3.9). The respective threshold cycles (C_T) were used as an indication of optimum concentration. Quantitative PCR was performed using 50 ng first strand cDNA synthesised from uninfested PI 137739 total RNA, 5 pmol of each Rubisco forward and reverse primers, LighytCycler – FastStart DNA Master SYBR Green I mix (LightCycler – FastStart DNA Master SYBR Green I Instruction Manual, Roche Applied Science, Germany) and the $MgCl_2$ concentration to be tested, in a 20 µl reaction. The cycling parameters used were a pre-incubation cycle at 95 °C for 10 minutes in order to activate the FastStart polymerase and denature the cDNA template. Amplification followed for 40 cycles where 1 cycle consisted of 10 seconds at 95 °C, 5 seconds at 60 °C and 10 seconds at 72 °C to allow extension of the fragment. Melting curve analyses commenced at 95 °C and consisted of a gradual increase in temperature from 65 °C to 95 °C. A cooling cycle of 30 seconds at 40 °C terminated the reaction. The data, detected as amount of fluorescence, was acquired during each extension phase (Deprez *et al.*, 2002). The optimum $MgCl_2$ concentration was found to be 3 mM per reaction (Fig. 3.9).

0.8 µg cDNA was used for subsequent real-time PCR (Wang and Brown, 1999) reactions. The PCR reactions further consisted of 2 µl FastStart DNA Master SYBR Green I, 3 mM $MgCl_2$ and 5 pmol each of the relevant forward and reverse primers. Reactions were compiled for uninfested first strand cDNA, as well as for day 2, day 3, day 4 and day 5 post-infestation first strand cDNA for each primer combination. An internal standard was also included for each primer pair that consisted of four reactions in order to generate a standard curve (Fig 3.10; Deprez *et al.*, 2002). Reaction 1 contained 800 ng, reaction 2 contained 80 ng (1:10 dilution), reaction 3 contained 8 ng (1:100 dilution) and reaction 4 contained 0.8 ng (1:1000 dilution) first strand cDNA template. The standards were included in order to quantify the concentrations of the uninfested and infested cDNA samples.

Quantitative PCR analyses were performed on a LightCycler Instrument using LightCycler Software Version 3.5 (Roche Applied Science, Germany). Cycling parameters were set as outlined in the LightCycler – FastStart DNA Master SYBR Green I Manual (Roche Applied Science, Germany). Pre-incubation consisted of 1 cycle at 95 °C for 10 minutes. Amplification followed for 40 cycles where 1 cycle consisted of 10 seconds at 95 °C, 5 seconds at the relevant primer annealing temperature and 10 seconds at 72 °C to allow extension of the fragment. Melting curve analyses commenced at 95 °C and was followed by a gradual increase in temperature from 65 °C to 95 °C. Lastly, a cooling cycle of 30 seconds at 40 °C was included. Quantitative and melting curve analyses were performed. The threshold cycle (C_T), the linear regression coefficient (r), slope and error rate for each reaction was calculated by the LightCycler software. The threshold cycle is an indication of the number of cycles necessary to detect a fluorescence signal from the amplified product; the regression coefficient is an indication of systematic errors within the experiment; the error rate should be ≤ 0.2 and reflect tube to tube variations; the slope is an overall indication of reaction efficiency, where a slope of -5.7 to -2.9 is ideal (LightCycler Operator's Manual, Version 3.5, Roche Applied Science, Germany).

4. RESULTS

4.1 RNA isolation and cDNA synthesis

The isolation of intact RNA and subsequent mRNA purification is imperative for successful cDNA synthesis and, thus, successful SSH studies. The concentrations of the total RNA isolated, the amount of mRNA purified from the total RNA and the final concentration of tester and driver cDNA synthesised are summarised in Table 3.2.

Table 3.2 A summary of the concentrations of the total RNA isolated from RWA infested and uninfested plants, the mRNA purified and cDNA synthesised from the total RNA samples.

Sample type	Tester concentration	Driver concentration
Total RNA	460 ng/µl	360 ng/µl
Purified mRNA	165 ng/µl	85 ng/µl
Synthesised cDNA	46 ng/µl	36 ng/µl

Figure 3.1 displays total RNA isolated from RWA infested wheat plants, and first and second strand cDNA synthesised from the relevant RNA.

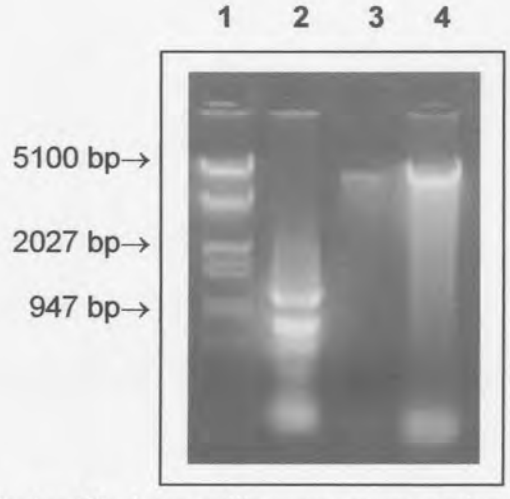

Figure 3.1 A 1% low-melting agarose gel containing ethidium bromide displaying total RNA extracted from RWA infested PI 137739 (lane 2), first strand cDNA (lane 3) and second strand cDNA (lane 4). Lane 1 = molecular marker III (λ DNA restricted with Eco RI and Hind III). Some genomic DNA is still present in the first strand and second strand cDNA samples.

4.2 Adapter ligation

Subtraction and normalisation greatly depend on the complete ligation of the adapters to the two tester cDNA samples. Since the adapters are designed without phosphate groups, the longer strand of each adapter can attach to the 5' end of the cDNA (Diatchenko *et al.*, 1999). Ligation of both adapter I and II to the two cDNA populations have been achieved since a smear ranging from 150 bp to 3 500 bp was obtained when the ligation test reactions were visualised on a 1% agarose gel (Fig. 3.2).

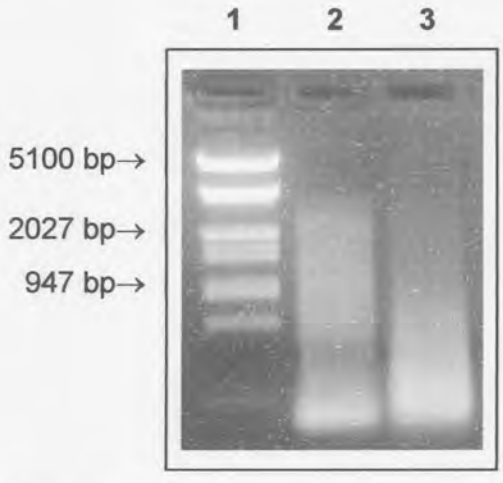

Figure 3.2 A 1% low-melting agarose gel image of the PCR assessing the adapter ligation to the two tester cDNA samples. Lane 1 = molecular marker III (λ DNA restricted with Eco RI and Hind III), lane 2 = adapter I ligated to tester cDNA (A1), lane 3 = adapter II ligated to tester cDNA (A2).

4.3 Subtraction, amplification and sequence analyses

Several sets of subtraction of the tester and driver cDNA, as well as subsequent PCR amplifications, were performed. Eighteen individual fragments were generated that were cloned and sequence characterised. These fragments were obtained from two different sets of template dilutions of the adapter I (A1a and A2a) and adapter II (A1b and A2b) cDNA samples. The first dilution set used for amplification was a 1:5 dilution for both the adapter samples (Fig 3.3); the second set consisted of a 1:1 dilution for adapter I (A1b) and a 1:3 dilution for adapter II (A2b; Fig 3.4).

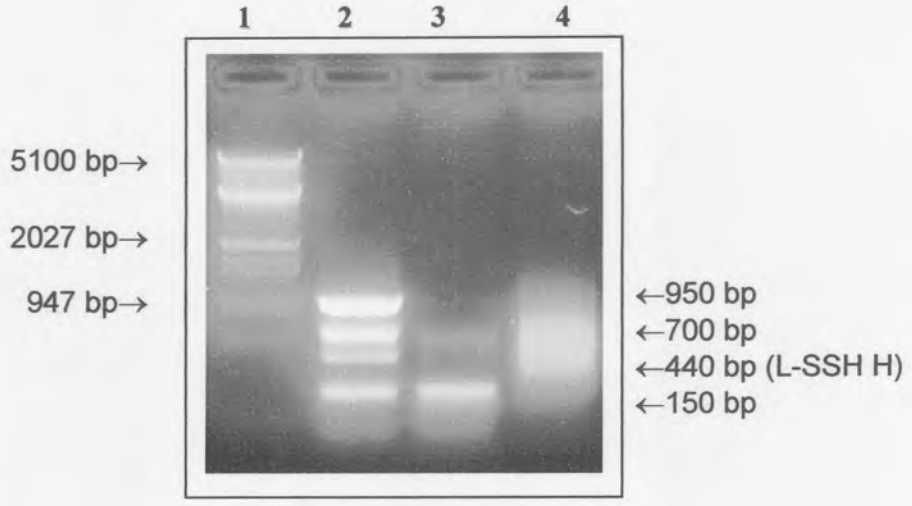

Figure 3.3 A 1% low-melting agarose gel presenting the products of a secondary SSH PCR using PN1 and PN2 primers. The adapter-ligated cDNA dilutions used for subtraction were 1:5 for both adapter samples. Lane 1 = molecular marker III (λ DNA restricted with Eco RI and Hind III), lane 2 = PCR using 3 µl primary PCR template, lane 3 = PCR using 3 µl diluted (1:10) primary PCR template, lane 4 = unsubtracted control.

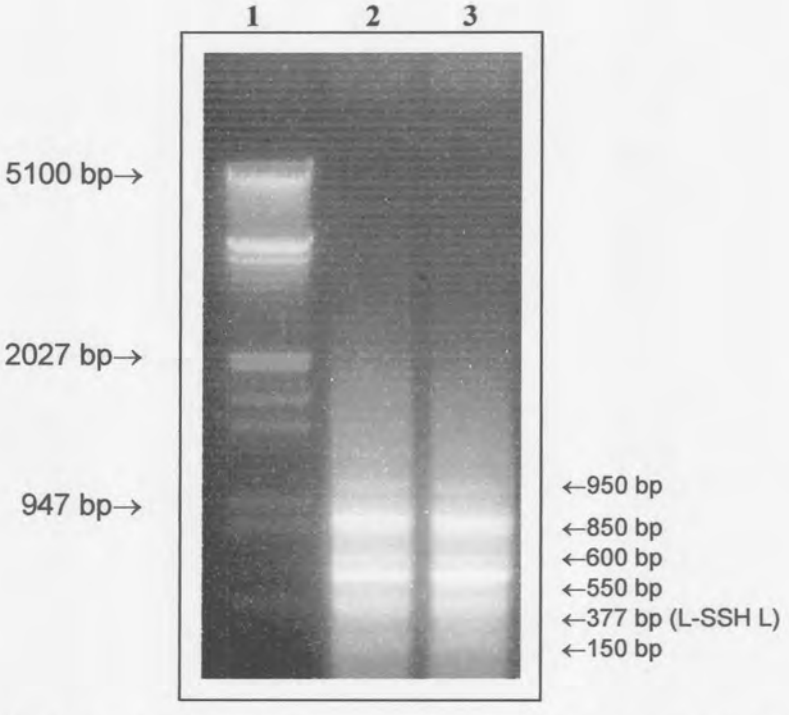

Figure 3.4 A 1% agarose gel presenting the products of a SSH secondary PCR using PN1 and PN2 primers. The adapter-ligated cDNA dilutions used for subtraction were 1:1 for adapter I and 1:3 for adapter II cDNA ligation samples. Lane 1 = molecular marker III (λ DNA restricted with Eco RI and Hind III), lane 2 and 3 = duplicate PCR samples.

Transformation efficiency of the cloned products ranged from 40 – 60% and inserts sizes from 125 bp to 1 000 bp were obtained. The clones that were further characterised, namely L-SSH G, L-SSH H, L-SSH J, L-SSH L and L-SSH E were 434 bp, 440 bp, 306 bp, 377 bp and 433 base pairs in length, respectively. These sequences were submitted to GenBank and the following accession numbers were assigned: CA798960 (L-SSH G), CA798957 (L-SSH H), CA798961 (L-SSH J), CA798962 (L-SSH L) and CA798959 (L-SSH E). All 18 isolated clones were found to contain fragments relevant to this study and aligned to 98 dbESTs during BLAST (Altschul *et al.*, 1990) analyses (Table 3.3).

Table 3.3 Data of the SSH sequence analyses displaying the clone identities of the sequences obtained during this study, the organisms and proteins to which homology was obtained, the amount of homologous sequences for each sequence, as well as the average expectancy (E) value. Values in brackets indicate the GenBank accession numbers to which homology was obtained and the accession numbers of the sequences submitted from this study (Lacock and Botha, 2002) are given in bold print.

Clone identity	BLASTN annotations	Nr. of homol.[a] ESTs	E-value
ABO 00049	26S rRNA 3' end: *Triticale cereale* (M37231)	3	0.0
ABO 00046	*Triticum aestivum* (AY049041.1)	2	0.0
ABO 00047	Proembryo mRNA: *Oryza sativa* (AF454918.1)	2	0.0
	25S rRNA: *Zea mays* (AJ309824.2)	3	0.0
	Oryza sativa (M11585.1)	1	0.0
ABO 00039	Ubiquitin carrier protein mRNA: *Triticum* (M28059.1) **CA798959**	1	e – 100
	Ubiguitin-conjugating enzyme: *Arabidopsis thaliana* (AY114061.1)	2	6e – 04
	Ay gene for HMW[b] glutenin: *Triticum aestivum* (X03042.2)	4	e – 137
ABO 00048	Mla locus: *Hordeum vulgare* (AF427791.1)	4	2e – 65
	WIS-2-1A Ty1-copia-like retrotr.[c]: *Triticum monococcum* (AF339051)	4	4e – 57
	Triticum aestivum (X57168.1)	6	3e – 55
ABO 00053	BARE-2 and BAGY-2 retrotr.[c]: *Hordeum vulgare* (AJ279072)	2	1e – 35
	Tonoplats intrinsic proteins 1 + 2: *Hordeum vulgare* (AF254799.1)	2	3e – 33
	BARE-1 copia-like retroelement: *Hordeum vulgare* (Z17327.1)	4	8e – 31
ABO 00052	Actin gene: *Triticum monococcum* (AF326781.1)	3	7e – 25
	NLL[d]-like protein: *Triticum monococcum* (AF326781.1)	3	7e – 25
	Glutathion-S-transferase 1 + 2: *Aegilops tauschii* (AY013753.1)	2	4e – 20
	Inverted terminal repeat1 gene: *Hordeum vulgare* (X65875.1)	2	2e – 19

Clone identity	BLASTN annotations	Nr. of homol.[a] ESTs	E-value
ABO 00055	CCF[e]: *Triticum monococcum* (AF326781.1)	3	3e – 24
	BARE-1 long terminal repeat: *Hordeum vulgare* (Z84569.1)	2	7e – 13
	T-DNA integration target sequence: *Oryza sativa* (U40814.1)	1	7e – 13
	Retrotransposon-like element: *Aegilops speltoides* (AJ300268.2)	1	e – 137
	Triticum tauschii (AJ300565.1)	1	e – 125
ABO 00051	DNA WIS2-1A retroelement: *S. cereale x T. turgidum* (AJ291717.1)	2	e – 134
	Aegilops squarrosa (AJ291716.1)	1	e – 125
	RCCR[f] gene: *Hordeum vulgare* (AJ243066.1)	1	5e – 04
ABO 00054	Telomere-associated DNA: *Triticum aestivum* (Z75576.1)	1	7e – 18
	Ty1-copia retrotransposon: *Secale cereale* (U88031.1)	4	9e – 37
	Hordeum vulgare (AJ241338.1)	3	8e – 31
ABO 00043	Chloroplast genes for LSU[g]: *Triticum aestivum* (X62117.1)	4	2e – 63
	Oryza sativa (D00207)	3	4e – 30
ABO 00044	Aminotransferase-like protein: *Oryza sativa* (AF324485)	2	1e – 66
	M[h] -induced giant cell protein: *Lycopersicon esculentum* (L24012.1)	1	0.007
	DNA: non-functional rpl23 homologue: *Triticum aestivum* (X12849.1)	2	2e – 63
ABO 00045	Rubisco rbcL gene: *Avena sativa* (L15300.1)	1	9e – 44
	Hordeum lechleri (Z49845.1)	1	2e – 32
ABO 00042	Leucine rich-like protein: *Aegilops tauschii* (AF497474.1) **CA798962**	8	e – 149
ABO 00037,40,41	LZ-LRR-NBS: *Aegilops tauschii* (AF446141.1) **CA798957,CA798960,CA798961**	4	e – 131
	Resistance gene analogue 2: *Triticum monococcum* (F326781.1)	2	7e – 25

[a] Homologous
[b] High molecular weight
[c] Retrotransposon
[d] Noduline-like-like protein
[e] Chromosome condensation factor
[f] Red chlorophyll catabolic reductase
[g] Large subunit
[h] Meloidogyne

The homological annotations of these 18 clones were classified into six functional groups (Fig. 3.7). The first group, the protein synthesis class (28%), consists of ribosomal RNA sequences, proembryo mRNA, chloroplast genes for the Rubisco large subunit, high molecular weight (HMW) and actin gene sequences, as well as aminotransferase-like proteins. The metabolic class (3%) consists of genes for the large subunit of Rubisco and red

chlorophyll catabolic reductases. The third class – structural sequences (7%) – contains tonoplast sequences, noduline-like-like sequences, telomere-associated DNA and giant cell proteins. The regulatory class (9%) is composed of ubiquitin-related sequences, glutathion-S-transferases, T-DNA integration factors and chromosome condensation factors. The fifth class consists of miscellaneous sequences (35%) such as retrotransposon-like elements, long terminal repeats, inverted terminal repeats and non-functional DNA sequences. The sequences assembled in the resistance class (18%) are that of the barley (*Hordeum vulgare*) *Mla* locus, leucine rich-like proteins, leucine zipper-leucine rich repeat-nucleotide binding site (LZ-LRR-NBS) sequences and resistance gene analogue sequences.

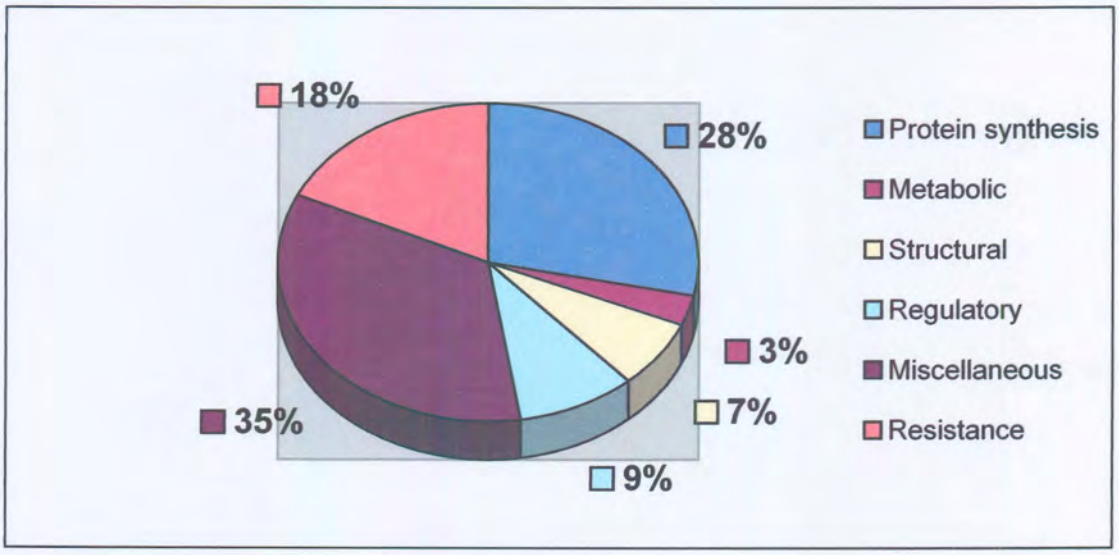

Figure 3.5 The proportional distribution of the acquired SSH ESTs presented as functional groups. Protein synthesis sequences constitutes 28% of the total amount of sequences analysed, the metabolic sequences 3%, the structural sequences 7%, the regulatory sequences 9%, miscellaneous sequences 35% and the resistance sequences 18%.

Apart from the fragments homologous to the *Mla* locus of barley, the remaining resistance fragments displayed homology to sequences identified in *Triticum monococcum* and *Aegilops tauschii*, which are the donors of the A (Kimber and Sears, 1987) and D (McFadden and Sears, 1944) genome of hexaploid wheat, respectively. The resistance gene analogue sequences (RGA2; Wicker *et al.*, 2001) were identified in *T. monococcum*, whereas the leucine rich-like proteins (Anderson *et al.*, 2002) and LZ-LRR-NBS (Brooks *et al.*, 2002) sequences were identified in *Ae. tauschii*. Figure 3.6 represents the nucleotide homology obtained between the L-SSH L fragment and a LZ-LRR-NBS sequence identified in *Ae. tauschii* (Brooks *et al.*, 2002).

```
Q: 29       cgcgagctttgctattatagttggatcctatgatgtttctcc--ccctctatctc-cttg 85
                      *           *              **       *  *
S: 71744    cgcgagctttgctactatagttggatcttatgatgtttctcctcccctcta-ctctcttg 71686

Q: 86       tgatgaattgagttttccctttgaagttatcttatcggcttgagtctttaaggattcgag 145
                                  *           *            *    *
S: 71685    tgatgaattgagttttccctttgaagttgtcttatcggattgagtctttaatgatttgag 71626

Q: 146      aacacttgatgtatgtcttgcatgtgcttatctgtggtgacaatgggatattcacgtgat 205
                         **  *                                *      **
S: 71625    aacacttgatgtatgtcttgcc-gtgtttatctgtggtgacaatgggatat-cacgtg-- 71570

Q: 206      ccacttgatgtatgttttggtgatcaacttgcgagttctgtgaccttgtgaacttatgca 265
                                           *   *  *****
S: 71569    ccacttgatgtatgttttggtgatcaacttgcgggttccg---cccatgaacctatgca 71514

Q: 266      tagggcttggcacacgttttcatcttgactatctggtagaaccttggggcactctttga 325
                *                 *       * *       *
S: 71513    tagggttggcacacgttttcgtcttgactctccggtagaaactttggggcactctttga 71454

Q: 326      agttgtttgtgtttgttgaatagatgaatctgagattgtgtgatgcatatcgtataatca 385
              **    *                        *
S: 71453    agtactttgtgttggttgaatagatgaatctgagattttgtgatgcatatcgtataatca 71394

Q: 386      tacccacagatacttgaggtgacattggagtatctaggtgacattagggttttgg 440
              *     *                *
S: 71393    tgcccacggatacttgaggtgacaatggagtatctaggtgacattagggttttgg 71339
```

Figure 3.6 The sequence of the L-SSH L fragment (S) exhibiting 90% homology to a LZ-LRR-NBS type of resistance gene analogue (Q; RGA2). The length of the sequence = 411 bp, the score of the sequence = 476 bits, the e-value = e-131 and the proportion gaps = 12/415 (2%) (Brooks *et al.*, 2002). S = subject, Q = query, * represent single nucleotide polymorphisms (SNPs).

