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Elucidation of *Diuraphis noxia* biotype-specific responses in *Triticum aestivum* (98M370 Dn7^{+})

By

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Declaration

The results presented here, follow from research carried out at the Department of Genetics, University of Pretoria, under the supervision of Prof. A-M Botha-Oberholster. These results are original and have not been submitted in any form to any other university.

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Soli Deo Gloria

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Chapter 1

Introduction

Diuraphis noxia (Mordvilko) (Russian wheat aphid, RWA) is a significant pest of wheat and barley, and has been reported in all wheat-producing countries by 1990, with the exception of Australia (Basky, 2003). Wheat production in South Africa and the United States has suffered the greatest losses, where the economic impact of the RWA on the USA alone reached $800 million from 1987-1993 (Morrison and Peairs 1998). The main symptoms that susceptible RWA-infested plants develop include white, yellow and purple longitudinal streaking patterns observed on leaf surfaces, as well as leaf rolling (Jyoti et al. 2006). In cases of severe infestation, aphid feeding ultimately leads to the death of the plant.

In terms of control of the RWA, the leaf rolling symptom provides the aphid with a degree of physical protection against the use of contact insecticides (Ma et al. 1998). Therefore, the application of numerous insecticides on a systemic level is required to achieve some degree of success in this regard. This however, is very detrimental to the environment and burdens the farmer financially (Mornhinweg et al. 2005). The use of biological control agents is continually being investigated, but leaf rolling again provides the aphid with suitable protection. As a result, effective control of the RWA will ultimately require an integrated pest management strategy – i.e. involving the use of chemical, biological and genetic resources. Exploiting the advantages of genetic resistance in plants however, requires a basic understanding of plant defence, when under threat from biotic factors in general (specific and non-specific), specifically the RWA.

Plants are sessile organisms that lack the benefit of a circulating immune system, and therefore they have to develop highly specific defence strategies against the threats posed by the RWA (and other pathogens and pests) (Botha et al. 2006). Plants therefore rely on passive preformed physical and chemical barriers that are non-specific (implying that these defence mechanisms are not directed at a specific pest or pathogen) (Jackson and Taylor, 1996), as well as specific resistance responses that

require the recognition of an eliciting agent in order to induce such a response (Botha et al. 2006). When considering preformed defence mechanisms, the protection provided by the plant cell wall can be regarded as the first line of physical defence, which consists of structural components such as cellulose, polysaccharides, proteins and a number of phenylpropanoid polymers (Botha et al. 2006). In addition to this, plants also produce a number of chemical compounds that posses anti-microbial properties and compounds that serve as deterrents against a number of herbivorous organisms (Menezes and Jared, 2002). Chemical mediated defence responses further include for example the production of proteins that directly interfere with the digestive system of invading insects, which includes proteinase inhibitors (Botha et al. 2006).

Phloem forms an important transport system for the distribution of organic compounds assimilated as a result of photosynthesis (Kehr, 2006), and aphids profit from this by extracting nutrients from the plant via a hollow feeding stylet inserted into phloem sieve tubes. As a preliminary response to injury, sieve tubes become plugged, preventing the aphid from meeting its nutritional demands – illustrating natural plant defence on another level (Will and van Bel, 2006). The aphid however, attempts to compensate for this, by injecting watery saliva into the plant during feeding, that amongst other functions, suppresses stress responses such as the closure (or plugging) of sieve tubes (Miles, 1999).

Phloem-feeders take up a large amount of phloem-sap during feeding, where the presence of proteins with insecticidal properties may potentially play a significant role in plant defence (Kehr, 2006). Protease inhibitors for example, are widespread in the phloem of various plant species. This abundance of protease inhibitors in phloem, and the associated scientific support for the importance of digestive proteases to phloem-feeders, implies an integral role of these protease inhibitors in further defence mechanisms (Kehr, 2006). Another group of defence proteins found in phloem sap are the lectins. In the case of insects, lectins are thought to upset feeding and digestion, interfering with insect growth

and development (Murdock and Shade, 2002). Therefore the inhibition of the plant's defences by the aphid is crucial to its survival, and an important role for the watery saliva in this regard is conceivable (Will and van Bel, 2006).

Host-mediated (genetic) resistance is regarded as important in plant defence against the RWA, since it is environmentally safe and provides an economically viable solution to control the RWA in the long run (Van der Westhuizen and Pretorius, 1996). Host-mediated resistance is based on the genetic model described for plant-pathogen interactions, and is termed the gene-for gene model (Flor, 1942). In order for defence to occur, the plant must possess a copy of a resistance (*R*) gene (in the dominant form), and the invading pathogen must carry a dominant avirulence (*Avr*) determinant (Keen, 1990). Should recognition of the pathogen occur, the plant normally activates the hypersensitive response (HR), which is visible as necrotic lesions around the area of infection preventing further spread (Pontier et al. 1998). Besides this HR response, a signal is generated at the initial site of pathogen infection that spreads systemically to healthy parts of the plant, providing the plant with a degree of protection against secondary infection. This is known as systemic acquired resistance (SAR), and it can last for weeks up to months after initial infection. Since the identification of this gene-based resistance as a sustainable solution to the problem of RWA infestation, eleven genes conferring resistance to the RWA (designated *Dn* genes) have been identified in wheat and its relatives. These have subsequently been incorporated into various wheat cultivars in extensive breeding programmes. These include *Dn1* and *Dn2* (du Toit, 1989), *Dn4* (Nkongolo et al. 1991b), *Dn5* (Marais and du Toit 1993), *Dn6* (Saidi and Quick, 1996), and *Dn8, Dn9* and *Dnx* (Liu et al 2001) all identified in wheat germplasm. A recessive resistance gene *dn3,* was identified in *Aegilops tauschii* (Nkongolo et al. 1991a) and *Dn7* was transferred from chromosome 1RS of rye into a wheat cultivar 'Gamtoos' (Marais et al. 1994). Another RWA resistance gene, *Dny* has also been recently incorporated into the 'Stanton' cultivar (Smith et al. 2004). Despite all the efforts to develop resistant wheat cultivars, breakdown of resistance

recently occurred in important wheat producing areas in the USA. The emergence of two new RWA-biotypes resistant to *Dn4* (Haley et al. 2004) and *Dny* (Jyoti et al. 2006), illustrate the importance of continuing research into furthering the development of resistant cultivars.