4.4 Southern blot analyses

The Southern blot analyses were performed using three different genome sets, namely AABBDD (lane 1; *Triticum aestivum*), AA (lane 2; *T. urartu*) and BB (lane 3; *Ae. speltoides*). Southern blot analyses confirmed that the five selected fragments obtained during SSH are indeed components of the PI 137739 (*Triticum aestivum*) genome (Lane 1, Fig 3.7). Fragment L-SSH E, the ubiquitin carrier protein, served as internal control indicating equal loading and exhibited equal representation in the three genomes investigated (PI 137739, *T. urartu* and *Ae. speltoides*). The three LZ-LRR-NBS fragments (L-SSH G, L-SSH H and L-SSH J) differed in their levels of abundance between the genomic DNA of these three cultivars. L-SSH G displays an equal representation in the genomes of PI 137739, *T. urartu* and *Ae. speltoides*. L-SSH H is equally abundant in *T. urartu* and in PI 137739 and altogether absent in *Ae. speltoides*. L-SSH J is equally abundant in PI 137739 and *T. urartu*, but much less abundant in *Ae. speltoides*. The leucine rich-like gene fragment L-SSH L is similar to L-SSH G, and are equally abundant in PI 137739, *T. uaratu* and *Ae. speltoides*.

It is clear therefore, that L-SSH G is equally abundant in the three genomes of hexaploid wheat. L-SSH H appears to be equally abundant in the AA and DD genome, but it is completely absent from BB genome. L-SSH J is present in equal copies in the AABBDD and AA genomes, but less abundant in the BB genome. Therefore, it can be assumed that this fragment is present in equal copies in the AA and DD genomes of hexaploid wheat.

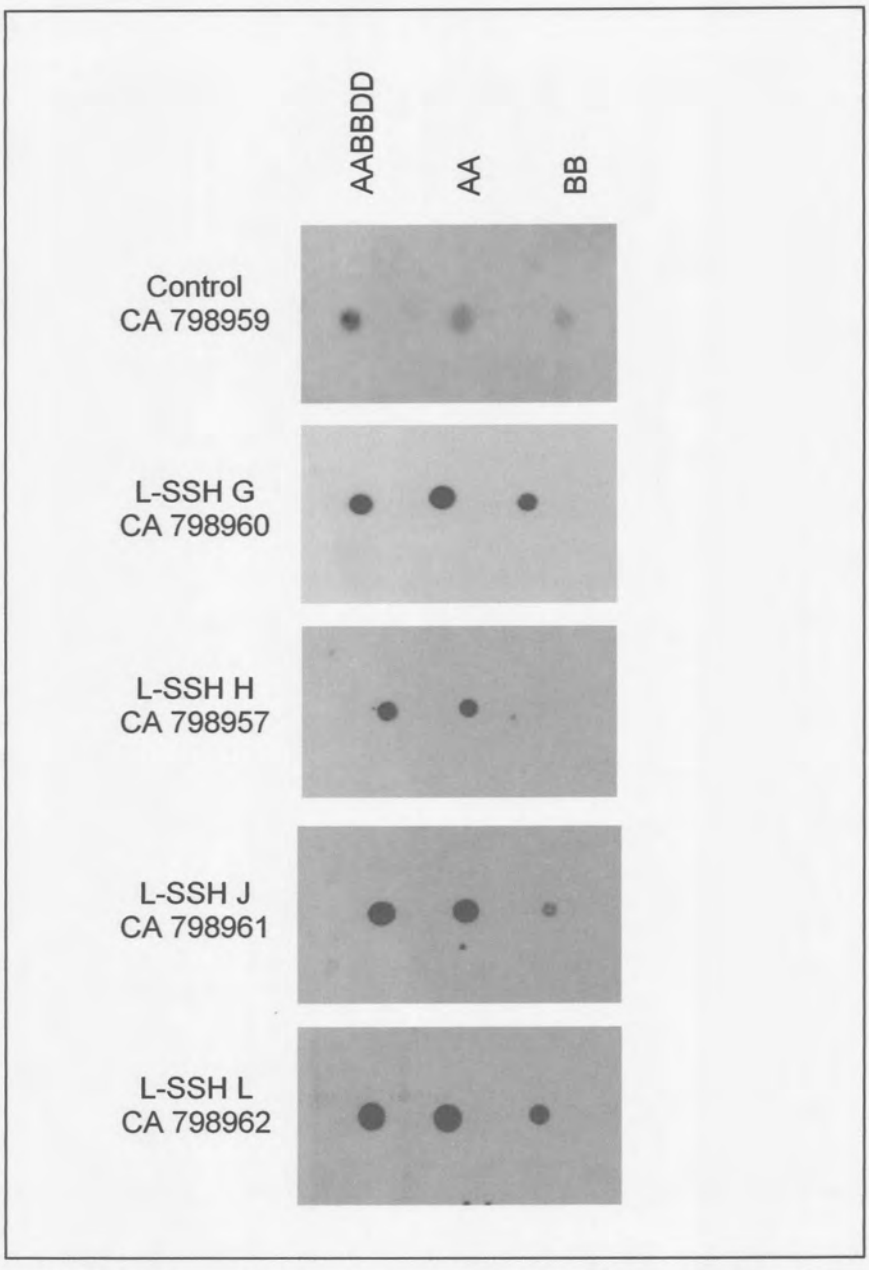

Figure 3.7 Southern blot analyses of the three LZ-LRR-NBS (L-SSH G, L-SSH H, L-SSH J), one leucine rich-like protein (L-SSH L) and one ubiquitin carrier protein (L-SSH E) clones. Each blot consists of 200 ng of *Triticum aestivum* (PI 137739; lane 1), *Triticum urartu* (lane 2) and *Aegilops speltoides* (lane 3) genomic DNA.

4.5 Northern blot analyses

Northern blot analyses (Fig. 3.8) were performed using total RNA extracted from uninfested PI 137739 plants (lane 1) and total RNA extracted from Russian wheat aphid infested plants at day 2 (lane 2), day 3 (lane 3), day 4 (lane 4) and day 5 (lane 5). The ubiquitin carrier protein served as internal control and this fragment (L-SSH E) exhibited equal expression in all the uninfested, as well as infested RNA populations.

It is noticeable that the LZ-LRR-NBS (L-SSH G, L-SSH H and L-SSH J) and leucine rich-like protein (L-SSH L) fragments exhibit an overall similar pattern of expression when the northern blots are compared. Expression of these fragments in uninfested plant tissue and expression during the second day of infestation were equal. Three days after RWA infestation a large decrease in expression was observed, since expression was almost undetectable. The expression of these fragments was slightly up-regulated during day 4 and significantly up-regulated during day 5 after RWA infestation. These results indicate a large up-regulation of these genes occurred five days after infestation with RWA.

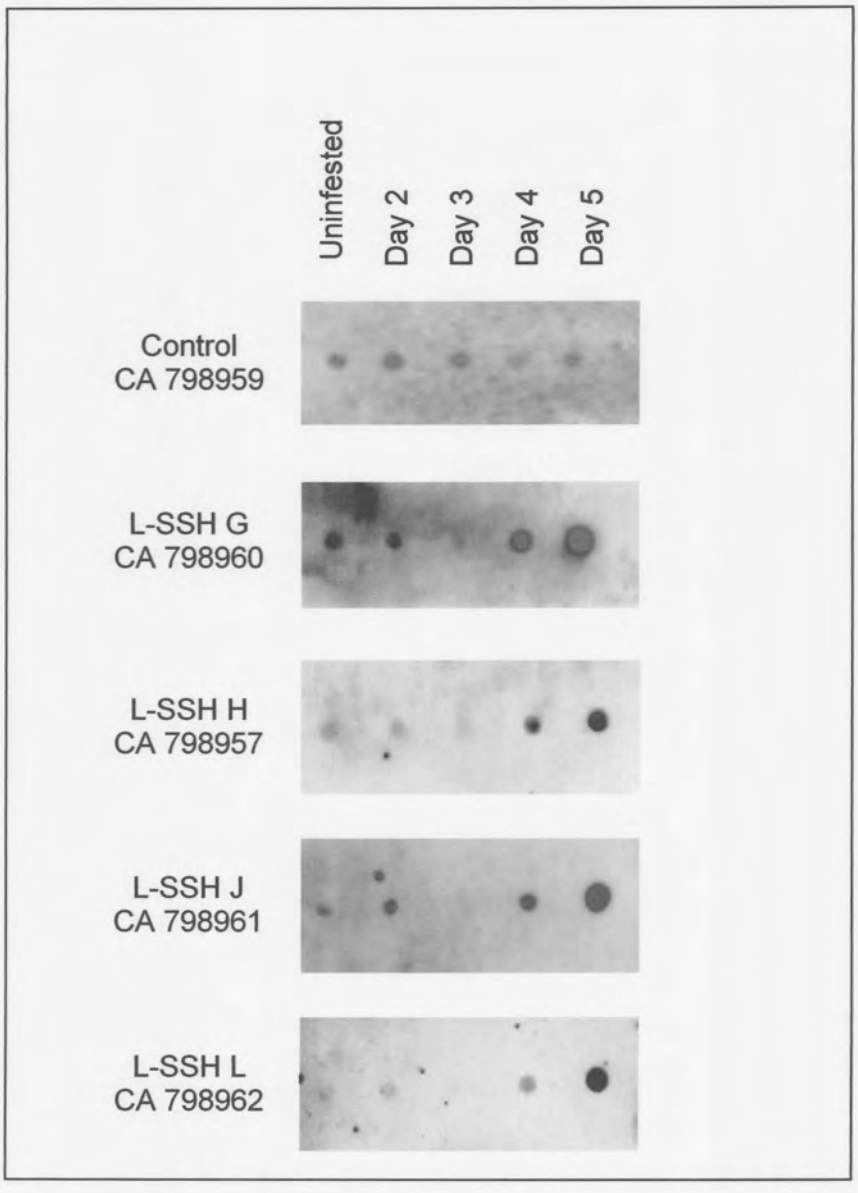

Figure 3.8 Northern blot analyses of the three LZ-LRR-NBS (SSH G, SSH H, SSH J), one leucine rich-like protein (SSH L) and the ubiquitin carrier protein (SSH E) gene fragments. Lane 1 represents total RNA extracted from uninfested wheat plants and lanes 2 to 5 represent total RNA extracted from RWA infested plants at days 2 to 5, respectively.

4.6 Quantitative PCR analyses

The $MgCl_2$ titration indicated that the optimum $MgCl_2$ concentration to be used during quantitative PCR reactions should be 3 mM (Fig. 3.9).

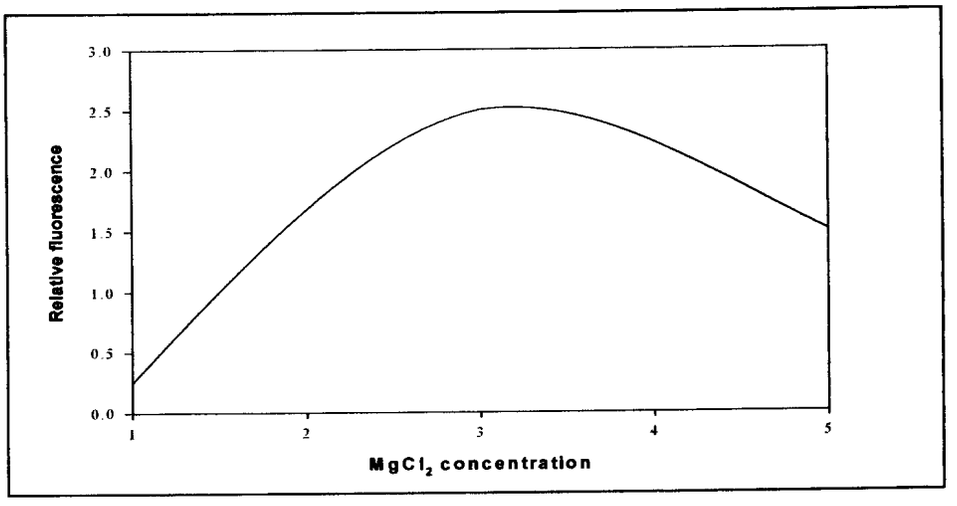

Figure 3.9 The $MgCl_2$ titration of 1 mM, 3 mM and 5 mM $MgCl_2$. The graph indicates that 3 mM $MgCl_2$ is the optimum concentration for subsequent quantitative PCR reactions.

A standard curve representing a typical quantitative PCR reaction is indicative of the efficiency of the PCR reaction (Fig. 3.10).

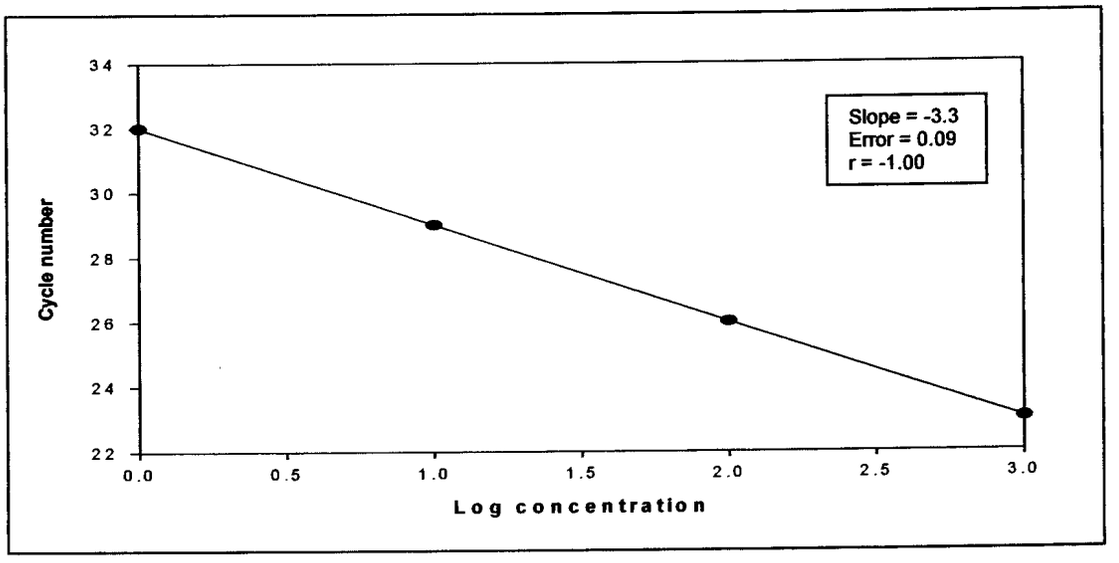

Figure 3.10 A standard curve indicating the efficiency of a quantitative PCR. The slope of the standard curve = -3.3, the error value = 0.09 and the linear regression coefficient = -1.00.

The quantitative PCR analyses of the four resistance-related SSH fragments (L-SSH G, L-SSH H, L-SSH J and L-SSH L) confirmed the results obtained with the Northern blot hybridisations. The melting curve analysis produced a single melting peak for each of the amplicons that represented the product formed during the PCR reactions (Deprez *et al.*, 2002). The data obtained from the PCR reactions of these fragments were compared with the data of the uninfested RNA samples (Table 3.4), as well as with the control fragment (L-SSH E; Table 3.5). Both data sets indicate that the expression of the resistance-related fragments increased slightly after two days of RWA infestation. Day 3 exhibits a down-regulation of these fragments and during day 4 and especially day 5, a drastic increase in expression of these fragments are observed. Table 3.4 also includes the threshold cycle (C_T) value, linear regression coefficient (r), error value and slope for each data set.

Table 3.4 Quantitative assessment of the expression levels of the LZ-LRR-NBS (L-SSH G, L-SSH H and L-SSH J), leucine rich-like protein (L-SSH L) and ubiquitin carrier protein (L-SSH E) gene fragments after Russian wheat aphid infestation when compared to the uninfested RNA expression levels. The amount of up- or down-regulation is presented as –fold increase or decrease, with the threshold cycles indicated.

Fragment	Day 2*	Day 3*	Day 4*	Day 5*	Slope	Error	r value
L-SSH E	1.4 C_T = 27	*1.4*# C_T = 28	2.3 C_T = 29	1.1 C_T = 27	-3.6	0.06	-1.00
L-SSH G	3.6 C_T = 22	*2.1*# C_T = 25	21.9 C_T = 20	988 C_T = 12	-3.5	0.11	-1.00
L-SSH H	3.1 C_T = 25	*12.8*# C_T = 30	17.4 C_T = 23	1218.9 C_T = 16	-3.5	0.06	-1.00
L-SSH J	10.1 C_T = 22	*9.2*# C_T = 29	38.6 C_T = 20	4466.4 C_T = 13	-4	0.3	-0.97
L-SSH L	15.7 C_T = 25	*5.5*# C_T = 28	798 C_T = 22	11218 C_T = 16	-2.6	0.13	-0.99

* Values were normalised with uninfested RNA expression levels
\# Values represent down-regulation

The leucine rich-like protein fragment (L-SSH L) exhibits by far the highest levels of induction at each point in the time study when compared to the expression in the uninfested samples. The LZ-LRR-NBS fragment that exhibits the highest levels of expression is L-SSH J. The expression levels of L-SSH H are 3.7 times less than that of L-SSH J and that of L-SSH G is 4.5 times less than L-SSH J. The expression of L-SSH L is 2.5 times higher than L-SSH G, 9.2 higher than L-SSH H and 11.3 times higher than L-SSH J.

Table 3.5 Comparison of the quantitative expression levels between the resistance-related fragments and the control fragment (ubiquitin carrier protein). The amount of up- and down-regulation is presented as –fold increase and decrease.

Fragment	Day 2*	Day 3*	Day 4*	Day 5*
L-SSH G	2.6	*1.5*#	9.5	898.2
L-SSH H	2.2	*9.1*#	7.6	1108.1
L-SSH J	7.2	*6.6*#	16.8	4060.4
L-SSH L	11.2	*3.9*#	347	10198.2

* Values were normalised with control (L-SSH E) expression levels
# Values represent down-regulation

5. DISCUSSION AND CONCLUSION

The study of differentially expressed genes has been simplified through the development of suppression subtractive hybridisation (SSH; Diatchenko *et al.*, 1996). Since SSH allows the detection of low-abundance differentially expressed genes (Birch *et al.*, 2000), the isolation of rare mRNA transcripts are possible.

During this study, several biologically important gene fragments were identified through the application of SSH. Apart from fragments related to resistance sequences, protein synthesis-encoding gene fragments and sequences with metabolic, regulatory and structural functions were also identified. These sequences were identified due to the presence of cDNA fragments in both the tester and driver cDNA populations after subtraction. This is possible since some differentially expressed mRNAs are present in both the cDNA populations (Gurskaya *et al.*, 1996; Diatchenko *et al.*, 1999). During an attempt by Caturla and his colleagues (2002) to isolate low-abundance genes involved in the activation of meristem cell division after water-logging, 192 different SSH clones were obtained. These included gene fragments for protein synthesis, primary and secondary metabolism, as well as cell wall proteins. They were, however, able to identify 66 transcripts (34.4%) relevant to their specific objectives (Caturla *et al.*, 2002). SSH was employed by Kloos and his colleagues (2002) as a means to isolate differentially expressed taproot genes and they were able to isolate six such sequences. A salt-stress study conducted by Zhang and his colleagues (2002) enabled them to isolate five differentially expressed cDNAs. During the present study, 18 fragments (18%) were identified that were linked to resistance gene sequences. The proportion of sequences identified involved in protein synthesis was 28%, the metabolic ratio 3%, the regulatory sequences 9%, structural sequences 7% and 34% were classified as miscellaneous fragments. Therefore, although SSH highly enriches the cDNAs of rare transcripts, unwanted genes fragments were also enriched and amplified (Diathchenko *et al.*, 1999).

Resistance (R) genes have been classified into four main groups (Hammond-Kosack and Jones, 1997) that consist of similar sequences and domain organisation (Young, 2000). Of these classes, the leucine rich repeat-nucleotide binding site (LRR-NBS) group is the largest (Tao *et al.*, 2000; Halterman *et al.*, 2001) and is involved in pathogen and pest resistance in plants (Michelmore, 2000). Twelve of the resistance-related fragments identified during this study are related to the LRR-NBS resistance class: eight are homologous to leucine rich-like proteins and four are homologous to leucine zipper-leucine rich repeat-nucleotide binding site (LZ-LRR-NBS) sequences.