In order to contribute to this endeavour, the broad aim of this project was to investigate the transcriptional responses activated in a resistant wheat line, 94M370 (Gamtoos *Dn7*), after infestation with three different RWA biotypes (US biotype 1, US biotype 2 and SA biotype 1). This was addressed by focusing on more specific objectives. Firstly, by clarifying the precise molecular mechanisms by which *Dn7* confers resistance to the RWA, and then to further determine how distinctive (or conserved) these responses are following infestation with different RWA biotypes. This involved RWA infestation of the resistant wheat line 94M370 ('Gamtoos'-R; $Dn7^{+}$) and its susceptible near-isogenic counterpart ('Gamtoos'-S; $Dn7^{-}$), after which responses where initially characterised on a molecular level with Suppression subtractive hybridization (SSH) (Chapter 3). Answers to these research questions however, were achieved with the aid of cDNA-AFLP (Amplified Fragment Length Polymorphisms) technology and Real-Time reverse transcription PCR (Chapter 4).

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Chapter 2

Literature Review

2.1 Introduction

The Russian wheat aphid, Homoptera: Aphididae (RWA, *Diuraphis noxia,* Mordvilko*)* is a major pest of wheat, barley and other cereal grains in countries such as the USA, South Africa, and South America (Liu et al. 2002), after having been introduced from countries in the Middle East, Asia Minor and Southern Russia (Marais et al. 1994). In the United States alone, economic losses suffered from a serious reduction in grain yield and increased production costs exceeded $485 million by 1995 (Miller et al. 2001). Such reductions in yield occur as a result of direct aphid feeding, leading to a loss of shoot and root biomass, slower growth rates, water imbalances and sterility (Kindler et al. 1992).

The use of pesticides has provided some degree of control over this pest, but this is not a sustainable solution, due to the adverse environmental effects and large expense associated with these chemical control agents (McDowell and Woffenden, 2003). For these reasons, the development of genetically resistant cultivars was identified as a promising solution to the problem. In depth research into the mechanisms by which current resistant cultivars function, may lead to the identification of novel defence genes and may contribute to our limited understanding of wheat-aphid interactions. By applying the information gathered from such studies, we might identify sustainable solutions for the resistant RWA problem.

2.2 Plant - pathogen interactions

2.2.1 Overview

Understanding plant-pathogen interactions is vitally important for the further elucidation of plant-insect interactions, due to their similar response activation. The discussion therefore that follows

focuses on the plant-pathogen perspective, but is also most likely to be true for plant-aphid interactions.

Plants are constantly under threat of attack from numerous different viruses, microbes and fungi. Because they lack the advantage of a proficient circulatory immune system as seen in mammals, every plant cell has to rely on a more evolutionary ancient means of defence. This strategy relies on preformed and/or inducible defence barriers, collectively referred to as innate immunity (Hammond-Kosack and Jones, 1997).

Despite these constant challenges by pathogens, the occurrence of disease is rare. Generally, there are three reasons for the failure of pathogens to cause disease. The plant may (i) not be able to support the requirements of the pathogen and is thus not a suitable host. The plant (ii) may possess various structural barriers or toxins that prevent colonization by the pathogen, or (iii) it mounts an active defence response (Hammond-Kosack and Jones, 1996). All of these interactions where disease does not occur are defined as being incompatible (Keen, 1990). When the potential pathogen successfully invades the plant host, it is due to unsuitable physical defence barriers, or the plant's active defence responses are ineffective, especially in terms of the magnitude of the induced response or the time needed for such a response to be activated. This will ultimately have plant disease as a result, and is referred to as a compatible interaction (Hammond-Kosack and Jones, 1996).

2.2.2 The gene-for-gene model

In order for a plant host to mount an active defence response against a pathogen, it must first be able to recognize this pathogen on molecular level. For this to occur the plant must possess a dominant form of a resistance (*R*) gene that is complementary to a corresponding dominant avirulence (*Avr*) gene in

the pathogen. If either host or pathogen lacks a functional copy disease occurs. Frequently plants may carry a number of different resistance genes directed at a single pathogen, and for a pathogen to escape recognition of so many *R* genes it must possess recessive alleles for all relevant avirulence (*Avr*) genes (Keen, 1990). This recognition system was first described by Harold Flor in the 1940's and is known as the gene-for-gene model (Flor, 1942). Successful recognition of the pathogen by the plant's *R* gene generally results in the hypersensitive response (HR), which is characterised by a controlled process of cell death around the area of pathogen infection (Pontier et al. 1998) and is visible as necrotic lesions on the plant surface. The main purpose of this response is to prevent further spread by the pathogen. The HR (and other important defence related responses) will be discussed in greater detail below.

2.2.3 Avirulence (*Avr*) genes

Although much time has been dedicated to understanding the function and biochemical nature of *avr* genes, little progress has been made in this regard (Bent, 1996). Plant viruses provide a potential exception, where genes encoding products such as structural coat proteins have been demonstrated as being avirulence determinants (Hammond-Kosack and Jones, 1997). Some *Avr* genes are thought to have important functional roles pertaining to pathogenesis, and loss of these genes might carry a significant fitness penalty for the pathogen, which explains why these genes provide a stable target for plant resistance genes (Bent, 1996). Exceptions do exist that illustrate that some pathogens can structurally modify or lose their avirulence genes with very little or no obvious loss of fitness. Clearly such cases have significant consequences in agriculture, as selection may favour pathogen populations that cannot be recognised by plant resistance genes (Bent, 1996).