The LRR structure of the LRR-NBS domain is thought to be involved with gene-for-gene recognition (Staskawicz *et al.*, 1995) and protein-protein interactions during plant infection (Kobe and Deisenhofer, 1994). The LRR structure is able to bind various ligands to its surface (Braun *et al.*, 1991) and probably interacts with other proteins during signal transduction (Hammond-Kosack and Jones, 1997). The leucine zipper (LZ)-containing sequences are classified as constituents of the non-toll/interleukin receptor (non-TIR) sub-class of the NBS domain (Lupas, 1996). The leucine zipper consists of repeating units of leucine residues that project from long, solvent-exposed α helices to facilitate the dimerisation of two molecules (Landschulz *et al.*, 1988). A putative function of the leucine zipper is the dimerisation of resistance-related proteins when they are activated during pathogen infection (Hammond-Kosack and Jones, 1997). The leucine zipper unit of the RPS2 protein that confers resistance to various pathogens in *Arabidopsis* is crucial for the function of the RPS2 protein (Tao *et al.*, 2000). NBS-LRR containing genes have also proved to provide resistance against several nematode species, as well as potato aphid resistance, in the wild tomato species, *Leucopersicon peruvianum* (Milligan *et al.*, 1998; Rossi *et al.*, 1998).

In the present study, two of the resistance-related sequences that were identified are homologous to some of the NBS class resistance gene analogues (RGAs) from *Triticum monococcum* identified by Seah and his

colleagues (1998). Nine RGA categories have been identified in rice based on their similarity to the NBS domain of disease resistance genes (Mago *et al.*, 1999). Further, RGA probes from maize, rice and wheat were used to map 17 loci in barley (Collins *et al.*, 2001). Some of these probes have been used to successfully map wheat RGAs, which is indicative of the conservation of RGAs between barley and wheat (Collins *et al.*, 2001). The homology of the RGA fragments identified from RWA resistant wheat to that of *T. monococcum* is indicative of the location of these gene fragments on the A genome of hexaploid wheat, since *T. monococcum* is considered as the donor of this genome (Kimber and Sears, 1987).

The close resemblance of RGA fragments in wheat with that of barley may explain the homology of the L-SSH G, H, J and L gene fragments in wheat to the *Mla* gene in barley (*Hordeum vulgare*). The *Ml* locus consists of 11 variants and confers resistance in barley to the powdery mildew fungus, *Erysiphe graminis* f. sp. *hordei*. One variant of this locus is the *Mla* locus (Wei *et al.*, 1999) that consists of 28 alleles and is interspersed within a 240 kilobase region (Ellis *et al.*, 2000). This 240 kb region contains multiple LRR-NBS gene clusters (Jones, 2001). Halterman and his colleagues (2001) identified three classes of cDNAs within this region, of which one class codes for coiled-coil (CC)-NBS-LRR proteins.

Accurate measurement of differential gene expression can easily be determined using quantitative real-time, fluorescent PCR (Muller *et al.*, 2002). The data can be collected in a log linear phase of the PCR reaction, the amount of cycles (threshold cycles, C_T) needed to detect amplification signals, as well as the initial amount of mRNA/cDNA can be determined (Deprez *et al.*, 2002). Since the C_T is inversely proportional to the log of the initial amount of target sequences, fewer C_T cycles will be needed when the initial amount of target sequence is high (Karsai *et al.*, 2002). The linear regression coefficient (r) is an indication of the quality of the standard curve for each experiment (Deprez *et al.*, 2002). The melting curve cycle is included since the fast loss of fluorescence of each amplicon takes place at its unique melting or denaturing temperature. During the present study quantitative real-time PCR

was used to confirm the data obtained through Northern blot analyses of resistance-related gene fragments. The Northern blot analyses indicated a slight induction in expression of the four L-SSH gene fragments after two days of Russian wheat aphid infestation. At day three a decrease in gene expression was observed, but during day four and especially day five, a drastic increase in gene expression was detected. The data generated during PCR support these findings and depicts the up- and down-regulation of gene expression even more accurately, since exact values were obtained for the levels of induction. Further, differences in gene expression between the various fragments were more pronounced than the visual evaluation of the Northern blots.

Findings parallel to those of the present study can be found in other studies concerned with insect infestation of plants. Distinct changes in infested plants take place after infestation: from differing oxidation reactions (Miles and Oertli, 1993) to the synthesis of certain pathogenesis related proteins (Broderick *et al.*, 1997; Van der Westhuizen *et al.*, 1998a, b). Miles and Oertli (1993) investigated the effect of sucking insects (e.g. *Therioaphis trifolii maculata*, *Acyrthosiphon kondo* and *Aphis pomi*) on infested plants and found that phenolic compounds are mobilised and oxidised. The initial phenolic molecules served as deterrents for the aphids and the phenolic compounds that form later on, closed off the damaged cells (Miles and Oertli, 1993). Broderick and his colleagues (1997) ascertained the induction of pathogenesis related (PR) proteins such as peroxidases, β-1, 3-glucanases and chitinases in clover after redlegged earth mite infestation. The induction of peroxidases, β-1, 3-glucanases and chitinases have also been observed in wheat plants infested with Russian wheat aphids (RWA; Van der Westhuizen and Pretorius, 1996; Botha *et al.*, 1998; Van der Westhuizen *et al.*, 1998a, b). The composition of intercellular proteins changed quantitatively in infested resistant wheat plants and two proteins similar to tobacco chitinases were isolated by Van der Westhuizen and Pretorius (1996). An increase in endochitinase activity in the intercellular washing fluids was also observed in wheat plants after RWA infestation (Botha *et al.*, 1995). The study conducted by Van der Westhuizen and his colleagues (1998b) further revealed the

gradual induction of β-1, 3-glucanases from day one to two after RWA infestation and a steep increase in β-1, 3-glucanase synthesis from two to ten days after infestation. The present study revealed equivalent induction of several resistance-related fragments two days after RWA infestation when compared to the induction of β-1, 3-glucanases and chitinases. The reduced induction of these fragments after three days of infestation is a unique finding, but the sharp induction that follows at day four and five corresponds to the induction of PR-proteins and oxidative processes.

The identification of resistance gene fragments through the past decade has enhanced the study of plant responses to pathogen and insect attack dramatically. New technological advances aid this movement towards unveiling even more about gene-for-gene and protein-protein interactions between plants and pests (Michelmore, 2000). The suppression subtractive hybridisation technique for expressed gene identification (Diatchenko *et al.*, 1996) has been employed successfully during the present study to identify differentially genes expressed in wheat after RWA infestation. Specific gene fragments related to plant resistance responses have been identified and their level of induction after infestation has been determined. Although SSH could be improved to obtain only the genes of interest, it proved useful during this study. The leucine rich-like fragments and leucine zipper-LRR-NBS fragments identified during this study leads the way for mapping of these fragments on the wheat genome, as well as for further studies related to resistance responses in wheat.

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Chapter 4

Transcriptome changes in response to *Diuraphis noxia* feeding in *Triticum aestivum* utilising a custom array

The data presented in Chapter 4 have been included in a journal publication entitled:

Gene expression profiling during *Diuraphis noxia* infestation of *Triticum aestivum* cv. 'Tugela DN' using microarrays.

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1. ABSTRACT

Diuraphis noxia (Russian wheat aphid; RWA) is a major pest on wheat in South Africa and many other wheat-growing countries. Many resistance (R) genes from various plant species have conserved amino acid domains, particularly the nucleotide binding sites (NBS) and leucine rich repeats (LRRs), which are consistent with their putative roles in signal transduction and protein-protein interactions. Previous studies on the intercellular washing fluid (IWF) of wheat cultivars resistant ('Tugela DN') and susceptible ('Tugela') to RWA showed alteration of some protein complexes within the first 12h period after RWA infestation in the resistant cultivar, but not in the susceptible near isogenic line (NIL). During these studies, both over-expression and down-regulation of proteins were observed in chitinase, ß-1, 3-glucanase and peroxidase activities after RWA infestation in wheat. Two responses, an initial hypersensitive response (HR) that decreases after approximately 24h, which was followed by systemic acquired resistance (SAR) that prevails in the tissue for an extended period of time, were observed previously in RWA infested wheat. During the present study, wheat sequences that were obtained using degenerate primer sets designed from the consensus NBS motif identified in other genomes (e.g. *Arabidopsis* and rice), subtraction suppression hybridisation (SSH), RACE (rapid amplification of 5' and 3' cDNA ends)-PCR and cDNA libraries, were examined. Selected wheat cDNA clones were spotted onto microarray slides. Purified mRNA from infested material, containing the RWA resistance gene *Dn1*, was isolated 0, 2, 5 and 8 days after infestation, post-labeled with Cy3- or Cy5- fluorescent dyes and hybridised to the microarray targets. Statistical analysis of the data using the Mixed Model approach revealed regulation of 12% of all the spotted gene fragments at a threshold log-2 expression ratio of 1.5 and $P \leq 0.05$. During this study, several gene fragments related to plant defence responses were identified. They include three resistance gene analogues (RGAs) and four leucine rich-like sequences.

2. INTRODUCTION

Differential gene expression patterns in living organisms result from their responses to external factors that influence their survival. The Russian wheat aphid (RWA; *Diuraphis noxia*), a serious threat to wheat (*Triticum aestivum* L.) crops in South Africa, is a pest who's infestation leads to changes in gene expression and protein synthesis patterns, specifically pathogenesis related (PR) proteins such as chitinases, β-1, 3-glucanases and peroxidases (Van der Westhuizen and Botha, 1993; Van der Westhuizen and Pretorius, 1996; Botha *et al.*, 1998; Van der Westhuizen *et al.*, 1998a, b). Results of chitinase profiling when employing intercellular washing fluid (IWF) and Western-blot analysis indicated that there are distinct differences between the obtained iso-enzymes and chitinase subunits after RWA infestation, wounding and exogenous ethylene treatments (Botha *et al.*, 1998). Studies conducted on the IWF of wheat resistant to the Russian wheat aphid ('Tugela DN'), showed that proteins were induced within six days of infestation.

The cloning of multiple resistance (R) genes from various plant species has revealed the existence of conserved domains at the amino acid level. The most notable is the presence of nucleotide binding sites (NBS) and leucine rich repeats (LRRs; Young, 2000). The presence of a NBS and a LRR are consistent with the protein products that play a significant role in signal transduction and putative protein-protein interactions (Whitham *et al.*, 1994; Jackson and Taylor, 1996; Pan *et al.*, 2000; Cannon *et al.*, 2002). The guard hypothesis proposes that NBS-LRR proteins guard plant targets against pathogen effector proteins; in this scenario, these pathogen products act as virulence factors to enhance susceptibility of the host plant in the absence of recognition (Van der Biezen and Jones, 1998; Dangl and Jones, 2001). The nucleotide binding sites are, further, required for ATP and GTP binding (Walker *et al.*, 1982; Saraste *et al.*, 1990). The ability of plant NBS-LRR proteins to bind nucleotides has been demonstrated for the tomato *Cf-2* gene (Dixon *et al.*, 1996) and the *Arabidopsis RPS2* gene (Tao *et al.*, 2000).

A method developed to improve the scientists' understanding of gene regulation and expression, especially plant defence responses, is microarray analysis (Baldwin *et al.*, 1999; Granjeaud *et al.*, 1999; Kehoe *et al.*, 1999). Microarray technology is based on well-known molecular techniques that underwent a series of modifications in order to adapt the technique for combining large sets of data from different experiments (Breyne and Zabeau, 2001). Microarrays have been described as second-generation dot-blots (Rockett and Dix, 1999), since both techniques involve the immobilisation of single-stranded DNA on a solid support (Southern, 1975) that is hybridised with a single-stranded DNA or RNA population (Hoheisel, 1997; Gerhold *et al.*, 1999; Rockett and Dix, 1999). Hybridisation is possible between two such populations since single-stranded DNA or RNA can bind to its single-stranded complement (Chee *et al.*, 1996; Brazma *et al.*, 2000).

Today, two methodically distinct techniques are employed to assemble microarrays. The first technique, namely oligonucleotide microarrays (Schena *et al.*, 1998), involves the synthesis of oligonucleotides that vary from 10 to 25 base pairs (Thieffry, 1999). The synthesis of these oligonucleotides takes place either *in situ* (Pease *et al.*, 1994; Schena *et al.*, 1998) or the amino acid chains are synthesised separately and then spotted onto a glass support (Marshall and Hodgson, 1998). One advantage of oligonucleotide microarrays is the detection of polymorphisms since the sequence of the target fragment can be determined (Hoheisel, 1997). Hacia and his colleagues (1998) utilised this characteristic during their examination of the breast and ovarian cancer gene, *BRCA1*, by carrying out mutational analysis of the 3.43 kb exon 11 of this gene. Further accomplishments have been the detection of single nucleotide polymorphisms (SNPs) in the human (Wang *et al.*, 1998) and yeast genomes (Winzeler *et al.*, 1998). More recently, Coller and her colleagues (2000) analysed 6416 genes and ESTs in an attempt to analyse changes in gene expression due to the activation of c-MYC (a proto-oncogene) in human fibroblast tissue. The second synthesis technique involves the exogenous spotting of long DNA fragments (from 50 base pairs to full-length cDNAs) onto solid supports (Schena *et al.*, 1998) and is known as DNA fragment-based or cDNA microarrays (Richmond and Somerville,

2000). The *Arabidopsis* genome, e.g., is being studied by employing cDNA microarray technology. Schenk and his colleagues (2000) have examined transcriptional changes in 2375 *Arabidopsis* ESTs that are involved in defence and regulatory responses to certain stress conditions.

A previous study conducted on the response of wheat to RWA infestation yielded several NBS-LRR gene fragments (Lacock *et al.*, 2003). This study utilised degenerate primer sets designed from the consensus NBS motif previously identified (Yu *et al.*, 1996), subtraction suppression hybridisation (SSH), RACE (rapid amplification of 5' and 3' cDNA ends)-PCR and cDNA libraries and was conducted on wheat cultivars containing the *Dn1* resistance gene. The feasibility of using the degenerate PCR approach was tested and it was found that 18% of all the obtained ESTs showed significant amino acid homology to resistance genes from other plants (E value $\leq 10^{-5}$; Kruger *et al.*, 2002), rendering the approach highly feasible if resistance gene analogues are the target of interest. From this previous study (chapter 2), numerous ESTs were isolated with no discernable function when compared to existing published data in GenBank, but homologues to NBS-LRR putative resistance genes were also isolated. The focus of the present study was to test the feasibility of utilising microarray technology in monitoring gene expression and regulation in RWA-infested wheat plants. Secondly, an attempt was made to identify specific genes involved in RWA resistance by incorporating microarray analysis. The previously isolated ESTs were used as template material in order to accomplish these goals. The expression profiles of these EST sequences were monitored by hybridising them against cDNA synthesised from leaves of the RWA resistant cultivar 'Tugela DN' pre- (day 0) and post-infestation (days 2, 5 and 8). The feasibility of utilising microarray technology in gene expression and regulation studies was compared to incorporating Northern blot analysis and quantitative real-time PCR strategies.

3. MATERIALS AND METHODS

3.1 Plant material

The Russian wheat aphid resistant cultivars 'Tugela DN' (Tugela*5/SA1684; *Dn1*) and PI 137739 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 24°C and the plants were watered daily.

3.2 RNA extraction and mRNA purification of probe material

Thirty 'Tugela DN' wheat seedlings were infested with RWA (10 aphids per plant) at the 3-4-leaf growth stage (Botha *et al.*, 1998). The aphids were removed from the infested leaves prior to RNA isolation through rinsing with water. RNA was extracted (Chomczynski and Sacchi, 1987) from 10 control (uninfested) plants using the second and third leaves, as well as from infested plants at day 2, 5 and 8 post-infestation – 10 plants for each infestation period – using the second and third leaves. The RNA samples were stored at -80 °C. mRNA was purified from the four RNA samples (mRNA Purification Kit, Amersham Pharmacia Biotechnology, USA) and cDNA synthesised (cDNA Synthesis System, Roche Molecular Biochemicals, Germany) from the mRNA. The obtained cDNA fragments were cloned (pGEM-T Easy Vector System, Promega Corporation, USA) and sequenced (BigDye Terminator Sequencing Reaction Kit, Perkin-Elmer). These cDNA samples served as the probe material to be hybridised with the ESTs/clones immobilised on the microarray slides.

3.3 Fluorescent probe preparation

Cy3- and Cy5-labelled cDNA probes were prepared from total RNA extracted from PI 137739 wheat leaves on days 0, 2, 5 and 8 post-RWA-infestation, according to the method of Chomczynski and Sacchi (1987). Poly A^+ RNA was purified from total RNA using the Oligotex mRNA spin-column

protocol (Oligotex™ Handbook 07/99, Qiagen, USA). 100 ng of the purified mRNA was used to prepare Cy3- and Cy5-labelled cDNA for microarray hybridisation using the Cyscribe Post-labelling Kit according to the manufacturer's instructions (Amersham Biosciences, Little Chalfont, UK). Unincorporated label and single stranded nucleotides were removed from the prepared labelled cDNA using the MinElute Cleanup Kit (MinElute™ Handbook 04/2001, Qiagen, USA). The probe samples for each of the individual time intervals were pooled for use during hybridisation.

3.4 Sequence analysis of target ESTs

Expressed sequence tags (ESTs) were obtained from previous studies through analysing cDNA of the relevant wheat cultivars, as well as from suppression subtractive hybridisation (SSH) and RACE-PCR of RWA infested and uninfested wheat material (Chapter 2 and 3; Lacock *et al.*, 2003). The identities of these ESTs were annotated by using the BLASTN (http://www.ncbi.nlm.nih.gov/BLAST/; Altschul *et al.*, 1997) algorithm and by aligning the sequences to other published sequences in GenBank. Functions were assigned to the dbESTs based on the BLAST results ($E \leq 10^{-5}$; Kruger *et al.*, 2002) and the sequences were classified into functional groups (Chapter 2 and 3; Lacock *et al.*, 2003). These ESTs/clones were spotted onto the microarray slides as target material.

3.5 Microarray slide preparation

The target cDNA (192 wheat ESTs, 55 flax and 33 banana clones) were amplified using standard PCR procedures (40 cycles; annealing at 64 °C; 2 ng plasmid template). The PCR products were purified using Multiscreen purification plates (Montage PCR_{96} Cleanup Kit, Millipore Corporation, USA), quantified by electrophoresis on a 0.8% (w/v) agarose gel and visualised by ethidium bromide staining on UV light. Microarray slides were printed on aminosaline slides using a BioRobotics Generation II Arrayer according to the manufacturer's instructions. The total amount of target DNA spots printed consisted of 192 wheat ESTs, 55 flax clones, 33 banana clones,

46 Lucidea Universal Scorecard clones and 8 internal control (actin gene fragment) clones. Figure 4.1 represents the dye emission output generated by the microarray software after hybridisation where each small block represents the data of one individual clone. This image serves as template for the data analysis of the various hybridisation experiments. Clone numbers are assigned to each individual spot in order to aid computational analysis of the various individual hybridisation events.

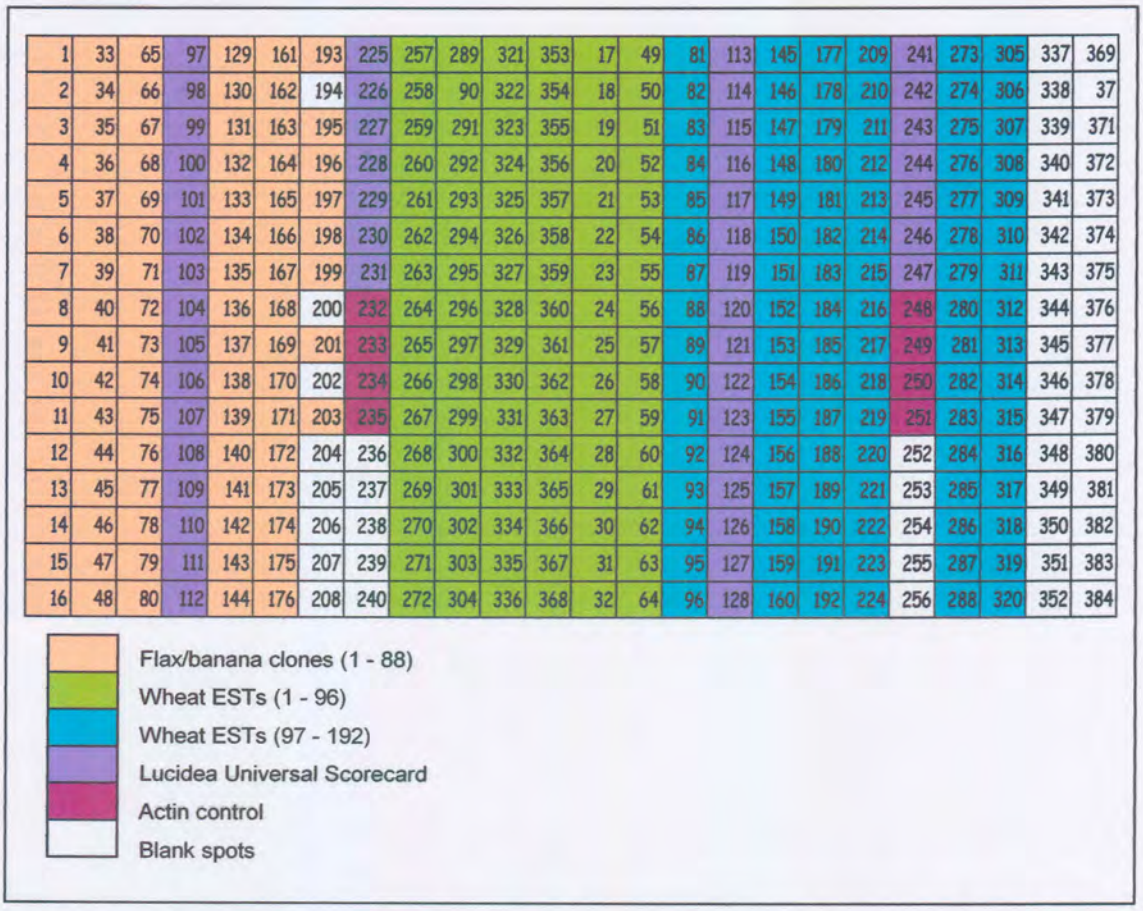

Figure 4.1 Representation of the dye emission image generated by the microarray software after hybridisation. The colour chart represents the 55 flax clones, the 33 banana clones, the 192 wheat ESTs, the Lucidea Universal Scorecard and the actin control clones (the clone numbers are indicated).