2.2.4 Resistance (*R*) genes: Structural and functional properties

Ever since the gene-for–gene model was proposed, scientists have made many attempts to isolate *R* genes in order to identify its structural and functional features. Success in this regard was delayed, until modern molecular techniques were developed that allowed for the cloning of plant genes of which the sequence or structure was unknown. Since then a large number of *R* genes have been successfully cloned and analysed, with results showing that *R* genes from various different plant species with specificity to a range of pathogens often have similar structural properties (Bent, 1996). This clearly suggests a high degree of conservation between R proteins in terms of function (Bent, 1996). Currently, four main classes of highly polymorphic, but structurally conserved R proteins have been identified. In plants, the most important of these, are the Nucleotide Binding Site – Leucine Rich Repeat (NBS-LRR) proteins and in addition to this, the less important Leucine Rich Repeat - Receptor Like Kinases (LRR-RLKs) and the LRR- Receptor Like Proteins (LRR-RLPs) (Jones and Takemoto, 2004). The latter two classes of R proteins are involved in extra-cytosolic perception, consisting of an extracellular LRR domain, connected to a membrane-spanning domain. These proteins either carry an intracellular (C-terminal) kinase effector domain (RLK), of which the rice gene *Xa21* is a good example, or they lack such a domain, such as tomato's *Cf-9* gene (RLP) (Jones and Takemoto, 2004).

The *Xa-21* gene, encodes a 1025 amino-acid protein that possesses 23 extracellular LRR domains, and is known to confer resistance to over 30 distinct strains of *Xanthomonas oryzae.* Although this gene shows homology to a similar gene found in *Arabidopsis*, it is still difficult to determine its exact function. This protein is important however, in the sense that it illustrates a link between R proteins that perform a receptor function, and downstream signalling pathways (reviewed by Hammond-Kosack and Jones, 1997). The *Cf-9* gene confers resistance to the leaf mould pathogen, *Cladosporium fulvum.* This gene encodes an 863 amino-acid glycoprotein, containing 27 LRR domains. In contrast to *Xa-21,*

Cf-9 does not have a kinase domain in the cytoplasm, but instead carries a small KKxx motif. This suggests that *Cf-9* must interact with various signalling components, probably kinases, in order to activate the defence response, which might not be necessary in the case of *Xa-21 (*Hammond-Kosack and Jones, 1997; Bent 1996).

The putative NBS-LRR class represents the largest group of R proteins (Holt et al. 2003). This class may also have structural variants, depending on the amino-terminal domain. A number of NBS-LRR proteins may have an N-terminal Toll and Interleukin-1 receptor like domain (TIR-NBS-LRR), whereas others have a coiled-coil (CC-NBS-LRR) motif at their N-termini (Holt et al. 2003).

The Toll and Interleukin-1 receptor like domains (TIR) are named as such, because of their homology with the cytoplasmic domain of *Drospohila's* Toll protein, as well as the mammalian interleukin-1 receptor protein. Because these domains exhibit homology with cytoplasmic signalling domains, it can be speculated that they are likely to be involved in signalling during plant defence and not pathogen recognition (Hammond-Kosack and Jones, 1997). The CC motif found in other R proteins, is a basic leuzine zipper domain, responsible for facilitating protein-protein interactions by allowing the formation of so called coiled-coil structures (Hammond-Kosack and Jones, 1997). Both forms of the intracellular group of R proteins have a NBS domain. These domains are found in a number of proteins that have been illustrated to have ATP or GTP binding activity. This suggests a possible role for the NBS domain in the activation of kinases, though no experimental evidence exists to support this theory (Hammond-Kosack and Jones, 1997). Bent (1996) therefore proposed that it would be an important future challenge to experimentally illustrate nucleotide triphosphate binding in intracellular R proteins. The LRR domain is arguably the most important component of R proteins as it is present in all classes. The domain is composed out of a number of structural units, each one consisting of a β-strand and an

α-helix interconnected by loops (Kobe and Deisenhofer, 1993). The structural units are arranged in such a manner that it forms a non-globular horseshoe type structure, with the β-strands lining the inner circumference of the horseshoe and the α-helices forming the outer circumference (Kobe and Kajava, 2001). The LRRs generally have repeats of 20-29 amino acid residues containing a conserved 11 residue fragment with consensus sequence LxxLxLxxN/CxL (where x is any amino acid, and L can be valine, isoleucine or phenylalanine), that corresponds to the segment surrounding the β-strands (Kobe and Kajava, 2001). These domains have been implicated in mediating protein-protein recognition events, possibly ligand binding in particular (Kobe and Kajava, 2001) but Moffet et al. (2002) has also experimentally illustrated a role for LRR domains in intramolecular interactions.

As discussed above, the various structural components of R proteins perform a number of specific functions, but it is clear that these various domains must act together in order for the R protein to perform its intended function, namely resistance. The exact mode of action (on biochemical level) for many R proteins remains to be determined, but work done by Moffet and colleagues (2002) may provide some useful insight into the mode of action of these R proteins. In their study, they addressed the function of a CC-NBS-LRR protein, Rx, which confers resistance to potato virus X (PVX). This was achieved by co-expression of various paired combinations of the Rx protein's three domains (CC, NBS, LRR), with a coat protein (CP) from PVX. Their results yielded the proposal of two possible models for the activation of Rx mediated resistance. A common feature identified in both these models, is that the viral coat protein (CP) interacts transiently with the R protein, initiating a two step conformational change. In step one, NBS-LRR interactions are disrupted, changing the nucleotide binding status of the NBS domain. This change consequently results in a disruption of the CC-NBS interaction. In model 1, an effector molecule associated with the Rx protein is released in order to

facilitate these changes. In model 2, inactive effectors are recruited to Rx for activation (Moffet et al. 2002).

2.2.5 The guard hypothesis

In its simplest form, the recognition of a pathogenic avr determinant by a plant *R* gene, is based on a receptor-ligand model, where direct interaction takes place between the *R* gene and the avr determinant (figure 2.2b – McDowell and Woffenden, 2003).

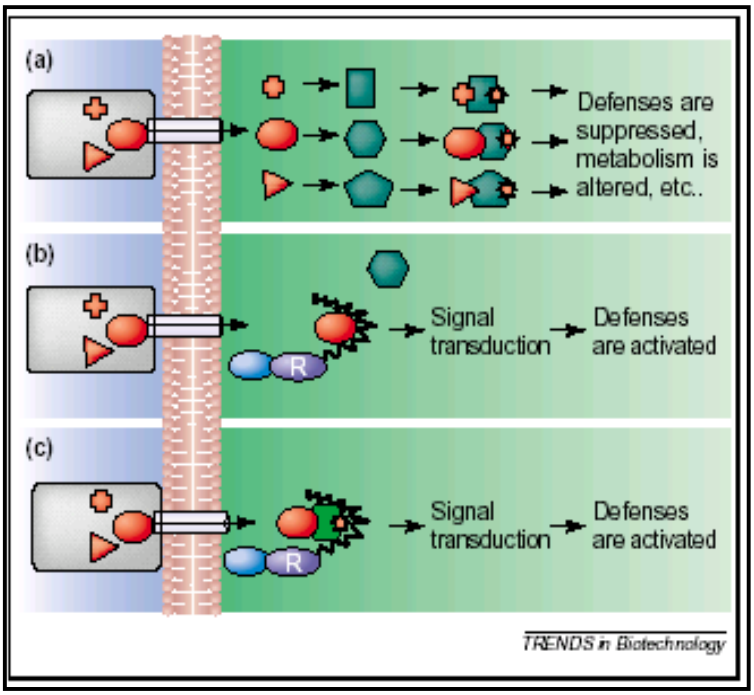

Figure 2.1:

Interactions between pathogen Avr determinants and plant R proteins. A pathogen (grey) attaches to the plant cell wall and expresses a number of virulence factors (red). (a) Because the plant does not posses the necessary *R* gene, it is incapable of recognizing the invading pathogen. The plant's defences, are therefore weakly induced or ineffective - resulting in disease. (b) The basic receptor-ligand model is illustrated. The R protein directly binds the pathogen's virulence factors, triggering an elaborate defence response. (c) The guard hypothesis. In this case the R protein (guard) detects a modified host protein (bait, green with red star), possibly as a complex with the virulence factors – thereby activating defence through indirect R-Avr interaction (McDowell and Woffenden, 2003).

Despite the extensive research on a large number of *R* and *avr* genes, direct interaction was only demonstrated in two cases. Jia et al. (2000) illustrated direct interaction between the rice CC-NBS-LRR gene (*Pi-ta*) and the Avr-Pita protein of the rice blast fungus *Magnaporthe grisea.* In the second case, Tang et al. (1996) demonstrated physical interaction between the Pto kinase protein in tomato, and the Avr-Pto protein in the *Pseudomonas* pathogen.

This lack of experimental evidence for the physical interaction between R and Avr gene products has lead to the formation of the guard hypothesis. This hypothesis postulates that the interaction between the R protein (guard) and its corresponding Avr determinant (in the pathogen) is mediated by a host protein (bait), which is targeted and modified by the Avr protein (Van der Biezen and Jones, 1998). This is illustrated in figure 2.2(c). A resistance response therefore results, when the R protein detects an attack on the bait protein. There is accumulating experimental evidence in support of the guard hypothesis.

One such case, describes the *Arabidopsis* protein RIN4 (RPM1 interacting protein 4). The RIN4 protein has been shown to mediate interaction (act as a bait protein) between RPM1 (a CC-NBS-LRR protein) and the AvrB and AvrRpm1 proteins from the leaf speck bacterium *Pseudomonas syringae* (Mackey et al. 2002). Some experimental results that appear to contradict the guard hypothesis, may in fact be interpreted in support of it. The RRS1-R R gene product in *Arabidopsis* has been shown to directly interact with PopP2, an Avr protein from the wilt bacterium *Ralstonia solanacearum* (Deslandes et al. 2003). The occurrence of a direct interaction between R protein and Avr determinant in this case, may be that the RRS1-R protein carries its own version of a bait protein, that facilitates the interaction with PopP2 (Jones and Takemoto, 2004).

2.3 Host defence responses

2.3.1 The hypersensitive response

The hallmark of gene based defence responses (mediated by gene-for-gene interaction) is the hypersensitive response (HR). The HR is generally visible as necrotic lesions on the plant's surface, as a result of programmed cell death induced around the area of pathogen invasion. One obvious function for the HR is to limit the spread and growth of the pathogen by breaking down the cell before it is colonised by the pathogen (Pontier et al. 1998). In addition to this localized HR response, plants also activate a systemic resistance mechanism, providing protection against secondary pathogen attack in uninfested parts of the plant. This is referred to as systemic acquired resistance (SAR), and the pathway is triggered by pathogenic invasion (compatible and incompatible) and generally appears after initiation of the HR. Initial reports hypothesized a connecting link between HR and SAR, but it has since then been determined that this connection is rather because of similarities in signalling mechanisms or certain steps in the signalling pathway (Pontier et al. 1998).

Cell death in particular is thought to be activated by two types of processes. Firstly, cell death arises due to a change in cellular metabolism, allowing for the accumulation of compounds toxic to both pathogen and plant cell (Pontier et al. 1998; Hammond-Kosack and Jones, 1996). Secondly, pathogen recognition causes genetic reprogramming of the cell, activating tightly controlled programmed cell death (Hammond-Kosack and Jones, 1996). A number of possible explanations have been given for this occurrence of cell death. In the case of biotrophic pathogens, cell death may be important to deprive the pathogen from obtaining further nutrients from the host (Hammond-Kosack and Jones, 1996). Necrotrophic and hemibiotrophic pathogens however, are still able to obtain nutrients from the plant even though the cells are dying, which gave rise to further proposed functions for cell death. The break up of cells during the cell death process, may in fact allow for the release of harmful substances

from the cell's vacuole (Osbourn, 1996). Hammond-Kosack and Jones (1996) suggested that cell death might also allow for the rapid accumulation of phytoalexins (antimicrobial peptides) to highly active concentrations, as they are usually rapidly metabolised in active plant cells. Further features of the HR response include the synthesis of pathogenesis related (PR) proteins, that have some damaging influence on the pathogen, and additionally the fortification of plant cell walls (Dixon et al. 1994). Defence responses related to signalling, such as various ion fluxes, the generation of reactive oxygen species (ROS) such as superoxide and changes in protein phosphorylation also occur (Dixon et al. 1994). Some of these components will be discussed in greater detail.