Figure 4.2 represents the complete layout of each microarray slide indicating the actual position of each spot. Each target DNA clone was printed four times in consecutive columns on the left side of each slide (Fig. 4.2; A) and this print pattern was repeated on the right side of the slide (Fig. 4.2; B). Thus, each spot was printed 8 times per microarray slide (e.g. eight number 1 spots, eight number 2 spots, etc.). A total of thirty-two microarray slides were spotted according to this layout. The Lucidea Universal Scorecard consisted of constitutively expressed and stress responsive genes.

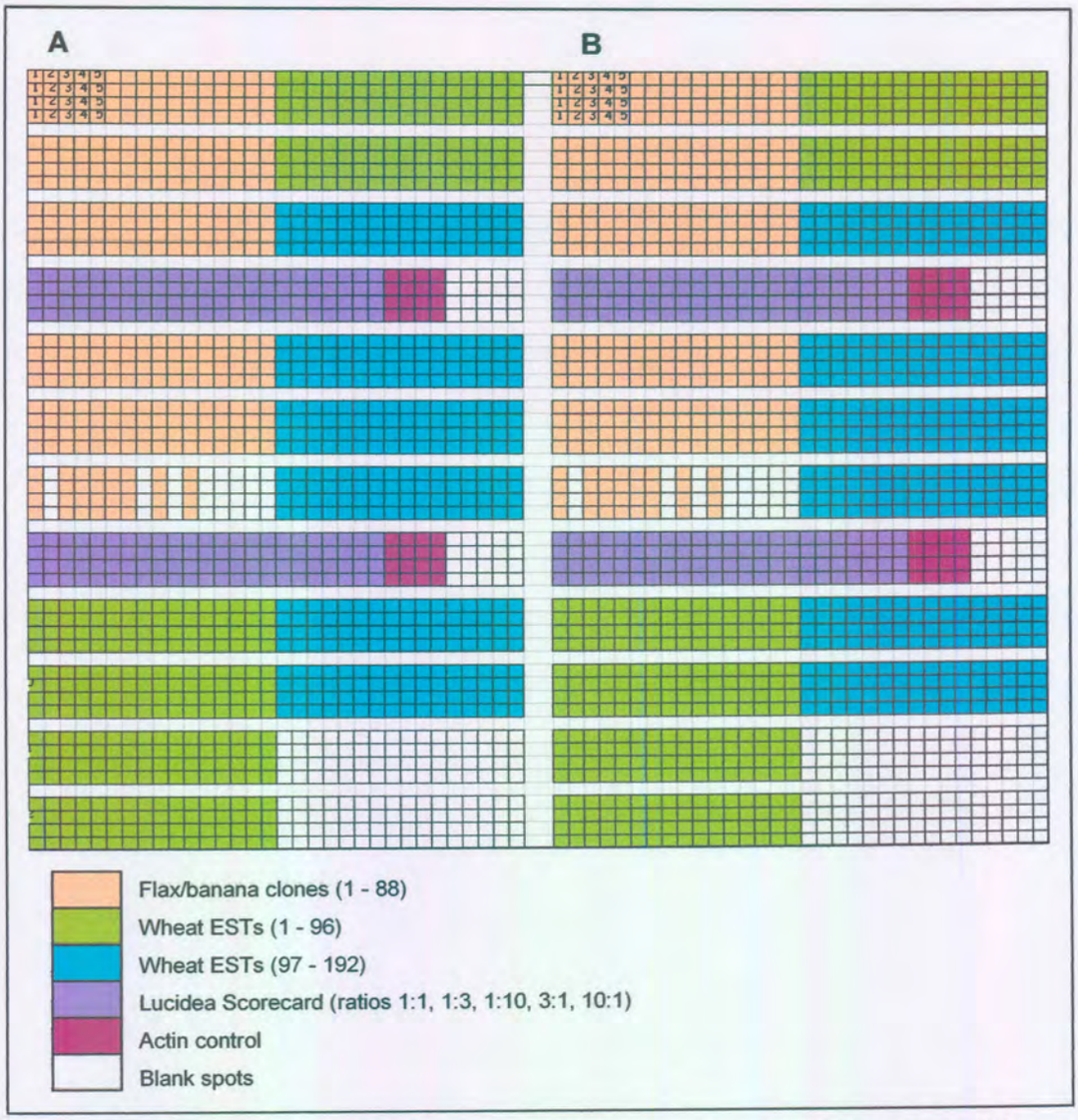

Figure 4.2 A diagram representing the layout of a complete microarray slide. Each clone was spotted 8 times – four times on each side of the slide. The colour chart represents the flax/banana clones, the wheat ESTs, the Lucidea Universal Scorecard and the actin control clones.

3.6 Microarray hybridisation

The microarray slides were pre-hybridised individually using a 35 µl pre-hybridisation solution (3.5 x SSC, 0.2% (w/v) SDS, 1% (w/v) BSA) for 20 min at 60 °C using a humidified hybridisation cassette. Slides were washed one by one in ddH2O for 1 min and air-dried using nitrogen gas. The spotted microarray slides were hybridised using combinations of probes (30 pmol per probe) of the following time-intervals: day 0 and 2, day 0 and 5, day 0 and 8, day 2 and 5, day 2 and 8, day 5 and 8. The experimental design used to assemble the microarray layout was a reference, 2x2 factorial design (Wang and Speed, 2002). Two biological repeats were performed, as well as reciprocal Cy3 and Cy5 experiments. Prior to hybridisation, the combined probe samples were dried in a 0.5 ml eppendorf tube, resuspended in 35 µl hybridisation solution (50% formamide, 25% hybridisation buffer, 25% milliQ H_2O) and denatured (98 °C for 2 min). The probe samples were pipetted onto the slides and hybridisation continued overnight at 42 °C. Each slide was washed three times at 42 °C for 4 min (once in 1x SSC, 0.2% (w/v) SDS, twice in 0.1 x SSC, 0.2% (w/v) SDS). This was followed by three washes at room temperature for 1 min each in 0.1 x SSC and drying with nitrogen gas.

3.7 Scanning and data analysis

An Axon GenePix 4000 A Microarray scanner and GenePix acquisition software (Axon Instruments, Incorporated, USA) were used in order to interpret the dye emission of each hybridisation event and to capture the data. After scanning and capturing of the data using the GenePix 3.0 software, the raw data was imported into Microsoft Excel for further analysis. Normalisation between Cy3- and Cy5-fluorescent dye emission intensities was achieved by adjusting the level of the photomultiplier gains ('global normalisation'). Background fluorescence values were automatically calculated by the GenePix program and subtracted from the obtained dye emission intensities before further calculations were performed. Two analytical approaches were employed to identify significant gene regulation and expression, namely ANOVA (analysis of variance; Dudoit *et al.*, 2001) and the Mixed Model

approach (Wolfinger *et al.*, 2001; Chu *et al.*, 2002); the computer software utilised was SAS/STAT software version 8 (SAS Institute Incorporated, 1999). Genes were organised and visualised by Cluster and Tree View (Eisen *et al.*, 1998). Spot to spot variation was determined and the expression profiles of the hybridisations with the infested probes were individually compared to the uninfested expression profiles and to each other.

3.8 Northern blot analysis

Northern blot analyses were performed using five randomly selected ESTs that exhibited high levels of regulation during microarray analysis in order to confirm the microarray data. Total RNA was extracted from uninfested (day 0) and RWA infested (day 2 and 5) wheat leaves. Probes were prepared according to the sequences of the five ESTs. Northern blots were also performed using total RNA extracted from wheat leaves and stems at day 0 and day 2 RWA-post-infestation. Probes were prepared from two RGA-2 gene fragments and two chloroplast ATP synthase gene fragments in order to compare differences in gene expression of these genes in different tissue types after RWA infestation. For each Northern blot, 200 ng RNA was transferred onto nylon membranes (Roche Diagnostic Corporation, Germany) and the RNA was UV-cross linked to the membranes (Sambrook *et al.*, 1989).

Probe labelling, using 50 ng of each fragment, was performed using the *Gene Images* Random Prime Labelling Module (Amersham Pharmacia Biotechnology, USA) according to manufacturer's instructions. Pre-hybridisation of RNA was preformed at 60 °C using the hybridisation buffer [5x SSC (75 mM NaOAc and 0.75 M NaCl), 0.1% (w/v) SDS, 5% Denhardt's solution] and a 20x dilution of Liquid Block (*Gene Images* Random Prime Labelling Module, Amersham Pharmacia Biotechnology, USA) for 3.5 hours. 15 µl of each probe was heat denatured for 5 min and added to the respective pre-hybridised membranes. Hybridisation was done overnight at 65 °C in a HB-1D Hybridiser (TECHNE, Cambridge, UK). Two stringency washes followed hybridisation. The membranes were washed once in 1 x SSC (15 mM NaOAc and 0.15 M NaCl) and 0.1% (w/v) SDS, followed by 0.1 x SSC

(1.5 mM NaOAc and 15 mM NaCl) and 0.1 % (w/v) SDS. The blots were incubated in buffer A (100 mM Tris-HCl and 300 mM NaCl) containing a 10 x dilution of Liquid Block (Amersham Pharmacia Biotechnology, USA) for 1 hour at room temperature. Thereafter, the blots were incubated in buffer A containing 0.5 % (w/v) BSA and a 1:5000 dilution of anti-fluorescein-AP conjugate for 1 hour. This was followed by three wash steps of 10 min each in buffer A and 0.1 % (v/v) Tween-20. 500 µl CDP-*Star*™ detection reagent was added to the blots for 5 min, before exposure to HyperFlim (Amersham Pharmacia Biotechnology, USA) for 30 min and development (Sambrook *et al.*, 1989).

3.9 Quantitative PCR

Two quantitative PCR experiments were performed as parallel experiments to each of the Northern blot hybridisations (i.e. one for the confirmation of the microarray data and one examining expression in different tissue types). The five ESTs selected as probes for Northern blot analysis served as primers during quantitative PCR analyses. First strand cDNA was synthesised for the relevant RNA populations and 70 ng was used for each real-time PCR reaction, as well as 10 pmol forward and reverse primers (designed from the above mentioned gene fragments), 3 mM $MgCl_2$ and LightCycler–FastStart DNA Master SYBR Green 1 Mix (FastStart Taq DNA polymerase, reaction buffer, dNTPs, SYBR Green 1 Dye and 10 mM $MgCl_2$) in a 20 µl reaction (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 10s, primer specific annealing T_m °C for 5s, 72 °C for 10s; followed by the melting curve analysis (95°C for 0s, 65°C for 15s, 95 °C for 0s), and cooling (40°C for 30s). A minimum of 7 reactions was performed for each fragment analysed, standard curves were generated using a dilution series (1:1, 1:10, 1:100, 1:1000) and each reaction was performed in triplicate. Results obtained were analysed using LightCycler Software version 3.5.

4. RESULTS

4.1 Statistical data analysis

The fluorescence data obtained from the different hybridisation experiments of each of the time trials were individually compared to the values of the uninfested templates and to each other. The data were analysed using ANOVA (Dudoit *et al.*, 2001), which indicated that 27% of the ESTs/clones were down-regulated and 28% up-regulated over the time period of eight days. ANOVA, however, does not take the presence of false positives into consideration and, therefore, the data was also analysed using the statistically rigorous Mixed Model approach (Wolfinger *et al.*, 2001). This model allows the identification of false positives, as well as the selection of genes with significant expression. The Mixed Model approach revealed that 12% of the data was significantly up- (7%) and down-regulated (5%) at a threshold of log-2 expression ratio of 1.5 and $P \leq 0.05$ (Fig. 4.3).

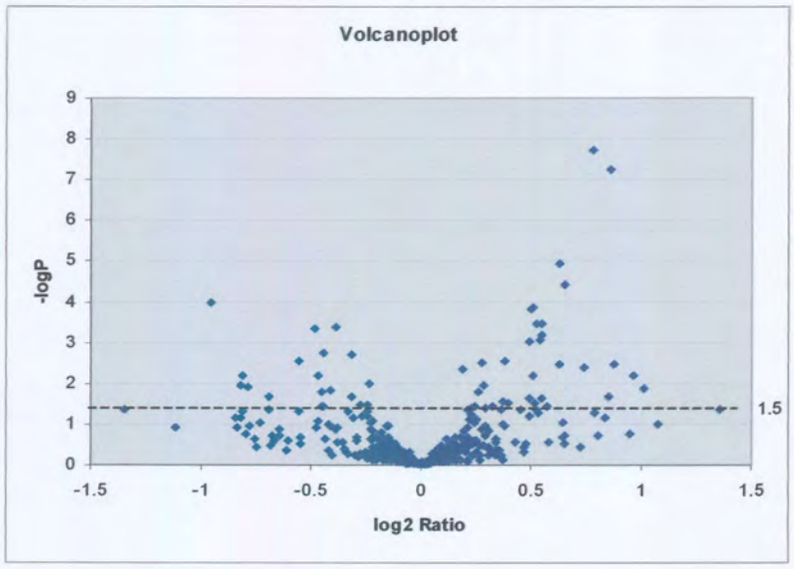

Figure 4.3 A volcano plot assembled from data obtained using the Mixed Model approach in order to visualise the significance of the RWA infested wheat, flax and banana data. The threshold value of 1.5 is indicated, the log2 values provide the estimated fold change (up- or down-regulation) of gene expression and the $-\log_{10}$ P value represents the level of regulation (i.e. whether a high amount or low amount of regulation takes place).

4.2 Expression profiling of microarray transcripts

The ESTs/clones with significant regulation were classified into four categories (Table 4.1), namely sequences involved in cell division, growth and organisation, chloroplast structure and function, defence related sequences and unclassified or unknown. The data from ESTs/clones with no significant change in expression (expression values < 1.5 at threshold of log-2) were not included in the table.

Table 4.1 Genes differentially expressed in response to RWA feeding. Relative fluorescence signals are shown for those genes whose ratios are significantly up- or down-regulated in response to RWA feeding. Values highlighted in red (up-regulation) or green (-; down-regulation) indicate differences in expression at a significance level of $P \leq 0.05$ (Mixed Model; Wolfinger *et al.*, 2001); values in black indicate differences at a significance level of $P \leq 0.05$ (ANOVA; Dudoit *et al.*, 2001).

GenBank accession numbers [a]	Annotation [b]	Expression ratios (fluorescence units)		
		Day 0/2	Day 0/5	Day 0/8
Cell division, growth and organisation				
	Xenopus laevis mRNA for KREMEN, complete cds (e-value = 9e-24)	-0.586	2.833	5.650
	Uncultured bacterium partial 16 rRNA gene(AF327894; e-value=4e-24)	-0.889	2.403	-0.531
	APC/C ubiquitin-protein ligase	-1.661	-0.351	1.812
	Beta nana Ty1-copia-like retrotransposon for putative reverse transcriptase (AJ489197.1; e-value = 3e-28)	-1.445	-0.332	2.753
	Beta nana Ty1-copia-like retrotransposon for putative reverse transcriptase-3 (AJ489202.1; e-value = 3e-13) **Clone #215**	9.541	0.842	3.895
	Hordeum vulgare BARE-1 long terminal repeat (Z84562.1; e-value = 5e-09) **Clone #182**	-1.652	-0.115	4.156
	Cicer arietinum Ty1-copia retrotransposon for putative reverse transcriptase-3 (AJ535884.1; e-value = 8e-43)	6.125	-8.058	-7.711
	Gene for Ribosomal DNA CON-2	-3.337	-0.871	5.853
	Gene for Ribosomal DNA CON-5	3.236	-0.341	1.040
	Atropa belladonna partial mRNA 3'UTR (AJ309392.1; e-value = 1e-19)	1.559	-0.285	0.048
	Oryza sativa T-DNA integration factor-2 (U40841.1; e-value = 2e-23)	-2.506	1.372	2.382
	Cicer arietinum Ty1-copia retrotransposon for putative reverse transcriptase-2 (AB086192.1; e-value = 2e – 42)	5.155	-0.347	-10.463
	Zea mays serine hydroxymethyl-transferase mRNA (AF439728.1; e-value = 6e-52)	-15.730	2.286	35.611
	Atropa belladonna partial mRNA 3'URT (AJ309392.1; e-value = 1e-16)	5.547	-1.526	-10.674
	Linum usitatissimum LIS-1 insertion sequence in genotrophs induced by the environment (AF104351; e-value = 0)	-6.398	1.239	9.017
	Linum usitatissimum LIS-1 insertion sequence (AF104351; e-value = 0)	1.130	-0.171	0.988
	Linum usitatissimum LIS-1 insertion sequence (AJ131994.1; e-value = 1e-149)	1.593	1.681	-3.808
	T-DNA integration factor (U40814.1; e-value = 3e-23)	1.101	0.014	0.540
	Beta nana Ty1 copia-like retrotransposon (AJ489200.1; e-value = 4e-13)	-0.969	-2.655	0.558
	Oryza sativa T-DNA integration factor-3 (U40841.1; e-value = 2e-23)	-2.256	0.334	-1.191

	Linum usitatissimum LIS-1 insertion sequence-2 (AJ131994.1; e-value = 0)	2.025	0.511	-2.840
	Linum usitatissimum LIS-1 insertion sequence-3 (AJ131994.1; e-value = 1e-102)	1.215	0.862	2.350
	Oryza sativa T-DNA integration factor (U40814.1; e-value = 6e-23)	-1.293	0.979	6.887
	Hordeum vulgare BARE-1 long terminal repeat-2 (Z84562.1; e-value = 9e-25)	2.433	0.179	-2.141
	Beta nana Ty1-copia-like retrotransposon for putative reverse transcriptase-2; (AJ489197.1; e-value = 7e-26)	-15.961	0.865	12.369
	Gene for ribosomal DNA CON-6	3.848	1.481	2.944
	Gene for ribosomal DNA CON-3	2.222	0.703	-1.957
	Genes for ribosomal DNA CON-1	-14.251	-2.334	28.515
	Genes for ribosomal DNA CON-4	2.664	0.432	0.838
	Hordeum vulgare BARE-1 long terminal repeat-3 (Z84562.1; 3-value= 1e-19) **Clone #317**	-0.268	-1.124	0.243
	Cicer arietinum Ty1-copia retrotransposon for putative reverse transcriptase (AJ535884.1; e-value = 9e-49)	-1.133	-0.997	-5.207
	Zea mays serine hydroxymethyl transferase mRNA (AF439728.1; e-value = 6e-52)	-1.328	0.467	1.627
	Chloroplast structure / function			
	Flax unknown chloroplast sequence (e-value = 1e-76)	2.361	-0.107	-2.887
	Dendrobium chrysotoxum trnK, matK pseudogene, chloroplast genes (AF448862.1; e-value = 2e-14)	-2.872	-1.468	14.494
CB412206	Wheat chloroplast – ATP synthase-7 (M16843.1 e-value = 0) [c]	1.165	0.024	-1.834
	Flax chloroplast gene for chloroplast product (e-value =1e-76)	2.007	0.905	-0.120
CB412223	Triticum aestivum chloroplast gene (AB042240.3; e-value = 0)	-1.055	-0.269	3.342
	Anthoceros punctatus chloroplast gene for photosystem I P700 apoprotein A1 (AB013664.1; e-value = 4e-16)	11.163	-1.840	14.849
CB412239	Wheat chloroplast-ATP synthase (M16843.1; e-value = 0) [c]	1.796	0.209	4.024
CB412251	Aegilops crassa chloroplast genes-ATP synthase (AEGATPS2; e-value = 0)	224.264	-63.190	463.415
	Maize chloroplast DNA for 4.5S rRNA (X01365.1; e-value = 8e-08)	2.153	1.134	-2.983
CB412240	Triticum aestivum chloroplast gene (AB042240.3; e-value = 0)	-0.933	3.773	-3.479
CB412217	Wheat chloroplast-ATP synthase-2 (M16843.1; e-value = 0) [c]	-1.209	-1.085	-0.653
CB412200	Wheat chloroplast- ATP synthase-6 (M16843.1; e-value = 0) [c] **Clone #29**	0.719	-1.015	-1.423
CB412218	TugelaDN chloroplast-ATP synthase-5 (M16843.1; e-value = 0) [c] **Clone #61**	9.171	-18.724	20.827
CB412238	Wheat chloroplast-ATP synthase-4 (M16843.1; e-value = 0) [c]	-0.156	-1.276	5.234
CB412237	Wheat chloroplast-ATP synthase-6 (M16843.1; e-value = 0) [c]	-1.177	0.330	-6.795
CB412222	Wheat chloroplast-ATP synthase-3 (M16843.1; e-value = 0) [c]	6.959	0.834	-10.864
	Defence related			
CB412258	T monococcum putative resistance protein (RGA-2; AF326781; e-value = 4e-93) **Clone # 271**	-46.583	25.112	132.091
CB412247	Aegilops tauschii putative resistance protein (RGA-2; AF326781; e-value = 2e-57)	22.777	2.133	-45.604
	Putative resistance protein (RGA-2; AF326781; e-value = e-35) **Clone #357**	-9.721	-5.733	1.050
CB412215	Aegilops tauschii leucine rich-like protein gene-3 (AF497474.1; e-value = 2e-44)	2.828	1.043	96.983
CB412254	Aegilops tauchii leucine rich-like protein gene (AF497474.1; e-value = 1e-89)	-2.687	1.827	-0.097
	Aegilops tauschii leucine rich-like protein gene-4 (AF497474.1; e-value = 1e-89)	14.886	0.063	9.260
	Aegilops tauschii leucine rich-like protein gene	1.315	-0.095	-1.060
	Unclassified or unknown			
	Linum usitatissimum clone 5-2 (AF074884.1; e-value = 1e-177)	-1.461	0.764	9.264
	Banana unknown protein-CC92	1.473	-0.103	-1.804
	Banana unknown protein-CC14	0.387	-2.235	2.700
	Banana unknown protein-CC15	-7.0775	4.238	14.461
	Flax unknown protein-CC59	9.155	-4.915	-15.889
	Banana unknown protein-CC91	9.155	-4.915	-15.889
	Flax unknown protein-CC65	14.349	7.279	-28.731