2.3.1.1 Reactive oxygen species

One of the earliest plant defence responses leading to the HR, is the rapid production of ROS, the so-called oxidative burst (Pontier et al. 1998). ROS plays a key role in plant defence – by causing cellular damage to both plant and pathogen, and may also be involved in initiating the HR (Hammond-Kosack and Jones, 1996). Plants rapidly produce large amounts of superoxide (O_2-), involving NAPDH oxidase. The superoxide generated is mostly dismutated (via superoxide dismutase - SOD) to form hydrogen peroxide (H_2O_2). Superoxide as well as hydrogen peroxide are however only moderately reactive, and are thus converted to more reactive derivatives (Hammond-Kosack and Jones, 1996). Protonation of superoxide leads to the formation of hydroperoxyl radicals (HO_2), which can effectively cross biological membranes. This could result in membrane damage and the formation of various signalling molecules during the defence response. In turn, hydrogen peroxide undergoes a reaction that yields the hydroxyl (OH^-) free radical. This particular derivative is extremely reactive, and together with other ROS products, is capable of considerable cellular damage (Hammond-Kosack and Jones, 1996).

In addition to its toxic effects, ROS also has a number of other functions related to plant defence. One important function is the role played by certain ROS in signalling. Hydrogen peroxide for example, leads to an increase in benzoic acid hydroxylase activity (Leon et al. 1995), which is required for the synthesis of an important signalling component, salicylic acid (SA). It is also plausible, that ROS may alter the redox balance in the cell. This can lead to induction of various enzymes and more importantly, may regulate the stability of defence related mRNA transcripts (Mehdy, 1994).

2.3.1.2 Ion fluxes

A rapid alteration of ion fluxes is another earlier change that occurs during the HR. This process is generally characterised by a rapid export of Cl^- and K^+ ions, as well as the uptake of Ca^{2+} and H^+ ions (Tavernier et al. 1995). These fluxes mainly serve a signalling purpose. Calcium in particular though, may have a critical signalling role in the latter stages of the HR, since calcium-dependent endonucleases that contribute to DNA breakdown are activated during this phase (Mittler and Lam, 1995).

2.3.1.3 Cell wall fortification

During the defence response, plant cell wall fortification may take place in order to prevent further penetration and spread by the pathogen. This strengthening of the cell wall is the result of the deposition of various molecules. These include rapid callose deposition by the plasma-membrane localized callose synthase enzyme, as well as the rapid cross linking of preformed hydroxyproline-rich glycoproteins (HRGP) and pathogenesis related proteins (Hammond-Kosack and Jones, 1996). In the case of certain biotrophs, such as *P. syringae,* fortifying the cell wall will prevent leakage of cytoplasmic contents and will thereby affect nutrient accessibility for the pathogen (Hammond-Kosack

and Jones, 1996). Necrotrophs, which destroy plant cells in order to extract maximum nutritional value, are dependent upon their toxins being able to penetrate plant cells. It is furthermore well established that microbial pathogens produce an array of different cell wall degrading enzymes, including cellulases, pectinases and xylanases - both examples further serving to illustrate the importance of cell wall modifications during the defence response (Hammond-Kosack and Jones, 1996).

2.3.1.4 Phytoalexins and lipoxygenases

Phytoalexins are antimicrobial compounds that rise in levels around the area of pathogen infection. The biosynthesis of these compounds occur via the activation of secondary pathways, which have diverted from primary metabolic precursors. It is obvious that the activation of such biosynthetic pathways requires the contribution of a number of key enzymes, indicating the need for tight regulation through various signalling events (Hammond-Kosack and Jones, 1996). It is thought that the role of phytoalexins in *R* gene based resistance is possibly to prevent or reduce the effect of secondary infection, or to retard the growth and progress of virulent pathogens. This was illustrated in one case when sulphur, present as cyclo-octasulphur (S_8), was shown to accumulate in xylem parenchyma cells and xylem vessels in resistant varieties of cocoa following infection with the fungus *Verticillium dahlia* (Hammond-Kosack and Jones, 1996). An increase in lipoxygenases (LOX) expression and activity is also frequently found to be associated with *R* gene mediated resistance. It is for example involved in the jasmonic acid (JA) biosynthetic pathway, which is an important signalling component of the defence response (Hammond-Kosack and Jones, 1996).

2.3.2 Systemic acquired resistance

Systemic acquired resistance (SAR), generally activated in association with the HR provides the plant with a higher level of protection against possible secondary infection in uninfested (healthy) plant tissue. SAR is activated following attack by a particular pathogen, but provides long term resistance (lasting for weeks to months following the initial attack) against a broader range of pathogens. Activation of SAR therefore makes the plant able to respond faster to another pathogenic invasion. A number of changes occur in these upper (unaffected) leaves after SAR activation. This includes the increased expression of various pathogenesis related (PR) proteins such as glucanases and chitinases (Metraux, 2001), and these areas of the plant are also able to more rapidly elicit an oxidative burst (such as those seen in the HR) at the new site of pathogen infection. Because of the systemic increase of various PR proteins, they can be assayed and used as molecular markers for the induction of SAR and the signalling pathways leading to it (Ryals et al. 1996).

Before SAR can be activated, some form of signal must be generated at the initial site of pathogen attack, which subsequently spreads systemically leading to the activation of the response (Metraux, 2001). The possibility that salicylic acid (SA) is the signalling molecule involved in this process has been extensively studied (Metraux, 2001; Ryals et al. 1996). Support for the correlation between SA and SAR has come from studies performed on transgenic plants. One such study used transgenic plants expressing the bacterial gene *nahG*, encoding salicylate hydroxylase. This enzyme catalyses the conversion of salicylic acid to catechol and these plants are consequently unable to accumulate SA. Plants carrying this gene were not only unable to accumulate SA, but they were also unable to activate the SAR response against viral, fungal and bacterial pathogens (Gafney et al. 1993). Further support for this, comes from work done on *Arabidopsis,* where it was demonstrated that the depletion of SA causes the breakdown of SAR. Using *nahG*-transformed *Arabidopsis*, it was found that severe disease

develops after inoculation with *Peronospora parasitica* or with *Pseudomonas syringae* carrying an avirulence determinant. This was in contrast to the response seen in wild-type plants, where pathogen colonization and disease did not occur (Delaney et al. 1994). Mauch-Mani and Slusarenko (1996), used an inhibitor of phenylalanine ammonia-lyase (PAL), to block phenylpropanoid metabolism and subsequent SA biosynthesis. After treatment of *Arabidopsis* plants with PAL, *P. parasitica* was able to cause disease in these plants. Such experiments strongly support the need of SA expression in disease resistance, but it leaves the question of whether or not SA itself is translocated to the distal parts of the plant, or if other signalling components are involved (Ryals et al. 1996).

Some reports publicised the notion of SA being transported to unaffected parts of the plant. Shulaev et al. (1995) demonstrated a significant increase of SA in uninfested tobacco leaves following infection with the tobacco mosaic virus, and furthermore showed that this was mainly due to phloem based transport of SA from the original area of infection. The rest of the SA increase was ascribed to transport of its precursor, benzoic acid. A number of other studies however, seem to contradict such findings, suggesting that SA is unlikely to be translocated (discussed in Ryals et al. 1996). These different results were consolidated into one hypothesis that suggests that SA is indeed essential for the induction of SAR, but that it probably forms part of a long distance signalling network involving a number of other molecules (Ryals et al. 1996; Metraux, 2001).

2.3.3 Pathogenesis related (PR) proteins

Much time has been dedicated to elucidating the contribution made by pathogenesis related (PR) proteins to the disease resistance response. These proteins are strongly expressed locally (at the site of pathogen infection) during the hypersensitive response, but also systemically during the systemic acquired resistance response (Fritig et al. 1998). Various reports have illustrated the requirement of

either Salicylic acid (SA) or Jasmonic acid/ethylene (JA/ET) signalling for the expression of specific PR proteins. This suggests that a specific combination of these signals are likely responsible for activating the most efficient response against a specific pathogen, and that PR proteins contribute differentially to resistance against different pathogens (van Loon and van Strien, 1999).

These PR proteins have been classified into different families, where members of each family have similar basic biological properties, but may differ with respect to some other properties such as substrate preferences or cellular localization (Fritig et al. 1998). These proteins are furthermore distinguished by class, based on their specific activities on a number of substrates, with class III for example (PR8 family) displaying lysozyme activity (van Loon and van Strien, 1999). A number of small antimicrobial peptides - thionins, defensins and lipid transfer proteins have also been identified that are induced following pathogen infection. These peptides generally target plasma membranes but probably function differently (Fritig et al. 1998). Because they share these characters with other well known PR protein families, Van Loon and Van Strien (1999) suggested the inclusion of them as three new PR protein families, two of which (the defensin and thionin) genes are JA dependent – see table 2.1. Since then, three more proteins have been classified as pathogenesis related, bringing the total to 17 families designated PR1 to PR17 (Christensen et al. 2002). In terms of the role played by PR proteins in defence, the vast majority of them are known to cause structural damage to the invading pathogen. β-glucanases (belonging to the PR2 family) and chitinases (PR3, PR4, PR8 and PR11) for example, have been implicated in causing damage to fungal and bacterial cell walls. Chitinases break down chitin, a polymer of N-acetylglucosamine, which is a structural component of most fungi (Fritig et al. 1998). Chitinases may also display lysosomal activity, capable of hydrolysing bacterial peptidoglycan, indicating numerous defence-related roles for such enzymes (Fritig et al. 1998).

Peroxidases, which belong to the PR9 family, likely has a more indirect role in defence, by catalysing oxidative crosslinking of various proteins in plant cell walls, leading to greater cell wall strength (Fritig et al. 1998). PR10 is a family of ribonuclease-like proteins, suggesting a possible role in RNA cleavage during viral infection, but this must still be experimentally demonstrated. PR1 is the only protein family to which no function has currently been assigned. PR1 proteins identified in tomato and tobacco show some antifungal activity, but the precise nature of their action remains to be elucidated (van Loon and van Strien, 1999). Interestingly, not all of the 17 suggested PR protein families have been identified in all of the various model plant systems. PR7, -10, -12, -13, -14 for example, is not known in tobacco. This may mean that only certain *PR* genes are present in different plants, or that only certain ones are expressed during pathogen attack (van Loon and van Strien, 1999). This shows that much work lies ahead, in terms of characterising these PR proteins, identifying new defence-related compounds as well as elucidating the signalling cascades by which they function (Fritig et al. 1998).

Table 2.1:

Families of pathogenesis-related proteins – showing type and enzymatic properties. The recently proposed families (15, 16 and 17) are not included (Van Loon and Van Strien, 1999).