	Flax unknown protein-CC266L1	1.172	-0.546	-12.219
	Banana unknown protein-CC11	4.138	-0.408	-5.355
	Flax unknown protein-CC31	-0.017	-2.694	6.121
	Flax unknown protein-CC14	-0.970	1.686	0.806
	Flax unknown protein-CC9	-3.193	-1.519	2.207
	Flax unknown protein-CC11	2.695	-0.431	19.00
	Banana Unknown protein-CCW74	-49.789	105.717	743.465
	Flax unknown protein-CC28	-3.470	2.197	51.017
CB412174	Unknown protein#307	1.022	1.210	0.186
CB412178	Unknown protein#309	-1.264	-0.565	0.668
CB412183	Unknown protein#311	16.472	14.401	5.099
CB412184	Unknownprotein#312	-1.111	-0.038	2.006
CB412192	Unknown protein#212	-1.316	0.359	0.941
CB412194	Unknown protein#277	-1.271	-0.661	0.813
CB412200	Unknown protein#285	3.691	-0.254	8.020
CB412207	PI137739 unknown protein-1	5.470	4.418	-4.709
CB412216	PI137739 unknown protein-2 **Clone #314**	8.628	2.272	-23.059
CB412238	PI137739 unknown protein-2	2.853	-1.039	-0.230
CB412239	PI137739 unknown protein-10	3.895	-0.050	-5.139
CB412254	PI137739 unknown protein-3	1.649	-0.451	-1.092
CB412257	PI137739 unknown protein-4	-1.478	-0.531	-0.237
CB412258	T aestivum line PI 137739 unknown protein	-7.879	-1.383	16.725
	TugelaDN unknown protein-4	-3.059	0.812	-3.621
CB412201	'Tugela DN' unknown protein-22	2.479	0.128	-2.063
CB412225	'TugelaDN' unknown protein-12	-4.0678	-2.206	-4.4274
CB412232	'TugelaDN' unknown protein-15	-15.641	-2.161	27.238
CB412197	'TugelaDN' unknown protein-21	-23.965	-8.792	88.234
CB412255	'TugelaDN' unknown protein-2	1.388	0.119	-0.311
CB412203	TugelaDN unknown protein-23	1.030	-0.384	-0.259
CB412214	'Tugela DN' unknown protein-7	1.891	0.217	-2.029
CB412208	TugelaDN unknown protein-3 **Clone #310**	8.740	2.903	25.424
CB412223	TugelaDN unknown protein-11	4.760	-0.223	-6.237
CB412209	TugelaDN unknown protein-21	1.993	0.220	-1.255
CB412196	TugelaDN unknown protein-24	3.835	-1.010	-7.001
	'TugelaDN' unknown protein-2	-1.708	0.243	1.962
CB412243	TugelaDN unknown protein-5	2.455	0.572	-0.830
CB412211	TugelaDN unknown protein-6	-3.795	1.370	5.640
CB412221	'Tugela DN' unknown protein-10	-0.560	-1.389	2.629
CB412227	'Tugela DN' unknown protein-14	-0.218	1.351	3.505
CB412235	'Tugela DN' unknown protein-18	-0.176	1.117	-0.165
CB412219	'TugelaDN' unknown protein-7	1.786	-2.084	17.748
CB412245	'Tugela DN' Unknown protein-1	-212.293	-10.013	1.697
CB412236	'Tugela DN' Unknown protein-13	9.804	5.988	-12.688
CB412246	'Tugela DN' Unknown protein-17	1.337	0.367	-4.082
CB412234	'Tugela DN' Unknown protein-16	-2.140	-0.505	-0.331
CB412241	'Tugela DN' Unknown protein#238	-10.936	-19.501	-15.971
CB412256	'Tugela DN' Unknown protein-3	-9.173	1.737	-1.887

[a] Accession numbers of the sequences submitted from this study to GenBank
[b] BLASTN annotations
[c] Wheat chloroplast-ATP synthase CF-1 gene, beta and epsilon subunit

The five ESTs selected for Northern blot analysis and quantitative PCR in order to confirm the microarray data, exhibited homology to a *Beta nana* Ty-1-copia-like retrotransposon, two *Hordeum vulgare* BARE-1 long terminal repeats and two sequences that could not be identified using the BLASTN annotation at a significance level of $E \leq 10^{-3}$. Each of the four functional categories contained sequences that were significantly up- or down-regulated. The sequences discussed here are examples where more than ten-fold regulation was observed. The cell division, growth and organisation class contained three sequences homologous to Ty1-copia retrotransposons of *Cicer arietinum* and four homologous to *Beta nana*. One of the *C. arietinum* clones was down-regulated > 10 times towards day 8 (-10.463) and one of the four *B. nana* clones were up-regulated towards day 8 (+12.369). An *Atropa bellabonna* mRNA untranslated region exhibited down-regulation from +5.547 to -10.674. Another gene that was significantly up-regulated was a ribosomal DNA gene (CON-1; -14.251 at day two increasing to +28.515 at day 8).

The chloroplast structure and function category consisted of three ATP-synthase genes (accession numbers CB412251, CB412218 and CB412222, respectively) displaying significant gene regulation. The *Aegilops crassa* ATP-synthase gene exhibited high initial regulation (+224.264), down-regulation at day 5 (-63.19) and during day 8 the up-regulation increased dramatically to 463.415-fold. The expression of a *Dendrobium chrysotoxum* chloroplast sequence gradually increased from two (-2.872) to five days (-1.468) after infestation, and exhibited a larger increase in expression during day eight (+14.494).

A sequence homologous to a *Triticum monococcum* RGA-2 was initially down-regulated (day 0/2; -46.583), up-regulated during the day 0/5 time interval (+25.112) and even more up-regulated during the last time interval (+132.091). Another putative RGA-2 gene was regulated similarly to the *T. monococcum* homologue, but exhibited less regulation on day eight (+1.050). An *Ae. tauschii* RGA homologue (CB412247) exhibited a contrasting regulation profile. It was initially up-regulated during day 2 (+22.777) and day 5 (+2.133) post-infestation, but down-regulated during

day 8 (-45.604). Four homologues to *Ae. tauschii* leucine rich-like proteins were identified. One of these (CB412215) was significantly up-regulated after eight days of RWA infestation (+96.983). The CB412254 homologue was initially down-regulated (-2.687), then slightly up-regulated (+1.827) and down-regulated again (-0.097). The third leucine rich-like fragment was up-regulated during early infestation (+14.886), down-regulated during day 5 (0.063) and late infestation triggered up-regulation (+9.260). The last leucine rich-like sequence exhibited down-regulation as the infestation period progressed.

Several EST annotations classified as unknown or without any discernable function exhibited significant response to RWA feeding. These include all types of regulation: mainly down-regulated (e.g. 'TugelaDN' unknown protein-4), mainly up-regulated (e.g. 'TugelaDN' unknown protein-3; CB412208), only down-regulated (e.g. banana unknown protein-CC91), only up-regulated (e.g. flax unknown protein-CC28), as well as a combination of down-/up-regulation (e.g. banana unknown protein-CC14).

The cell division, growth and organisational class contained 28% of the total amount of functional annotations, the chloroplast structure and functional class consisted of 14%, the defence related contained 6% and the unclassified/unknown class 52% of the sequences (Fig. 4.4).

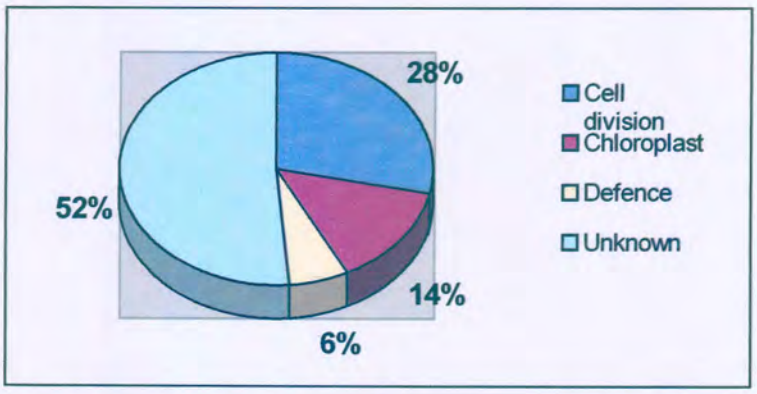

Figure 4.4 The functional distribution of the analysed microarray clones. The cell division, growth and organisation class consists of 28% of the sequences, the chloroplast structure and function class of 14%, the defence related class of 6% and the unclassified group of 52% of the sequences.

Hierarchical clustering (Eisen *et al.*, 1998) was performed in order to group the clones with similar expression profiles obtained from the Mixed Model approach, in clusters (Fig. 4.5).

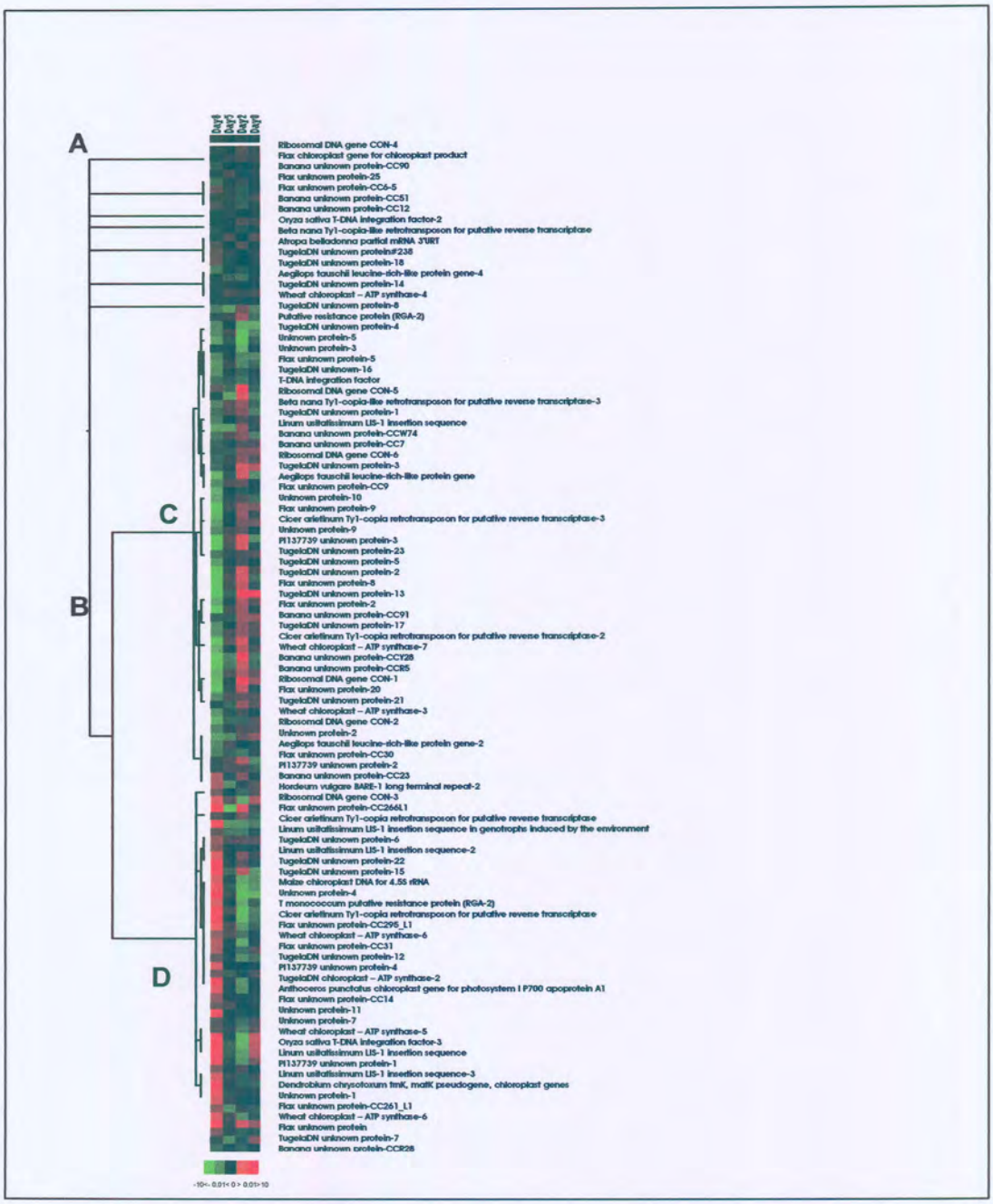

Figure 4.5 Hierarchical clustering of the clones using Cluster and Tree View. The red data points are an indication of up-regulation and the green data points indicate down-regulation. Two distinct groups are visible: group A exhibits little or no regulation and group B exhibits significant regulation. Group C exhibits initial up-, then down-regulation; group D exhibits initial down-, then up-regulation.

The hierarchical cluster revealed two categories of regulation where one group of genes exhibits little or no regulation (A) and the other group exhibits significant up- or down-regulation (B). The latter consisted of two major clusters, representing the genes that are initially either significantly up-regulated and then down-regulated (D), or *vice versa* (C). Six groups could further be discerned, based on their expression profiles, from the hierarchical classification of groups C and D: (1) up-regulated through the time trial, (2) initially up-regulated (day 0 and 2) and then down-regulated (day 5 and/or 8), (3) initially down-regulated (day 0 and 2) and then up-regulated (day 5 and/or 8), (4) down-regulated through the time trial, (5) up-regulated (day 0 and 2), down-regulated (day 5) and then up-regulated (day 8) and (6) down-up-down-regulation throughout the time trial. The data points indicated in red (up-regulation) and green (down-regulation) are in agreement with the values presented in Table 4.1.

4.3 Confirmation of microarray quantification

The microarray data obtained from the day 2 and day 5 post-infestation treatments were compared (Fig. 4.6). Microarray analysis indicated higher expression of EST #317 (BARE-1 long terminal repeat) throughout the infestation period and this is comparable the Northern blot results. The quantitative PCR data indicates a slight decrease in expression at day 2 and an increase in expression on day 5, but the general trend for this EST exhibits an increase in expression throughout the period of infestation. Clone #314 (unknown protein) exhibits higher expression over the infestation period when the Northern blot and microarray data are considered, but the quantitative PCR data indicate that this gene was initially up-regulated and then down-regulated. The microarray and quantitative data for clone #182 (BARE-1 long terminal repeat) revealed a decrease in expression during day two and an increase in expression on day five, while the Northern blot indicates increased expression from day 2 onwards. Northern blot analysis of clone #310 (unknown protein) suggests that a gradual increase in expression takes place during the period of infestation. The quantitative data indicates that a relative high amount of up-regulation occurred at day two and that the regulation

decreased after five days of infestation, whereas the microarray data suggests down-regulation at day 2 and up-regulation at day 5. The Northern blot, microarray and quantitative PCR data of clone #215 (Ty1-copia-like retrotransposon) are comparable: an increase in expression occurred throughout the infestation period. The global trends in gene expression observed when considering these three techniques are in agreement with each other.

	Northern blot analyses	Microarray data		Quantitative PCR data	
	Day 0 Day 2 Day 5	Day 2	Day 5	Day 2	Day 5
BARE-1 long terminal repeat #317		+0.906	+4.773	-1.5	+8.2
Unknown protein #314		+2.173	+4.613	+77.7	+1.4
BARE-1 long terminal repeat #182		-0.654	+5.415	-3.5	$+5.249 \times 10^3$
Unknown protein # 310		-0.225	+10.211	$+2.2 * 10^3$	$+1.09 \times 10^2$
Ty1-copia-like retrotransposon #215		+1.231	+11.465	+32	$+2 \times 10^5$

Figure 4.6 A comparison between the microarray data, Northern blot analyses and quantitative PCR data utilising the five selected ESTs. The Northern blots represent data from uninfested material (day 0) and infested material from day 2 and day 5 post-RWA infestation; the microarray and quantitative PCR data are given for day 2 and 5 post-infestation. Clone numbers are indicated for each EST investigated. Microarray and quantitative data represent the fluorescence values obtained after the respective experiments.

4.4 Analysis of tissue-specific microarray quantification

The results of the tissue specific Northern blot analyses in order to confirm the differential expression of ESTs in response to RWA feeding (Fig. 4.7) indicated a significant higher expression of the selected ESTs in tissues of resistant 'Tugela DN' than in the tissues of the susceptible 'Tugela' cultivar (Fig. 4.7). However, RWA feeding did not enhance the expression of these ESTs, but rather down-regulated the expression in 'Tugela DN' at day 2 after infestation, when compared to uninfested leaf material.

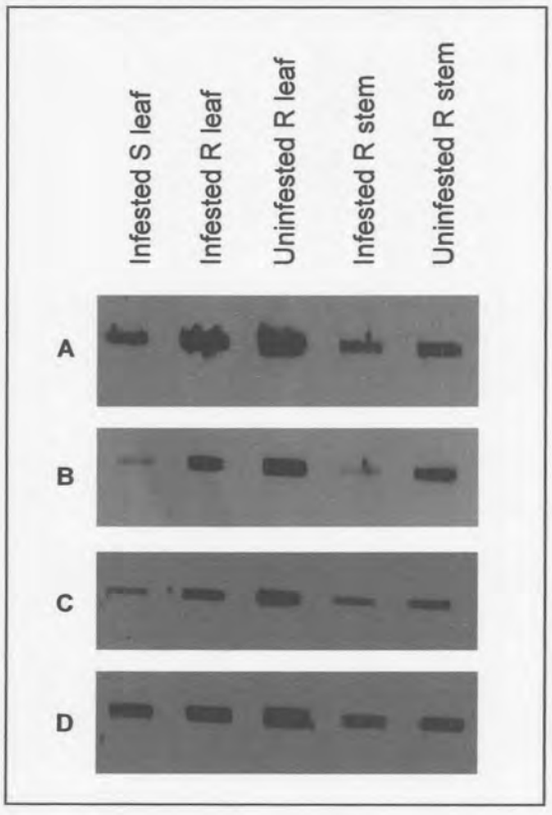

Figure 4.7 Northern blot analysis indicating differential gene expression in leaf and stem tissue. A represents the *T. monococcum* RGA-2 homologue (clone #271), B represents the putative RGA-2 resistance gene, (clone #357), C represents the ATP synthase-6 (clone #29) and D the ATP synthase-5 (clone #61) ESTs. S = susceptible ('Tugela'), R = resistant ('TugelaDN').

Quantitative PCRs were also performed on RNA extracted from leaf tissue at day 0, 2 and 5 after RWA infestation using the above-mentioned four probes (Fig. 4.8). Some of the data sets contradicted the microarray results, while others confirmed them. The microarray data indicated that the *T. monococcum* RGA-2 fragment (clone #271) was dramatically down-regulated after two days of infestation and then significantly up-regulated five days after infestation. The quantitative PCR data showed gradual down-regulation for this fragment of the infestation period. Both the microarray and quantitative PCR data indicated similar trends of down-regulation for the RGA-2 gene fragment (clone #357). The ATP-synthase-6 fragment (clone #29) exhibited slight up-, then down-regulation according to the microarray data; the quantitative PCR data exhibited a gradual up-regulation throughout the infestation period. The microarray and quantitative PCR both indicated the same activity for the ATP-synthase-5 fragment (clone #61), namely initial up-regulation and then down-regulation over the infestation period.

	Microarray data		**Quantitative PCR data**	
	Day 2	**Day 5**	**Day 2**	**Day 5**
T. monococcum RGA-2 homologue #271	-46.583	25.112	-2	-8
Putative RGA-2 resistance protein #357	-9.721	-5.733	-2.5	-7.8
Wheat chloroplast-ATP synthase-6 #29	0.719	-1.015	+1.5	+3.7
'Tugela DN' chloroplast-ATP synthase-5 #61	9.171	-18.724	+1	-8

Figure 4.8 A comparison between the microarray data for leaf tissue, two and five days after infestation, and the quantitative PCR data. The microarray data represents the fold up- or down-regulation.

5. DISCUSSION AND CONCLUSION

This study represents the initial experiments performed on Russian wheat aphid infested wheat material in order to test the feasibility of using microarray technology in gene expression profiling. The validity of the microarray data was monitored by incorporating Northern blot and quantitative PCR analyses.

One advantage of microarray analysis is that gene expression and regulation patterns can be monitored on a large scale over several biological experiments (Quackenbush, 2001). A further advantage is that the putative function of an unknown gene can be determined when such a gene clusters together with a group of genes of known function (Kerr and Churchill, 2001). The present study revealed that a known function could not be assigned to 52% of the clones. 28% of the sequences were found to be involved in cell division, growth and organisation, 14% were involved in chloroplast structure and function, and 6% of the sequences were homologous to resistance gene fragments. The functions of the unknown group of sequences can, however, be determined by hybridisation with probes of known sequence and function (Hoheisel, 1997). The large amount of unknown sequences is an indication that many genes of the wheat genome still have to be identified and characterised.

An informative approach for microarray analysis is the incorporation of different time points as a means of studying gene regulation and expression. DeRisi and his colleagues (1997), for example, studied gene expression profiles during the metabolic shift from fermentation to respiration in yeast and were able to identify previously uncharacterised genes. The present study indicated that gene regulation of specific genes has to be studied over time, since it aims at monitoring the plant's continual reaction to prolonged insect feeding. The numerous regulation patterns observed are indicative of the large amount of organisation executed by an infested plant over time.

Statistical analysis using the ANOVA method as suggested by Dudoit and his colleagues (2001), indicated that 27% of the ESTs were down-regulated and 28% up-regulated, but the presence of false positives was not taken into consideration. This model is based on the normalisation of log ratios and then permutation-based t-statistics for testing the significance of each gene, and *p*-values are suitably adjusted for multiplicity. After fluorescence the data was subjected to the statistically rigorous Mixed Model approach (Wolfinger *et al.*, 2001), only 12% of the transcripts were regulated at a significance level of $P \leq 0.05$. These included the ESTs previously identified as significantly regulated. The latter model centres around two interconnected ANOVA models, namely the Normalisation Model and the Gene Model. The analysis corrects, amongst others, for spot position and pen position differences, fluorescence bias and differences due to experimental design and biological repeats. The Mixed Model was found to be very rigorous and it also excluded all false positives.

Assembling the data into a hierarchical cluster has the advantage of simplifying and visualising the data (Eisen and Brown, 1999). The aim of such clustering is, further, the organisation of single expression profiles in groups of similar expression (Qauckenbush, 2001). The hierarchical cluster generated during this study organised the data into three main clusters, namely one cluster that is not significantly regulated, one group of genes that is initially up- and then down-regulated, and one group that is initially down-, then up-regulated.

Three distinct techniques, all based on the hybridisation of a probe to a target sequence, were employed to investigate the regulation of five randomly selected ESTs. Mainly, microarray technology was tested to determine the feasibility of using this technique as analytical method, and Northern blots and quantitative PCRs were employed to monitor the validity of the microarray data. The expression profiles of the Northern blot analyses of these ESTs all exhibited up-regulation during the five-day infestation period. Although small differences between the actual microarray and quantitative PCR values were obtained, the global picture of gene regulation observed were similar for the

two methods. Differences in actual values are to be expected, since quantitative PCR is a very sensitive procedure and experiments were performed using different biological material over time.

It was observed that the five selected ESTs are involved in different cellular functions. Little is known regarding the function of the BARE-1 long terminal repeats and Ty-1-copia-like retrotransposons, apart from their evolutionary significance and their involvement in genome organisation (Hanson *et al.*, 2000; Katsiotis *et al.*, 2002). Chloroplast-ATP synthases are key enzymes in plant metabolism, providing cells with energy in the form of ATP. The enzyme is located in the thylakoid membrane and synthesises ATP from adenosine diphosphate and inorganic phosphate at the expense of the electrochemical proton gradient formed by light-dependent electron flow (McCarty *et al.*, 2000; Mellwig and Bottcher, 2003). It is interesting to note that one of the two mentioned ATP-synthase genes are only regulated slightly, while the other ATP-synthase gene exhibits much higher levels of regulation. The RGA-2 fragments are constituents of the NBS-LRR resistance gene family and have a putative receptor-like function; they are also thought to be involved in signal transduction (Whitham *et al.*, 1994; Jackson and Taylor, 1996; Pan *et al.*, 2000; Cannon *et al.*, 2002) and pathogen/pest-plant recognition (Van der Biezen and Jones, 1998; Dangl and Jones, 2001). These two ESTs also exhibited differing levels of regulation during the microarray analyses; one EST was regulated slightly, while the other was regulated significantly.

The analysis of the two ATP-synthase and RGA-2 ESTs indicated significantly higher expression in the infested resistant cultivar ('Tugela DN') than in the infested susceptible cultivar ('Tugela'). Expression of these gene fragments is, further, higher in leaf tissue than in stem tissue. However, RWA feeding did not enhance the expression of these ESTs, but rather down-regulated the expression in the resistant cultivar after two days when compared to uninfested leaf material. It was also revealed that some of the microarray data sets of the ATP-synthase and RGA-2 ESTs agree with that of the quantitative PCR data, but other data was contradictory.

The varying patterns of gene regulation exhibited by several of the ESTs during this study still have to be examined further. The role of the resistance gene analogues, e.g., during RWA infestation has to be clarified, especially after the observation that the regulation of the various RGA genes differs. Previous studies suggested that RWA feeding causes a decrease in effective leaf area (Walters *et al.*, 1979). RWA feeding also destroys the cell membranes, the chloroplasts are damaged and, thus, the effective photosynthetic capacity declines (Fouché *et al.*, 1984). This decline in chlorophyll is visible only in the RWA susceptible cultivar 'Tugela' and does not occur in the RWA resistant 'Tugela DN'. Chlorophyll loss was also observed after feeding of RWA and greenbug (*Schizaphis graminum*) on susceptible TAM107 (Deol *et al.*, 2002). The observed loss in chlorophyll content after feeding by the greenbug was correlated with a decrease in photosynthetic rate (Nagaraj *et al.*, 2002). Thus, the role of other significantly regulated genes, such as ATP-synthase, should be investigated further in order to understand the significance of these enzymes during RWA feeding.

The study of gene regulation in RWA-infested wheat plants proved to be a successful one. This study allowed the establishment of the microarray procedures and protocols in our laboratory. The global view of gene regulation in wheat challenged with insect infestation revealed that up- and down-regulation of a vast amount of genes occur in response to RWA infestation. The data obtained from the Northern blot and quantitative PCR analyses indicated that the microarray data was valid and that the incorporation of microarray analysis in such a study is highly feasible.

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Chapter 5

Summary

The investigation of differential gene expression in Russian wheat aphid (RWA) infested bread wheat is imperative for breeding RWA resistant lines. The wheat genome is such a large and complex one that it creates a mayor obstacle in studying differential gene expression in wheat. Differential gene expression profiling, however, was achieved during this study by combining several molecular tools.

Bioinformatic functional gene analysis was accomplished by screening the genomic, as well as the complementary DNA, of a resistant wheat line, PI 137739. The general response of expressed genes during infestation was assessed and genes specifically related to defence reactions was determined. PCR-based approaches, aimed at isolating nucleotide binding site (NBS) sequences, were utilised during the screening procedures and proved to be effective for such purposes. The data obtained from the gene fragments obtained from genomic, as well as cDNA, revealed that 30% of these sequences are involved in metabolic activities, 28% are involved in protein synthesis, 10% exert regulatory activities, 4% has structural functions and 18% were classified as miscellaneous sequences. The PCR-based approaches that were utilised also proved to be feasible for the identification of resistance-related fragments (10%). Although sequences containing NBS domains were not isolated, resistance gene analogues (RGAs), leucine rich repeat regions, receptor-like kinases and a WIR pathogen resistance fragment were identified. These genes are all thought to be involved in plant defence responses. In order to conduct a thorough investigation regarding gene expression during plant infestation, a large amount of clones should be examined. Thus, this study can be refined by including even more clones generated from genomic, as well as cDNA, when time and financial constraints are not considered as impediments. The ESTs identified during this study opens the way for further research concerning the exact role of RGAs and NBS-LRRs during the plant's defence responses. One question to be addressed is whether RGAs, as well as the leucine rich repeats and leucine zipper-NBS-LRR sequences, are involved in signal transduction, ligand biding, energy transfer reactions or possibly another defence response.

Differential gene expression profiling was continued by employing suppression subtractive hybridisation (SSH). SSH involves the comparison of the defence responses in RWA infested plants with the gene expression in uninfested plants. The SSH procedure revealed that 28% of the identified expressed sequence tags (ESTs) are involved in protein synthesis, 3% is involved in metabolic activities, 9% is regulatory sequences, 7% is related to structural functions and 35% was classified as miscellaneous. A relative high amount (18%) of resistance-related sequences were identified in the resistant PI 137739 wheat line. These fragments exhibited similarity to a resistance gene locus in barley (*Mla*; 2e - 65), leucine rich-like fragments (e - 149), leucine zipper-nucleotide biding site-leucine rich repeats (LZ-NBS-LRRs; e - 131) and resistance gene analogues (7e - 25). The SSH procedure aims at identifying novel fragments, but this was unfortunately not achieved. However, the isolation gene fragments related to resistance responses in infested wheat plants that were absent in the uninfested plants, was accomplished. This is indicative of the feasibility of employing SSH for studying differential gene expression. The probability of obtaining more resistance-related gene fragments can be increased by incorporating more stringent hybridisation procedures, as well as by adding a higher concentration of denatured driver during secondary hybridisation in order to enrich even more for the single-stranded tester fragments. It is also recommended that a susceptible near isogenic line (NIL) should be included in SSH studies. The introduction of a susceptible NIL would result in the normalisation of the two different genomes and highlight novel gene fragments. The resistance-related SSH fragments obtained during this study have to be characterised further in order to determine their exact role during plant defence responses. Once these fragments have been characterised, their linkage to existing resistance genes can be determined and they can be mapped on the wheat genome.

Microarray technology was implemented in order to test the feasibility of utilising this technique for investigations of differential gene expression and regulation in plants challenged with insect infestation, specifically the RWA. A series of ESTs obtained throughout this study was used as target material

and hybridised with probes prepared from mRNA of uninfested and infested wheat material at different time points post-RWA infestation. Several patterns of gene regulation were observed and differential expression of selected ESTs was obtained in leaf and stem tissue. It was, further, found that the gene regulation of functionally similar genes (e.g. RGA-2 homologues, ATP-synthases and the retrotransposon sequences) differs.

Since the microarray study was performed as an introductory investigation, some recommendations should be considered for future studies. Firstly, more clones that are known to be involved in plant defence responses should be included, since this would provide a more comprehensive view of gene regulation exerted by infested wheat plants. The results from a more comprehensive study would also prevent bias towards certain gene fragments, e.g. the gene regulation of resistance-related fragments was highlighted during this study since probes were prepared from RWA-infested material. A valuable confirmation gained from this study is the importance of applying correct statistical analysis. It was revealed that different statistical procedures provide different results and, thus, different interpretations of the obtained data. The Mixed Model approach proved to be more effective since it allowed for more thorough, precise analysis.

Several questions came forth from the microarray study. Firstly, the sequences identified as being involved in defence responses such as the resistance gene analogues and leucine rich repeats, have to be characterised further. Their exact role during the plant's resistance reactions, i.e. are they involved in signal transduction or another more specific response, has to be determined. Since it was observed that various ATP-synthases are regulated differently, the exact role of each of these during the infested plant's metabolism has to be established. Similar questions have to be examined regarding the retrotransposable elements, as well as the terminal repeat elements, since differing patterns of gene regulation was also observed for these genes. In retrospect, this study revealed that the microarrray procedure is trustworthy and provides a vast amount of information regarding specific scientific investigations.

Appendices

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Functional and comparative analysis of expressed sequences from *Diuraphis noxia* infested wheat obtained utilizing the conserved Nucleotide Binding Site

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Russian wheat aphid (*Diuraphis noxia*, Morvilko; RWA) is a major pest on wheat, barley and other triticale in South Africa. Infestation by the RWA results in altered protein expression patterns, which is manifested as differential expression of gene sequences. In the present study, Russian wheat aphid resistant (Tugela DN, Tugela*5/SA2199, Tugela*5/SA463, PI 137739, PI 262660, and PI 294994) and susceptible triticale (Tugela) were infested and cDNA synthesized. A PCR based approach was utilized to amplify the nucleotide binding site conserved region to obtain expressed sequence tags (ESTs) with homology to resistance gene analogs (RGAs). The approach proved highly feasible when the isolation of RGAs is the main objective, since 18% of all obtained ESTs showed significant hits with known RGAs, when translated into their corresponding amino acid sequences and searched against the nonredundant GenBank protein database using the BLASTX algorithm.

Key words: Resistance gene analogs, degenerate PCR, nucleotide-binding site-leucine rich repeat resistance genes, *Aegilops tauschii*.

INTRODUCTION

Russian wheat aphid (*Diuraphis noxia*, Morvilko; RWA) is one of the most adaptable insects that is recognized as a pest of wheat, barley and other triticale (Bryce, 1994; Walters et al., 1980). Infestation can occur shortly after the emergence of the wheat plants and the aphids are found on the newest growth and the axils of the leaves, but damage is greatest when the crops start to ripen. This is due to the twisting and distortion of the heads and the resulting failure to emerge properly (Unger and Quisenbury, 1997). Further symptoms of RWA feeding on susceptible cultivars include longitudinal streaking and leaf rolling, which under severe infestation leads to a drastic reduction in effective leaf area (Walters et al., 1980). Infestation by the RWA also results in altered protein expression patterns, which is manifested as differential expression of total proteins, and specific pathogenesis-related proteins like chitinases, ß-1,3-glucanases and peroxidases (Bahlmann, 2002; Botha et al., 1998; Van der Westhuizen et al., 1998a,b, 2002; Van der Westhuizen and Botha, 1993; Van der Westhuizen and Pretorius, 1996). The use of RWA-resistant cultivars, however, may reduce the impact of this pest on wheat production and in the same time reduce environmental risks and control costs due to chemical spraying (Tolmay et al., 1999). The need for more RWA tolerant plants places emphasis on obtaining resistance candidate genes, as well as on the understanding of the underlying mechanisms of defense against the RWA.

Disease resistance genes have been isolated and characterized at the molecular level in several plant species such as *Arabidopsis*, tobacco, tomato and wheat (Jones and Jones, 1997; Cannon et al., 2002). Resistance gene products specifically recognize and provide resistance towards a large number of pests and pathogens (Seah et al., 1998; Pan et al., 2000). These genes can be divided into four broad, structurally distinct classes. The first class of resistance genes belongs to the serine-threonine kinases (Martin et al., 1993; Ritter and Dangl, 1996). The protein kinases phosphorylate serine/threonine residues and thus control certain signaling networks during the resistance response. The second class of resistance genes encodes putative transmembrane receptors with extracellular leucine rich repeat (LRR) domains (Jones et al., 1994; Dixon et al., 1998). The third class encodes for a receptor-like kinase and combines qualities of both the previous classes. Both the LRR domain and the protein kinase regions are encoded in the same protein. The fourth class, which

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represents the majority of plant disease resistance genes cloned so far, is the nucleotide-binding site-leucine rich repeat (NBS-LRR) resistance genes. The NBS-LRR class of genes is abundant in plant species. In *Arabidopsis*, it has been estimated that at least 200 different NBS-LRR genes exist making up to 1% of the genome (Ellis et al., 2000; Sandhu and Gill, 2002).

The NBS-LRR genes contain three distinct domains: a variable N-terminus, a nucleotide-binding site and leucine rich repeats. Two types of N-termini are present in NBS-LRR. One kind contains a leucine zipper or coiled-coil sequence that is thought to facilitate protein-protein interactions. The coiled-coil motif has been found in the N terminus of both dicotyledons and cereals (Pan et al., 2000; Cannon et al., 2002). The second kind of N-terminus has been described only in dicotyledons and is similar to the cytoplasmic signaling domains on the *Drosophila* Toll- or the mammalian interleukin receptor-like (TIR) regions (Whitham et al., 1994; Cannon et al., 2002). These NBS regions are found in many ATP and GTP-binding proteins that act as molecular switches (Jackson and Taylor, 1996). These genes regulate the activity of proteases that can initiate apoptotic cell death. Since defense mechanisms in plants include the hypersensitive response, which is very similar to apoptosis, the common occurrence of NBS domains in both plants and animals could be an indication of similar functioning (Cannon et al., 2002).

NBS-LRR homologues encode proteins that are structurally closely related. This suggests that they have a common function in signal transduction pathways, even though they confer resistance to a wide variety of pathogen types. The conservation between different NBS-LRR resistance genes enables the use of polymerase chain reaction (PCR)-based strategies in isolating and cloning other R gene family members or analogs using degenerate primers for these conserved regions. Strategies using degenerate primers have been successfully utilized in the cloning of other putative NBS-LRR resistance gene analogs (RGA) from potato (*Solanum tuberosum* L.)(Leister et al., 1996), soybean (*Glycine max* L. Merr.)(Yu et al., 1996) and citrus (Deng et al., 2000).

The identification and analysis of expressed sequence tags (ESTs) provide an effective tool to study thousands of genes expressed during plant development and their response to varying environmental conditions (Gyorgyey et al., 2000; White et al., 2000; Yamamoto and Sasaki, 1997) in complex genomes like wheat. The development of EST databases further provides a resource for transcript profiling experiments and studies of gene expression (Mekhedov et al., 2000; Schenk et al., 2000).

The aim of this study was to survey the expressed sequence tags obtained through PCR-based strategies utilizing the conserved nucleotide binding site motifs in an effort to increase the efficacy of isolating resistance gene candidates, from the complex hexaploid wheat genome.

MATERIALS AND METHODS

Plant Material

The plant materials in the study were *Aegilops tauschii*, the near isogenic lines 'Tugela DN' (Tugela*5/SA1684, *Dn1*), Tugela *Dn2* (Tugela*5/SA2199), Tugela *Dn5* (Tugela*5/SA463) and Tugela (RWA susceptible), as well as RWA tolerant lines PI 137739 (SA1684, *Dn1*), PI 262660 (SA2199, *Dn2*) and PI 294994 (SA463, *Dn5*). The plants 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 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. The second and third leaves from uninfested and infested plants were removed after one week for analysis. The aphids were removed from the infested leaves under running water to prevent aphid derived nucleic acid contamination during the RNA isolation. The leaves were dried and used immediately for total RNA isolation.

Treatment of glassware, plastic ware and solutions

All glassware, plastic ware and solutions used, up to the second strand cDNA synthesis, were treated and then kept free of RNases. The glassware was treated overnight in 0.1% (v/v) diethyl pyrocarbonate (DEPC), autoclaved for 20 min at 121°C and baked at 200°C for 3-4 hours (Sambrook et al., 1989). The mortars and pestles were washed in 0.25M HCl for 30 min, prior to DEPC treatment, autoclaving and baking. All plastic ware and solutions, except those containing Tris (2-Amino-2-(hydroxymethyl)-1,3-propandiol), were DEPC treated and autoclaved.

Total RNA Isolation and cDNA synthesis

Total cellular RNA was extracted using an acid guanidium thiocyanate-phenol-chloroform extraction method described by Chomczynski and Sacchi (1987). The RNA samples were stored at -80°C for further use. The RNA concentration was determined on a Beckman DU®-64 spectrophotometer, by reading the absorbance at 260 nm. The 260/280 ratio was determined to indicate the level of protein contamination (Sambrook et al., 1989). The integrity of the RNA was confirmed by analyzing both the infested and uninfested total RNA on a 2 % (w/v) agarose gel (Sambrook et al., 1989). The molecular mass standard used was λ DNA digested with *Eco*RI and *Hin*dIII (Sambrook et al., 1989). Isolated RNA was electrophoresed at 100 V for 30 min and visualized under UV light with ethidium bromide (EtBr) staining.

mRNA Isolation

The mRNA was purified from the total RNA using Oligo(dT) Cellulose affinity chromatography (GibcoBRL, Life Technologies). The synthesis of cDNA was carried out using either the Roche Molecular Biochemicals cDNA Synthesis System according to manufacturers specifications, or the RLM-RACE system (GeneRacer Kit, Invitrogen). Both the uninfested and the infested wheat mRNA were used as the substrate for the cDNA synthesis reaction. The ds cDNA was purified by the QIAquick Spin Purification Procedure (QIAGEN). The cDNA was eluted with water and the concentration determined spectrophotometrically and stored at -20°C.

When making use of the RLM-RACE system, the mRNA was dephosphorylated with calf intestinal phosphatase to remove the 5' phosphates and decapped with tobacco acid pyrophosphatase

Table 1. Functional annotation of expressed sequence tags (ESTs) that produced BLASTX hits.