Family	Type	Properties
PR-1	Tobacco PR-1a	Unknown
PR-2	Tobacco PR-2	β-glucanase
PR-3	Tobacco P, Q	Chitinase I, II, IV, V, VI, VII
PR-4	Tobacco 'R'	Chitinase I, II
PR-5	Tobacco S	Thaumatin-like
PR-6	Tomato Inhibitor I	Proteinase inhibitor
PR-7	Tomato P69	Endoproteinase
PR-8	Cucumber chitinase	Chitinase III
PR-9	Tobacco 'lignin-forming peroxidase'	Peroxidase
PR-10	Parsley 'PR1'	'Ribonuclease-like'
PR-11	Tobacco class V chitinase	Chitinase I
PR-12	Radish Rs-AFP3	Defensin
PR-13	Arabidopsis THI2.1	Thionin
PR-14	Barley LTP4	Lipid-transfer protein

2.4 Signalling mechanisms
2.4.1 Salicylic acid mediated signalling

Activation of the elaborate array of plant defence responses is dependent on a number of signalling molecules that are involved in two major defence signalling pathways. These are SA-dependent and JA/ET dependent (SA independent) pathways. These pathways, instead of functioning completely independently, form complex interactions with various regulatory consequences (Kunkel and Brooks, 2002). As discussed above, it is now widely accepted that SA accumulation is crucial for the activation of localized defence responses, as well as the induction of SAR. Genetic studies performed on mutant model plant systems, have greatly contributed to our understanding of plant defence signalling and of the role played by SA (Feys and Parker, 2000). Some of the well-studied, key signalling components will be discussed here.

Zhou and colleagues (1998) showed that a mutation in the *Arabidopsis* gene *PAD4*, shows a reduction in resistance capability to *P. syringae.* This was due to a reduction in the synthesis of SA, in turn required for production of the antimicrobial peptide camalexin, and for the expression of SA dependent PR proteins (Zhou et al. 1998; Ryals et al. 1996). Because of the reduction in SA synthesis of *PAD4* mutants, it leads to the conclusion that *PAD4* specifically acts upstream of SA in the defence signalling pathway (figure 2.3). Plants deficient in the *EDS1* gene (a signalling component), showed altered responses to pathogenic invasion, particularly against *P. parasitica* and *P. syringae.* This was because the mutated *EDS1* caused an early breakdown of *R* gene mediated signal transduction (Dong, 1998), see figure 2.3. Although *EDS1* is placed upstream of SA in the proposed signalling cascade, mutants of another gene, *SID2* (together with *SID1*) also show increased susceptibility to *P. parasitica* and *P. syringae,* similar to *PAD4* and *EDS1*. In contrast to *PAD4* for example, *SID2* does not affect camalexin synthesis, suggesting that it does not play a role in regulating

SA accumulation as *PAD4* does. *SID2* contributes to the signal transduction cascade by being involved in the biosynthesis of SA (Feys and Parker, 2000).

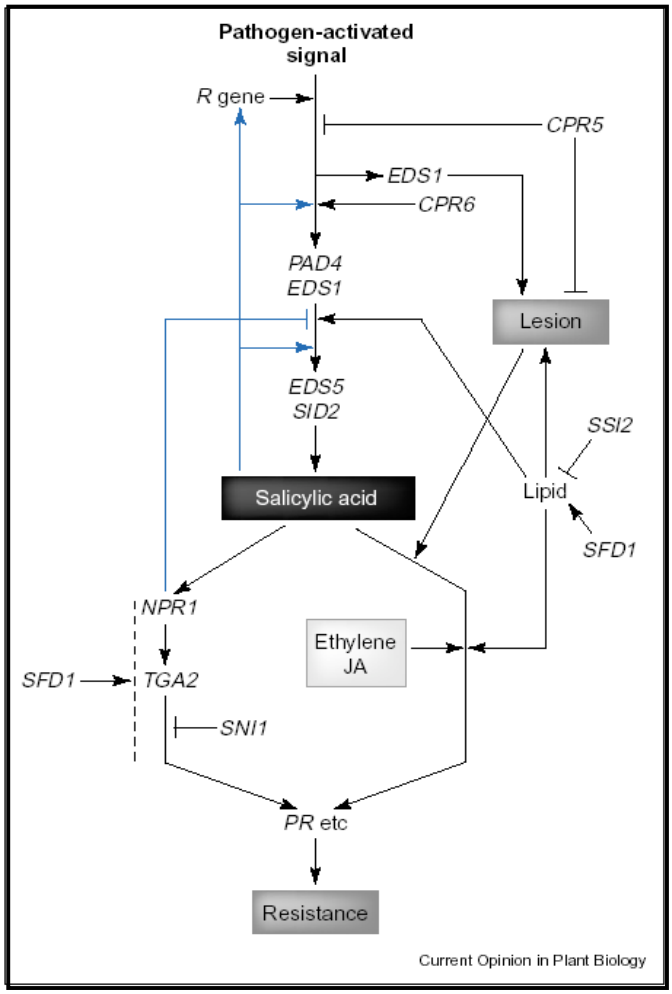

Figure 2.2:

Proposed model of salicylic acid signalling in plant defence. Pathogen recognition results in the activation of *R* gene mediated resistance responses and SA synthesis, resulting in a signalling cascade involving a number of genes culminating in resistance. An SFD-1 signal is thought to be involved, but its exact position in the pathway is unknown (broken line). The blue lines indicate feedback regulation. Lines ending in arrows show positive effects, whereas lines ending in bars show inhibitory effects. Refer to text for further details (Shah, 2003).