BLASTX annotation	E-value	No. of dbESTs hits
Mitochondrial 26S rRNA (*Triticum aestivum*, Z11889; *Zea mays*, K01868; *Beta vulgaris*, AP000397; *Arabidopsis thaliana*, Y08501)[a]	0.0	20
Mitochondrial DNA (*Secale cereale*, Z14059)	0.0	2
Mitochondrial 18S rRNA (*Zea mays*, X00794)	0.0	4
Mitochondrial 23S rRNA (*Beta lupin*, X87283)	4.00E-13	1
Mitochondrial 16S rRNA (*Beta japonicum* , Z35330)	0.0	2
SSU[b] rRNA (*Drimys winteri*, AF197162; *Tetracentron sinense*, AF193998;*Grevillea robusta*, AF193995; *Trochodendron aralioides*, AF161092; *Nelumbo nucifera*, AF193983; *Platanus occidentalis*, AF161090)	2.00E-87	6
S7 ribosomal fragment (*Triticum aestivum*, X67242)	1.00E-03*	1*
S13 ribosomal fragment (*Triticum aestivum*, Y00520)	0.085*	1*
Chloroplast DNA (*Triticum aestivum*, AB042240; *Oryza sativa*, AB042240.3; *Zea mays*, X15901; *Hordeum vulgare*, X86563; *A. crassa*, X86563.2; *A. columnaris*, X00408; *Clinostigma savoyanum*, X00408.1,)	0.0	15
Long terminal repeat (*Hordeum vulgare*, Z84569)	7.00E-03*	2*
Actin gene (*Triticum monococccum*, AF326781)	8.00E-31	8
Resistance gene analogue2(*Triticum monococccum*, AF326781)	7.00E-13	6
Retrotransposon, MITE[c] (*Hordeum vulgare*, AB022688)	3.00E-76	5
Tonoplast DNA (*Hordeum vulgare*, AF254799)	6.00E-50	3
HMW[d] glutenin gene (*Aegilops tauschii*, AF497474)	1.00E-116	2
Noduline-like-protein (*Triticum monococcum*, AF326781)	2.00E-43	2
Chromosome condensation factor (*Triticum monococcum*, AF326781)	2.00E-43	2
mRNA for SSU, Rubisco (*Triticum aestivum*, K01229; *Secale cereale*, M37328; *Hordeum vulgare*, AJ131738; *Triticum timopheevii*, U43493; *Triticum urartu*, AB020955; *Oryza sativa*, AB020954; *Avena strigosa*, AF052305; *Avena maroccana*, AF097360; *Aegilops squarrosa*, AF104250; *Aegilops bicornis*, AB020938; *Aegilops sharonensis*, AB020936; *Aegilops longissima*, AB020935)	6.00E-58	24
ATP synthase β subunit (*Clinostigma savoryanum*, AB020933; *Elaeis oleifera*, AF449171; *Cyphophoenis nucele*, AY012452; *Howea belmoreana*, AY012445; *Phoenix canariensis*, AY012435; *Linospadix longicruris*, AF209652)	1.00E-168	6
Aldehyde dehydrogenase (*Zea mays*, AF449172)	3.00E-14	1
Microsatellite fragment (*Oryza sativa*, AF348415)	3.00E-07*	2*
Receptor-like kinase (*Triticum aestivum*, AY021654)	6.00E-07*	2*
LRR[e] 19 (*Triticum aestivum*, AF325196)	6.00E-65	2
WIR pathogen R[f] gene (*Triticum aestivum*, AF325196)	1.00E-125	3
Leucine-rich-like protein (*Aegilops tauschii*, X87686)	1.00E-55	2
RGA link to resistance loci in rice (*Oryza sativa*, AB022168)	4.00E-16	1
actin (ACT-1) gene, partial cds (*Triticum monococcum*, AF326781)	4.00E-16	1
putative chromosome condensation factor (CCF) (*Triticum monococcum*, AF326781)	4.00E-16	1
putative resistance protein(RGA-2) (*Triticum monococcum*, AF326781)	4.00E-16	1
putative nodulin-like protein (NLL) gene (*Triticum monococcum*, AF326781)	6.00E-26	1
chloroplast matK gene for maturase (*Cycas pectinata*, AB076238.1; *Zamia angustifolia*, AB076567.1)	6.00E-26	2
clone tac 923.8 3' Ac insertion site sequence (*Zea mays*, AY065582.1)	1.00E-29	1
Genomic seq. BAC F27F5 (*Arabidopsis thaliana*, AC007915.3)	0.011*	1*
Germline Ig heavychain var. region (*Macaca mulatta*, U57580)	0.043*	1*
Genomic DNAChr. 1 BAC clone: OJ1294_F06 (*Oryza sativa*, AP004326.3)	0.0	1
Wheat chloroplast ATP synthase CF-1 gene, beta and epsilon subunits, complete cds, and Met-tRNA gene (*Triticum aestivum*, M16843)	7.00E-29	1
Ty1-copia-like retrotransposon partial pol pseudogene, clone Tbn-1 (*Beta nana*, AJ489202)	2.00E-32	1
microsatellite DNA, CA-repeat (AC) (*S. salar*, Y11455)	0.0	1
Predicted membrane protein (*Clostridium acetobutylicum*, AE007615-4)	1.00E-03*	1*
Nucleotide binding site LRR protein-1 (*Oryza sativa*, AY043283)	4.00E-22	1
Nucleotide-binding leucine-rich-repeat protein 1 (*Oryza sativa*, AF271293)	1.00E-12	1
Nucleotide-binding leucine-rich-repeat protein-like (*Oryza sativa*, AP003802)	4.00E-14	2
Putative NBS-LLR type resistance protein (*Oryza sativa*, AC097447)	3.00E-13	1
Putative disease resistance protein (*Oryza sativa*, AC087181)	1.00E-55	
Resistance gene candidate CC-NBS-LLR Class (*Arabidopsis thaliana*, NM_175742.1)	1.00E-33	1
F12M16 (*Arabidopsis thaliana*, AC008007)	4.00E-43	1
Disease resistance complex protein NBS-LRR class (*Arabidopsis thaliana*, NP_188065.1)	1.00E-43	1
Putative disease resistance protein CC-NBS Class (*Arabidopsis thaliana*, NM_104655)	1.00E-43	1
PRM1 homolog (*Arabidopsis thaliana*, AB028231)	1.00E-43	1
Putative RGA PIC23 -(*Lactuca sativa*, AF017751)	3.00E-16	1
Resistance complex protein I2-C-2 (*Lycopersicon peruvianum*, AF004879)	7.00E-13	1
NBS-LRR resistance protein candidate (*Lactuca sativa*, AF113949)	7.00E-13	1
Serine/threonine kinase protein (*Triticum aestivum*, *Pseudoroegneria*)	1.00E-18	2
Conserved hypothetical protein (*E. coli*, NC_002655, NC_003047)	6.00E-31	2
NBS-LRR type protein (r15) gene (*Oryza sativa*, AF032702)	6.00E-13	1

BLASTX annotation	E-value	No. of dbESTs hits
Resistance protein candidate RGC2A pseudogene (*Lactuca sativa*, AF072268)	1.00E-14	1
Polymyxin ß-resistance protein (*Saccharomyces*, S569090)	1.00E-04*	1*
Receptor like protein (*Arabidopsis thaliana*, NP_190339)	3.00E-13	1
Thioredoxcin (*Triticum aestivum*, AJ005840)	1.00E-66	1
18S ribosomal RNA (*Triticum aestivum*, AY049040)	0.440*	1*
Intergrase/recombinase (*Brucella melitensis*, AE009541)	1.00E-03*	1*
Intergrase-like protein (Bacteriophage H191, AJ236875)	0.61*	1*
Microsatellite DNA (*Entandrophragma*, AJ420885, *Cocos*, AJ458311)	3.00E-48	9
mRNA sequence (*Zea mays*, AY105736)	2.00E-287	2
Aminotransferase-like protein (*Oryza sativa*, AF105736)	5.00E-52	3
Genomic DNA chromosome 4 (*Oryza sativa*, AL662950)	9.00E-27	2
Giant Cell protein mRNA (*Lycopersicon esculentum*, L24012)	7.00E-03*	1*
RbcL for Rubisco (*Oryza sativa*, X62117;*Triticum aestivum*, X62119; *Aegilops tauschii* , X04789; *Avena*, L15300)	9.00E-44	9
Mitochondrial gene for tRNA-His (*Oryza sativa*, D13101)	2.00E-35	1

a. NCBI accession number of homologous sequence
b. Small subunit
c. Miniature inverted terminal repeat element
d. High molecular weight
e. Leucine rich-repeat
f. Resistance
* Protein with discernable function

(TAP) to remove the 5' cap. The dephosphorylated, decapped mRNA was ligated to a GeneRacer™ RNA oligo using the GeneRacer Kit (Invitrogen). The ligated mRNA was reverse-transcribed using SUPERSCRIPT™ II RT (Invitrogen) and the GeneRacer™ Oligo dT Primer to create RACE-ready cDNA with known priming sites at the 5' and 3' ends. The 5' ends were amplified using a reverse degenerate nucleotide-binding site primer and the GeneRacer™ 5' Primer. The degenerate oligonucleotide primers were based on the amino acid sequences of two highly conserved motifs of the NBS in the tobacco *N* and *Arabidopsis RPS2* genes (Yu et al., 1996). The 3' ends were amplified using a forward degenerate nucleotide-binding site primer and the GeneRacer™ 3' primer (GCTGTCAACGATACGCTACGTAACGGC ATGA CAGTG(T)$_{18}$). The cycling parameters used for the GeneRacer™ reactions were five cycles consisting of 94°C for 30 sec and 72°C for 1 min, five cycles of 94°C for 30 sec, 70°C for 30 sec and 72°C for 1 min and twenty cycles of 94°C for 30 sec, 68°C for 30 sec and 72°C for 1 min.

Degenerate NBS-PCR

For the amplification of NBS sequences from the synthesized cDNA the following degenerate primers was applied: NBS-F1 (GGAATGG GNGGNGTNGGNAARAC); NBS-R1 (YCTAGTTGTRAYDATDAYY YTRC), where R = A/G, Y = C/T, D = A/G/T, H = A/C/T, N = A/G/C/T. The PCR reaction consisted of 50 µM of each primer, 50 ng of the RT template, 1X reaction buffer (Promega), 2.5 mM $MgCl_2$, 0.2 mM of each dNTP and 2.5U of *Taq* DNA polymerase, and 1.3 M betaine to increase primer annealing. Thirty cycles of PCR, consisting of 95°C for 1 min, 55°C for 1.5 min, and 72°C for 1 min, were performed in a Perkin-Elmer GeneAmp PCR System 9700 DNA thermal cycler (Applied Biosystems).

Cloning and Analysis of NBS-PCR Products

The PCR products were purified from an agarose gel slice using a Geneclean III Kit (Bio101). These fragments were cloned into the pGEM®-T Easy vector system (Promega). Ligation mixtures were used to transform competent *E. coli* (JM109) cells. Plasmid DNA was isolated from candidate clones and purified. Sense and antisense strands of the clones were used in cycle sequencing using the dideoxy-DNA chain-termination method with the BigDye Terminator Cycle Sequencing Reaction kit (Perkin-Elmer) on the ABI-3100 Prism Automated sequencer (Perkin Elmer).

Sequence identity and functional annotation

The sequence identities were obtained after BLAST searching and alignment to other published sequences in GenBank (Altschul et al., 1997). Functions were assigned to ESTs based on the results returned from searches using the BLASTX algorithm. Any ESTs that did not produce a BLASTX hit were considered to have an unknown function. Sequences that produced hits to proteins with E values greater than 10^{-5} were also considered to have an unknown function. Sequences with hits to proteins with no discernable function were placed into the miscellaneous category. Sequences with hits to plant defense (pest and pathogen) were placed into the Secondary metabolism category. The remaining sequences were placed into five broad functional categories: protein synthesis and modification, metabolism, regulatory, structural and genes of unknown function (miscellaneous).

RESULTS

We constructed cDNA libraries from Russian wheat aphid infested wheat leaves at the 3-4-leaf growth stage. The average titer of the cDNA libraries collectively were approximately 2 x 10^6 CFU, and with the average cDNA insert size of approximately 1kB. Following a single-pass, 5'-end sequencing approach, we obtained a total of 207 ESTs with sizes that ranged from 230 to 772 bases, and an average size of 489 bp.

To assign function to the proteins encoded by nonredundant sequences, the DNA sequences were translated into their corresponding amino acid sequences and searched against the nonredundant GenBank protein database using the BLASTX algorithm. A maximum probability threshold for a sequence match was set at 10^{-5}. Following this approach we obtained a total of 194

ESTs with significant E-values already present in GenBank (Table 1).

After the sequence identities were obtained from GenBank, functions were assigned based on the results returned after BLAST searching of the obtained ESTs (Figure 1). The annotated functions comprise of 25% of sequences involved in protein synthesis and modification, such as the translation factors, tRNA ligases, protein kinases and hydrolases; 25% of the sequences were involved in structural functions, such as membrane-bound and cytoskeleton proteins; 22% of the sequences were involve in the general metabolic activities required for energy production. Only 3.5% of the obtained sequences represented hits with regulatory function. Of the obtained sequences, 6.5 % failed to give a significant hit with any known protein function and thus represent the miscellanous portion. Following this approach we obtained 18% sequences with functions assigned to the secondary metabolism, and most of these had significant hits to either specific resistance gene analogs or putative RGAs.

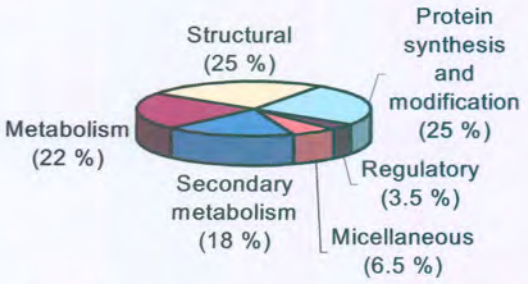

Figure 1. Percentage of nonredundant sequences grouped as genes of unknown function and genes classified into functional groups. Protein synthesis and modification: translation factors, tRNA ligases, protein kinases and hydrolases; Metabolism: proteins with a defined metabolic function like those involved in energy, redox, lipid, or carbohydrate metabolism; Structural: membrane-bound, cytoskeleton, and ribosomal proteins; Regulatory: kinases, transcription factors and proteins involve in cell cycle control; Secondary metabolism: pathogenesis-related proteins; Miscellaneous: proteins with no discernable function. Expressed sequence tags (ESTs) that did not produce a BLASTX hit, or with hits with E-values greater than 10^{-5}, were considered to have an unknown function.

The obtained RGAs were grouped accordingly to the main resistance gene classes (Table 2), and represent the major groups of resistance resistance genes, which include the serien/threonine kinases (2), transmembrane receptors (2); leucine-rich repeats (2); nucleotide binding sites (10) and leucine zippers (2). No hits were obtained that fall within the grouping of toll/interleukin-1. A further 18 sequences gave significant hits with functions either defined as putative resistance proteins or proteins with known linkages to pathogen resistance, but which does not fall within the assigned groupings.

Table 2. Expressed sequence tags (ESTs) that produced BLASTX hits with significance to resistance (R) genes.

Structural domain classes	E-value	No. of dbESTs hits
Serine/threonine kinases		
Serine/threonine kinase protein	1.00E-18	2
Transmembrane receptor		
Receptor-like kinase	6.00E-07*	2
Leucine-Rich Repeats		
LRR19	6.00E-65	2
Nucleotide binding sites		
NBS-LRR resistance protein candidate	7.00E-13	1
NBS-LRR type protein (r15) gene	6.00E-13	1
Putative NBS-LLR type resistance protein	1.00E-55	1
Nucleotide binding site LRR protein-1	4.00E-22	1
Nucleotide-binding leucine-rich-repeat protein 1	1.00E-12	1
Nucleotide-binding leucine-rich-repeat protein-like	4.00E-14	2
Resistance gene candidate CC-NBS-LLR Class	1.00E-33	1
Disease resistance complex protein NBS-LRR class	1.00E-43	1
Putative disease resistance protein CC-NBS Class	1.00E-43	1
Resistance complex protein I2-C-2	7.00E-13	1
Toll/interleukin-1		
Leucine Zipper		
Leucine-rich protein	1.00E-55	2
Other[a]		
Resistance gene analogue2	7.00E-13	6
WIR pathogen R gene	1.00E-125	3
RGA link to resistance loci in rice	4.00E-16	1
Putative resistance protein(RGA-2)	4.00E-16	1
Putative disease resistance protein	1.00E-55	1
Putative RGA PIC23	3.00E-16	1
Resistance protein candidate RGC2A pseudogene	1.00E-14	1
Polymyxin ß-resistance protein	1.00E-04*	1
Thioredoxcin	1.00E-66	1
PRM1 homolog	1.00E-43	1

[a] Structural class is not well defined
* Protein with discernable function

DISCUSSION

The majority of plant disease resistance genes cloned so far contain nucleotide-binding sites (NBS) and leucine-rich repeat (LRR) domains. This class of R genes belongs to a superfamily that is present in both dicotyledons and monocotyledons as suggested from

sequence comparisons made between these isolated genes (Bent et al., 1994; Lagudah et al., 1997; Meyers et al., 1998).

The use of PCR based approaches with degenerate oligonucleotide primers designed from the NBS region of cloned disease resistance genes has led to the cloning of resistance gene-like sequences in several plant species (Leister et al., 1998; Seah et al., 1998; Garcia-Mas et al., 2001). Co-segregation of some of these sequences with known disease resistance gene loci has been reported.

In the present study we tested the feasibility of using such a PCR-based approach. The degenerate oligonucleotide primers designed from conserved motifs in the NBS domain, was used to clone several disease resistance gene homologues from wheat lines. Out of the 207 ESTs obtained, 37 gave hits with significant homology to plant defense (E-values < 10^{-5}). In the present study, a clear bias for obtaining resistance gene analogs were found, when compared to other similar but randomized studies (Kruger et al., 2002; White et al., 2000; Yamamoto and Sasaki, 2000). In a similar study, where the expressed genes from *Fusarium graminearum* infected wheat spikes were analyzed, most of the obtained nonredundant ESTs were of miscellaneous nature, followed by sequences related to general metabolism and of importance to cell structure (Kruger et al., 2002).

The NBS and LRR domains are conserved amongst several disease resistance genes and this has led to the hypothesis of cloning additional resistance genes based on the homology to these conserved sequences. The procedure can be complicated by an excess of genes that contain the NBS region, but are not related to resistance genes (Yu et al., 1996). This is also true for this study, as only 8% of the RGAs could be linked to specific resistant genes, and 50% could be assigned to specific groupings, whereas the others contained only the specific conserved motif. Also many homologous resistance genes may be located throughout the genome in a plant species. Thus, the sequence homology among these genetically independent and functionally distinct disease-resistance genes will present a difficulty in isolating individual clones, which correspond to a specific resistance gene by hybridization. However, it proved useful in the present study, as these isolated clones will be utilized in a gene expression study approach in a future study.

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We wish to express our sincere gratitude to 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 financial support and provision of infrastructure.

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A LEUCINE RICH HOMOLOG TO *AEGILOPS TAUSCHII* FROM BREADWHEAT LINE PI 137739 OBTAINED BY SUPPRESSION SUBTRACTIVE HYBRIDISATION SHOW LINKAGE TO RUSSIAN WHEAT APHID RESISTANCE GENE *DN1*

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ABSTRACT

Large-scale damage to small-grain crops caused by infestation with Russian wheat aphid (*Diuraphis noxia* Mordvilko, RWA) has been reported since its introduction to South Africa in 1987. This has resulted in a consorted effort to control the damage caused by this pest. These include searches to identify sources of pest resistance that originate from the centre of origin. In an effort to characterise gene sequences that contribute towards the host defence response we employed suppression subtractive hybridisation using cDNA, synthesised from infested and control wheat line PI 137739. We obtained a SSH clone of 411 bp in size with significant homology to a leucine rich-like protein from *Aegilops tauschii* (GenBank accession # AF4446141.1; *AMO00SSHL1*). A second clone (NBS-RGA2), homologous to a *Triticum monococcum* RGA2 (368 bp; GenBank accession # AF326781; 7e-13; NBS-RGA2) was amplified from genomic DNA using NBS-specific primers. After the expression of the fragments were confirmed in wheat after infestation using RT-PCR and Northern blot analysis, we tested linkage of the fragments to RWA resistance using a segregating $F_{2/3}$ 'Tugela' x 'Tugela' DN population. We observed co-segregation of *AMO00SSHL1* and NBS-RGA2 with the RWA resistance gene *Dn*1; linkage distance of 7.41 cM and 3.15 cM were obtained, respectively.

INTRODUCTION

Severe yield losses of wheat (*Triticum aestivum*), barley (*Hordeum vulgare*) and other Triticale crops in South Africa are caused by Russian wheat aphid infestation. RWA outbreaks have necessitated the application of undesirable and expensive insecticides. Therefore, the development of cultivars resistant to the RWA is considered to be a more effective means of controlling the RWA. Breeding programmes in South Africa aims at developing such wheat cultivars. In order to facilitate breeding programmes DNA-based molecular markers and genetic maps of these markers are developed in aid of marker assisted selection (MAS; Melchinger, 1990). Ten genes for RWA resistance have already been identified in wheat, namely *Dn*1 to *Dn*9 and *Dnx*. The aim of the present study was to determine the linkage of a SSH fragment (*AMO00SSHL1)* and a nucleotide binding site-related fragment (NBS-RGA2) to the *Dn*1 RWA resistance. Co-segregation with the *Xgwm111* and *Xgwm635* microsatellite markers was also tested (Liu *et al.*, 2001).

subtracted products were P[illegible]l fragments were cloned, sequenced and analysed using the BLASTN annotation (Altschul *et al.*, 1990). Northern blot analysis was performed using uninfested RNA and RNA extracted at day 2, 3, 4 and 5 post-infestation. Hybridisation probes were prepared from fragments identified after sequence analysis: three probes from leucine zipper-nucleotide binding site-leucine rich repeat (LZ-NBS-LRR) fragments (A, B, C), one from a leucine rich-like protein fragment (D) and an ubiquitin protein fragment (E) that served as control. Southern blot analysis was performed on PI 137739 genomic DNA using the above-mentioned probes. The probes were labelled and hybridisation was performed according to the instructions of the *Gene Images* Random Prime Labelling Module (Amersham Pharmacia Biotechnology, USA). Quantitative RT-PCR analysis was performed on a LightCylcer Instrument using the above-mentioned RNA populations for first strand cDNA synthesis. The primers used were designed according to the sequence identities of the above mentioned SSH fragments.

RESULTS

The SSH procedure resulted in the identification of 18 clones that contained fragments of relevance to this study and these sequences aligned to 98 dbETSs during BLASTN analysis. The sequences were categorised into functional groups: 28% was involved with protein synthesis, 3% was metabolic sequences, 9% consisted of regulatory sequences, 7% was involved with structural functions, 35% was classified as miscellaneous and 18% of the sequences displayed homology to resistance-related fragments (Fig. 1). Four sequence alignments exhibited homology to the *Mla* locus of barley (*Hordeum vulgare*; ~380 bp; 2e-65), eight sequences exhibited homology to leucine rich-like proteins (e-149; ~ 377 bp) and four were homologous to LZ-NBS-LRRs (e-131; ~440 bp) of *Aegilops tauschii*, and four sequences aligned with resistance gene analogs (RGAs) of *Trticum monococcum*.

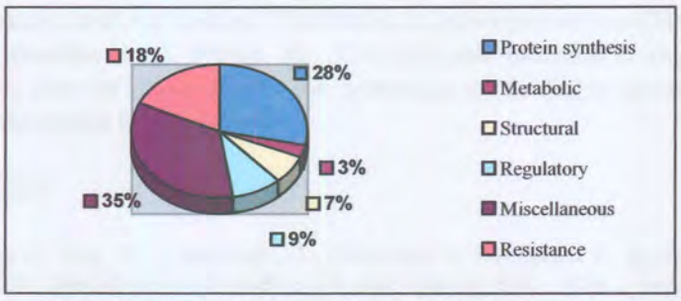

Figure 1 The proportional distribution of the acquired SSH ESTs presented as functional groups.

Northern blot analyses (Fig. 2; lane 1 = uninfested RNA; lane 2 = day 2, lane 3 = day 3, lane 4 = day 4 and lane 5 = day 5 post-infestation RNA) revealed that the levels of expression of the resistance-related fragments (three LZ-NBS-LRRs and one leucine rich-like fragment) after two days of RWA infestation are equal to the expression in uninfested material. Expression during day 3 post-infestation is significantly down-regulated. Higher expression levels are observed during day 4, and day 5 exhibited a significant increase in expression of these gene fragments. The results obtained after quantitative PCR confirmed the up- and down-regulation of these fragments (Table 1). The quantitative analysis of the resistance-related fragments indicates that the leucine rich-like fragment is induced at much higher levels (~10200 times) than the LZ-NBS-LRR fragments (890 – 4000 times). Southern blot analysis confirmed the identified resistance-related fragments originate from PI 137739 genomic DNA.

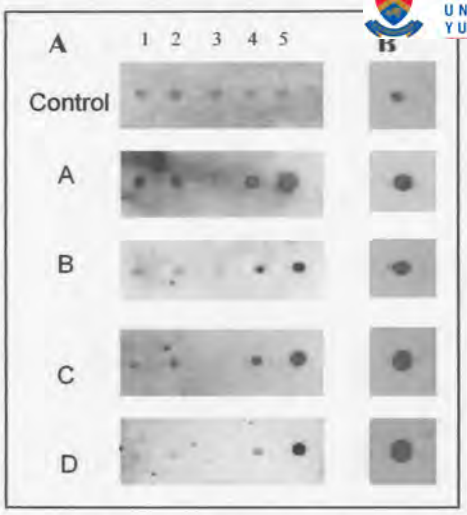

Figure 2 Northern blot (A) and Southern blot (B) analyses of the resistance-related and control fragments.

1 Expression levels of the resistance-related SSH fragments (A, B, C and D) compared to the control fragment as –fold increase or decrease.

Fragment	Day 2*	Day 3*	Day 4*	Day 5*
A	2.6	-1.5#	9.5	898.2
B	2.2	-9.1#	7.6	1108.1
C	7.2	-6.6#	16.8	4060.4
D	11.2	-3.9#	347	10198.2

* Values were normalised with control (E) expression levels
\# Values indicate down-regulation

DISCUSSION AND CONCLUSION

Suppression subtractive hybridisation proofed useful during this study in order to isolate gene fragments related to resistance in wheat against RWA infestation. The leuicne rich-like fragments, as well as the LZ-NBS-LRRs are constituents of the largest class of plant resistance (R) genes, namely the NBS-LRR group. The RGA fragments identified during this study are also classified as part of the NBS-class of R genes. The induction of the resistance-related fragments after RWA infestation can be compared to the induction of pathogenesis-related (PR) proteins such as chitinases, β-1, 3 glucanases and peroxidases illustrated in previous studies (Botha *et al.*, 1998; Van der Westhuizen *et al.*, 1998a, b). The dramatic increase in expression of these fragments can also be compared to the induction of oxidative processes after insect infestation (Miles and Oertli, 1993).

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SUPPRESSION SUBTRACTION (SSH) EMPLOYED TO INVESTIGATED GENE EXPRESSION AFTER RUSSIAN WHEAT APHID INFESTATION

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ABSTRACT

The Russian wheat aphid (RWA; *Diuraphis noxia* Mordvilko) is a major pest on wheat, barley and other Triticale in South Africa. During this study, SSH was employed to isolate gene sequences that are involved in wheat resistance against RWA infestation. Gene fragments related to several resistance gene families were identified and constituted 18% of the isolated SSH fragments. This includes a leucine rich-like fragment and leucine zipper-nucleotide binding site-leucine rich repeats (LZ-NBS-LRR) homologous to *Aegilops tauschii*. The expression of these fragments during plant infestation was confirmed through Northern blot hybridisations and quantitative RT-PCR. Hybridisations and quantification were performed using uninfested wheat RNA and RNA extracted at day two, three, four and five after infestation. On average, the resistance fragments exhibited increased expression after two days of infestation (2 - 11 fold), less expression at day three (2 - 9 fold down-regulation) and gradual up-regulation of the gene expression during day four (8 - 350 fold) and day five (890 - 10 200 fold).

INTRODUCTION

Insect infestation in plants results in altered gene expression patterns and protein synthesis as a means of self-defence. The study of these differentially expressed genes has been simplified through the development of suppression subtractive hybridisation (SSH). SSH is based on the selective amplification of target cDNA while simultaneously suppressing the amplification of non-target cDNA (Diatchenko *et al.*, 1996). During the present study, SSH was employed in an attempt to isolate gene sequences that are involved in wheat resistance against the Russian wheat aphid (RWA). The RWA is an important pest of wheat crops in South Africa and has caused severe yield losses since 1978. The wheat line PI 137739, that is known to exhibit resistance against the RWA and contains the *Dn*1 resistance gene, was used as RNA source. Tester cDNA was prepared from RWA infested leaf material and driver cDNA from uninfested tissue, and subtractively hybridised. The SSH fragments were cloned and sequenced in order to compare them with putative resistance gene fragments. The level of induction of the resistance-related fragments was assessed through Northern blot analyses, as well as quantitative PCR reactions.

MATERIALS AND METHODS

The wheat cultivar used was PI 137739. RNA was isolated from the second and third leaves of uninfested and RWA infested plants, two to five days after infestation. Tester and driver cDNA were synthesised using 1.6 µg infested and 0.85 µg uninfested mRNA, respectively, and adapter ligation was carried out according to the directions of the CLONTECH PCR-Select System (CLONTECH Laboratories Incorporated, USA). Primary and secondary hybridisation was performed, the

MATERIALS AND METHODS

The wheat lines used during this study, was the resistance lines 'Tugela' DN and SA 1684, and the susceptible line 'Tugela'. The F_2 population consisted of 92 individuals that were evaluated for phenotypic segregation of resistance after RWA infestation. The disease severity scale that was used scored susceptible plants as 1-6 and resistance plants as 7-10 (Tolmay, 1995). The microsatellite markers also tested on this population were *Xgwm111* (linked with Dn1) and *Xgwm635* (linked with *Dn*8). DNA extraction was performed according to the method of Edwards and co-workers (1991). A resistance-related fragment (homologous to a leucine zipper-NBS-LRR) was generated using SSH and designated *AMO00SSHL1*. A second fragment was generated through PCR of genomic DNA of the wheat line PI 137739. This fragment exhibited homology to a resistance gene analogue (RGA2) sequence and was designated NBS-RGA2. Primers were designed for these clones and used during PCR screening of the F_2 population. The linear linkage of the *AMO00SSHL1* and NBS-RGA2 markers to the Dn1 gene were determined using MAPMAKER (Lander *et al.*, 1987). The linkage distances of these fragments to *Dn*1 were calculated according to Kosambi (1944).

RESULTS

During this study 92 individuals, of which 72 were resistant and 20 susceptible to RWA, were screened. Phenotypic segregation ratio of the *Dn*1 gene was calculated as 3.6:1 (resistant: susceptible) and the χ^2 value 0.96. This ration is an indication that *Dn*1 is a single dominant gene. The primer pair NBS-RGA2 produced three specific PCR amplification bands, a 280 bp, a 220 bp and a 130 bp bands (Fig. 1).

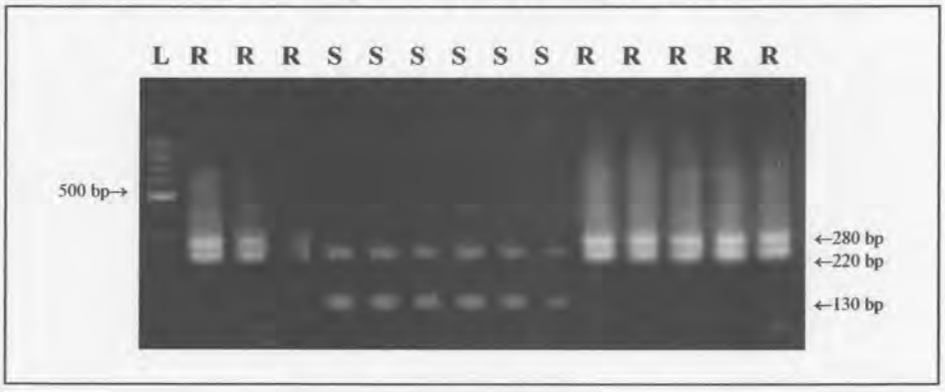

Figure 1. PCR bands amplified from the DNA of the F_2 population using the NBS-RGA2 primer pair. R = RWA resistant, S = RWA susceptible, L = 100 bp ladder.

The recombination fraction and linkage distance of the *AMO00SSHL1* and NBS-RGA2 fragments were calculated (Table 1). The linkage map (Fig. 2) of the *AMO00SSHL1* and NBS-RGA2 fragments indicates linkage distances of 7.14 cM and 3.154 cM, respectively, to the *Dn*1 gene.

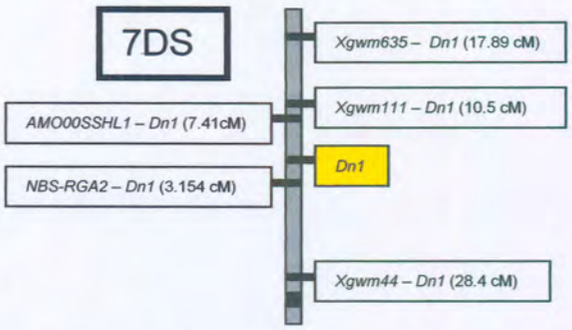

Figure 2 Linear position of the *AMO00SSHL1* and NBS-RGA2 markers on the short arm of the 7D gene of *Triticum aestivum*.

Table 1 Summary of the markers linked to RWA resistance gene *Dn*1.

Marker	Gene	Recombina -tion (%)	Linkage distance (cM)
AMO00SSHL1	*Dn*1	7.38	7.41
NBS-RGA2	*Dn*1	3.15	3.154

DISCUSSION AND CONCLUSION

The present study clearly exhibits the linkage of a leucine zipper-leucine rich repeat-nucleotide binding site (LZ-LRR-NBS) fragment and a resistance gene analogue (RGA2) fragment to the RWA resistance gene *Dn*1.

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Gene expression profiling during *Diuraphis noxia* infestation of *Triticum aestivum* cv. 'Tugela DN' using microarrays.

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INTRODUCTION

Diuraphis noxia (Mordvilko, Russian wheat aphid, RWA) are probing and sucking insects, which feed on wheat, barley and other triticale (Bryce, 1994). In a study on feeding behaviour of RWA it was confirmed that the RWA probed more and fed less on resistant cultivars, resulting in the formation of more lesions on the resistant cultivars (Bahlmann *et al.*, 2003). Infestation by the RWA results in altered protein expression patterns, which is manifested as differential expression of total proteins, and specific pathogenesis-related proteins (PR) like chitinases, ß-1,3-glucanases and peroxidases (Botha *et al.*, 1998; Van der Westhuizen *et al.*, 1998a,b). Feeding by RWA also wounds the tissue. Results on chitinase profiling (IWF and Western-blot analysis) indicated that there are distinct differences between the obtained isoenzymes and chitinase subunits after RWA infestation, wounding and exogenous ethylene treatments (Botha *et al.*, 1998). Studies conducted on the intercellular washing fluid of wheat (*Triticum aestivum* L.) resistant to the Russian wheat aphid ('Tugela DN'), showed that proteins were induced within six days of infestation. The response is induced within the first 12h after infestation by the RWA in resistant cultivar 'Tugela DN', but not in the susceptible near isogenic line 'Tugela' (Botha *et al.*, 1998; Van der Westhuizen *et al.*, 1998a,b).

Recently, cloning of multiple R genes from various plant species has revealed conserved domains at the amino acid level. The most notable being the presence of nucleotide binding sites (NBS) and leucine rich repeat regions (LRR). The presence of a NBS and a LRR 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). In the comparative analysis using infested material containing the RWA resistance genes *Dn1*, *Dn2* and *Dn5*, and utilizing degenerate primer sets designed from the consensus NBS motif from other genome studies (e.g. *Arabidopsis* and rice), suppression subtractive hybridization (SSH), RACE-PCR and cDNA libraries, several NBS-LRR sequences were obtained (Lacock *et al.*, 2003). The feasibility of using the degenerate PCR-based approach was tested, and it was found that 18% of all the obtained ESTs showed significant homology to resistance genes from other plants on amino acid level (E value $< 10^{-5}$) rendering the approach highly feasible if resistance gene analogs are the target of interest.

In this study, we have analyzed the expression profiles of selected gene sequences obtained from our previous study (Lacock *et al.*, 2003), as well as from other studies. To follow the expression profiles of these gene sequences, we hybridized the

microarray against cDNA synthesized from leaf tissue of RWA resistant cultivar 'Tugela DN' pre- (day 0) and post-infestation (days 2, 5 and 8), in an effort to identify gene sequences with significance to the RWA defense response.

MATERIALS AND METHODS

Russian wheat aphid resistant cultivar 'Tugela DN' (Tugela*5/SA1684, *Dn1*) was infested with RWA (10 aphids per plant) at the 3-4-leaf growth stage (Botha *et al.*, 1998). Total RNA isolation, purification of mRNA, cDNA synthesis, cDNA library construction and sequencing was performed as previously described (Lacock *et al.*, 2003). After sequencing of 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).

Target cDNA 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). PCR products were quantified by electrophoresis on 0.8% agarose gels (w/v) and visualized by ethidium bromide staining. Microarrays were printed on a BioRobotics Generation II Arrayer according to the manufacturer's instructions. Arrays were printed on aminosaline slides and each target DNA were spotted 8 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.

For Cy3- and Cy5-labelled cDNA, total RNA was isolated from wheat leaves on days 0, 2, 5 and 8 post-infestation by the RWA, as previously described. Poly A^+ RNA was purified from total RNA using the Oligotex mRNA spin-column protocol (Oligotex™ Handbook 07/99, Qiagen). 100 ng of the purified mRNA was used for the preparation of Cy3- and Cy5-labelled cDNA for microarray hybridization using the Cyscribe Post-labeling kit according to the manufacturers instructions (Amersham Biosciences, Little Chalfont, UK). Unincorporated label and single stranded nucleotides were removed from the prepared labeled cDNA using the MinElute cleanup kit according to the manufacturers protocol (MinElute™ Handbook 04/2001, Qiagen).

Microarrays were pre-hybridized by adding 35 µl pre-hybridization solution (3.5 x 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 ddH2O 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% formamide; 25% hybridization buffer; 25% mQ) and denatured (98 °C for 2 min). The slides were hybridized overnight for 12-18 h at 42 °C. Slides were washed three times at 42 °C for 4 min (once in 1xSSC/0.2% (w/v) SDS, twice in 0.1 x SSC/0.2% (w/v) SDS). This was followed by three washes at room temperature for 1 min each in 0.1 x SSC and dried with nitrogen gas. Hybridization was repeated over biological material, made use of direct comparisons, and a reference, 2x2 factorial design (Yang and Speed, 2002).

An Axon GenePix 4000 A 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 the mixed model approach (Wolfinger *et al.*, 2001; Chu *et al.,* 2002) and SAS/STAT software version 8 (SAS Institute Inc. 1999). Genes were also organized and visualized by Cluster and Tree View (Eisen *et al.*, 2000).

RESULTS AND DISCUSSION

The custom-designed microarrays used in this analysis were screened with cDNA synthesized from mRNA samples derived from wheat leaves at the three to four leaf stage, pre- (day 0) and post-infestation (days 2, 5 and 8). Previous studies on RWA feeding induced responses, indicated the induction of PR-proteins and other defense related proteins, e.g. chitinases, peroxidases, ß-1,3-glucanases (Botha *et al.*, 1998; Van der Westhuizen *et al.*, 1998a,b), lipoxigenase, ROS (Mohase and van der Westhuizen, 2002) 3 to 12 days post-infestation. Two hundred and fifty-six wheat NBS-LRR and other sequences were obtained using degenerate primer sets designed from the consensus NBS motif from other genome studies (e.g. *Arabidopsis* and rice), suppression subtractive hybridization (SSH), RACE-PCR and cDNA libraries (Lacock *et al.*, 2003). Selected wheat cDNA clones were spotted onto microarrayer slides. Purified mRNA from infested material, containing the RWA resistance gene *Dn1* was isolated 0, 2, 5, and 8 days after infestation, post-labeled with Cy3- or Cy5-fluorescent dyes and hybridized to the arrays.
Statistical analysis of the expression data revealed the up-regulation and down-regulation of 5% of all the spotted gene fragments (Fig. 2) at a threshold log-2 expression ratio of 1.5 and $P \leq 0.05$ (Fig. 1).

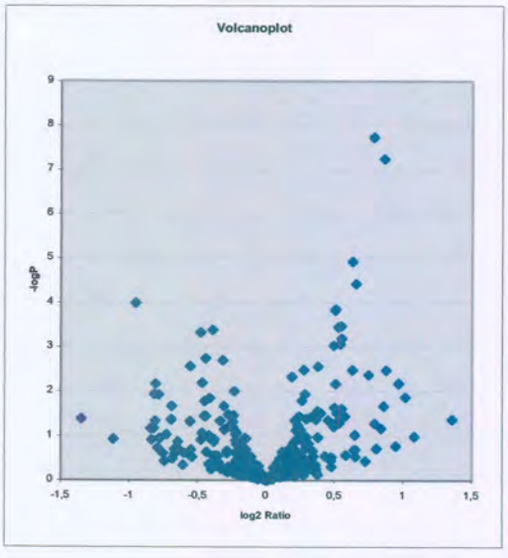

Figure 1. Significance plot for data set.

Several unknown ESTs obtained from a previous study (sequences with no significant homology to published dbESTs in GenBank at a E value $< 10^{-5}$, Lacock *et al.*, 2003) showed significant regulation in response to RWA feeding (Fig. 2, 3, Table 1). Wheat homologs to *RGA*-2 and chloroplast-ATP synthase genes were down-regulated in response to RWA feeding on days 2 and 5 post-infestation (Table 1), whereas wheat homologs to BARE-1 long terminal repeat and Ty1-copia-like retrotransposon for putative reverse transcriptase were significantly up-regulated on days 2 and 5 post-infestation (Figure 3, Table 1).

Figure 2. Hierarchical clustering of data to demonstrate regulation of genes upon RWA feeding (Days 0, 2, 5 and 8 post-infestation; Green = down-regulation; Red = up-regulation; Black = zero regulation).

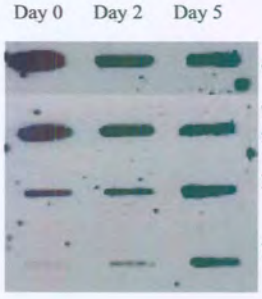

Figure 3. Northern blot analysis of cDNA clones spotted on the Microarray. Control demonstrating equal loading of cDNA.

Table 1. Real time quantitative PCR of selected cDNA clones to quantify the observed regulation of data.

Clone ID[a]	Day 2*	Day 5*
Unknown protein #23	+ 2.6	+ 4.4
Triticum monococcum putative resistance protein (RGA-2)	+ 1.2	- 8.0
Wheat chloroplast – ATP synthase	- 2.1	- 11.3
Unknown protein #57	- 2.3	- 3.8
BARE-1 long terminal repeat	+ 3.5	+ 5 200.0
Unknown protein #310	+ 2200.0	- 109.0
Ty1-copia-like retrotransposon for putative reverse transcriptase	+ 32.0	+ 20 000.0
Unknown protein #314	+ 77.7	+ 1.4

[a]EST (array spot #) with no significant homology (E value $<10^{-5}$) to dbEST in GenBank
*times up (+) or down(-) regulation

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Profiling of gene expression in bread wheat (Triticum aestivum L.) line PI 137739 in response to Russian wheat aphid (Diuraphis noxia Mordvilko) feeding

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The investigation of differential gene expression in Russian wheat aphid (RWA) infested bread wheat is imperative for breeding RWA resistant lines. Profiling differentially expressed genes and gene regulation was achieved by combining several molecular techniques.

Functional gene analysis was assessed by screening the genomic, as well as the complementary DNA, of a resistant wheat line, PI 137739, for resistance-related gene fragments. PCR-based approaches, aimed at isolating nucleotide binding site (NBS) sequences, were utilised during the screening procedures. The data obtained from the identified gene fragments revealed that 30% of these sequences are involved in metabolic activities, 28% are involved in protein synthesis, 10% exert regulatory activities, 4% has structural functions and 18% were classified as miscellaneous sequences. The PCR-based approaches that were utilised proved to be efficient for the identification of resistance-related fragments (10%). Although sequences containing NBS domains were not isolated, resistance gene analogues (RGAs), leucine rich repeat regions, receptor-like kinases and a WIR pathogen resistance fragment were identified. These genes are all thought to be involved in plant defence responses.

Differential gene expression profiling was continued by employing suppression subtractive hybridisation (SSH). SSH involves the comparison of

the defence responses in RWA infested plants with gene expression in uninfested plants. The SSH procedure showed that 28% of the identified expressed sequence tags (ESTs) are involved in protein synthesis, 3% is involved in metabolic activities, 9% is regulatory sequences, 7% is related to structural functions and 35% was classified as miscellaneous. A relative high amount (18%) of resistance-related sequences were identified in the resistant PI 137739 wheat line. These fragments exhibited similarity to a resistance gene locus in barley (*Mla*), leucine rich-like fragments, leucine zipper-nucleotide biding site-leucine rich repeats (LZ-NBS-LRRs) and resistance gene analogues.

Microarray technology was implemented in order to test the feasibility of utilising this technique for investigations of differential gene expression and regulation in plants challenged with insect, specifically the RWA, infestation. A series of ESTs obtained throughout this study was used as target material and hybridised with probes prepared from RNA of uninfested and infested wheat material at different time points post-RWA infestation. Several patterns of gene regulation were obtained and differential expression of some ESTs was obtained in leaf and stem tissue. Further, the validity of the microarray data was monitored by incorporating Northern blot, as well as quantitative PCR, analysis. The data of these three techniques presented a global overview of the gene regulation executed by an infested wheat plant. The data obtained from the Northern blot and quantitative PCR analyses indicated that the microarray data was valid and that the incorporation of microarray analysis in such a study is highly feasible.