The *Arabidopsis* gene, *NPR1* (for non-expressor of PR1) has been identified as forming an important part of SA signalling (Shah, 2003). Analysis of this gene revealed that it carries a functionally significant repeat domain, thought to be involved in protein-protein interactions, as well as a nuclear localization signal (NLS) at its carboxyl end. These structural properties suggest that *NPR1* may receive pathogen-derived signals that lead to the interaction between NPR1 and other proteins in order to further transduce the required signal down the pathway, eventually culminating in the appropriate defence response (Feys and Parker, 2000). The NPR1 protein acts downstream of SA (Dong, 1998), as illustrated in figure 2.3. Experimentation on *NPR1* over-expressing plants has shown that the expression of *PR* genes in general is stronger in *NPR1* over-expressing mutants as compared to wild-type plants. These findings make *NPR1* a suitable candidate for genetic engineering of new resistant crops (Dong, 1998). SA mediated signalling can however, also take an *NPR1* independent route. Reuber et al. (1998) studied the activation of defence genes such as PR proteins (particularly family 1, PR1) in *Arabidopsis* mutants after infection by the fungal pathogen *Erysiphe orontii. NahG* transgenic plants deficient in SA, showed a considerable reduction in PR1 protein expression following fungal infection. However, in plants carrying a mutated version of *NPR1*, this reduction in *PR1* mRNA expression after infection was modest. These findings show that signalling follows this *NPR1* independent route, in order to produce defence proteins – figure 2.3 (Reuber et al. 1998).

2.4.2 Jasmonic acid/Ethylene mediated signalling

Another main signalling pathway that may also lead to the induction of a local- and sustained systemic resistance response, involves JA/ET instead of SA. Wounding primarily activates the JA/ET pathway, resulting in the synthesis of JA and subsequent activation of defence related genes, but this response can also be activated by microbial pathogens (Dong, 1998). The responses induced in this case, differ from SA mediated signalling in that different defence proteins are expressed. JA activates the

expression of PR proteins such as defensins and thionins, as well as proteinase inhibitors. In addition, ET is also rapidly induced in leaves following physical injury to the plant, or after the elevation of JA levels. Its importance in the JA pathway has been shown where various inhibitors of ET negatively affected JA mediated signalling (Dong, 1998).

The particular involvement of ET in this pathway has recently been proposed to contribute more to the control of disease symptoms, rather than being a deciding factor of susceptibility or resistance (Feys and Parker, 2000). For example, in ethylene-insensitive soybean mutants, reduced levels of ET might have detrimental or positive effects on the plant in terms of symptom development, depending on the type of pathogen (Hoffman et al. 1999). When the mutant plants were exposed to *Septoria glycines* the ET insensitive mutants seemed to be more susceptible. In contrast to this, after *P. syringae* infection, the disease symptoms were found to be less severe in mutants as compared to wild type plants (Hoffman et al. 1999).

A number of studies have previously illustrated the now well-established link between JA and ET. One such case studied the response by *Arabidopsis* to infection by the fungal pathogen *Alternaria brassicicola.* Analysis of different genes with anti-fungal activity (defensins) revealed that their induction (expression pattern) differs distinctly from *PR1*, for example. Because *PR1* is dependent on SA, the conclusion that these defensin genes function through a different signalling network (being JA) can be supported (Penninckx et al. 1996). The expression of these genes was increased by the exogenous application of JA or ET, but not by application of SA. In addition, expression of defensin gene *PDF1.2* was not altered in transgenic plants unable to accumulate SA (Dong, 1998). The link between JA and ET can thus be established in the fact that the pathogen induced expression of *PDF1.2* required the presence of JA as well as ET driven pathways (Penninckx et al. 1996). This was demonstrated in *Arabidopsis* mutants where both JA-insensitive plants (*coi1*) and ET-insensitive

mutants (*ein2*) failed to induce *PDF1.2* expression after *A. brassicicola* infection (Dong, 1998; Penninckx et al. 1996).

2.4.3 Interactions between different signalling pathways

Evidence is continually mounting to suggest that SA and JA/ET mediated signalling pathways are involved in complex interactions, rather than functioning completely independently (Kunkel and Brooks, 2002). These interactions allow the plant to modify or adapt its responses to function optimally against any particular pathogen, by appropriately activating different signalling events, and ensuring that inappropriate or unnecessary responses are not activated or suppressed (Feys and Parker, 2000; Kunkel and Brooks, 2000). Interactions between SA and JA controlled pathways mainly appear to be mutually antagonistic (Kunkel and Brooks, 2002). Doares et al. (1995) studied the expression levels of JA dependent proteinase inhibitor (*PI*) genes, after exposure to SA in tomato, *Lycopersicon esculentum.* Northern blot analysis revealed a significant decrease in *PI* gene expression induced by exposure to methyl-JA after treatment with SA. It was found that SA reduced induction of *PI* gene expression to less than 50% of its levels before treatment, suggesting the inhibition of JA by SA (Doares et al. 1995). A different study showed that this inhibitory effect on *PI* genes could be overcome in part, by the treatment of plants with JA and ET. This response was not generated by the treatment of either JA or ET alone (Odonnel et al. 1996), further strengthening the proposal that JA and ET act together in defence signalling.

Experimental support for the antagonistic effect of JA on SA is also accumulating (Kunkel and Brooks, 2000). *Arabidopsis coi1* mutants deficient in JA signalling, confer high levels of resistance to various isolates of *P. syringae,* showing no symptoms of disease. This enhanced level of resistance was ascribed to the hyperactivation of *PR1* gene expression and increased levels of SA, a correlation that is

to be expected, as *PR1* is a family of defence genes up-regulated after SA induction. This enhanced SA mediated response can be ascribed to the suppression of JA signalling in these mutants, illustrating the negative influence of JA on SA driven defences (Kloek et al. 2001).

Limited evidence supports the interaction (negative or positive) between ET and SA signalling (Kunkel and Brooks, 2000). Tomato plants infected with *Xanthomonas campestris* require the expression of SA as well as ET, as SA was found to increase in expression level associated with defence responses in wild type plants, but failed to accumulate in ET-insensitive plants (Odonnell et al. 2001). Our understanding of the truly complex nature of signalling mechanisms involving JA, ET and SA is still very limited. In future, the application of modern molecular biology techniques will be required to identify additional signalling components, and to gain a greater understanding of the molecular interactions taking place between them (Kunkel and Brooks, 2000).

2.5 Russian wheat aphid

2.5.1 Russian wheat aphid feeding induced symptoms

A greater comprehension of the nature of plant-pathogen interactions (as discussed above) forms the basis for addressing the Russian wheat aphid problem, because of overlapping mechanisms. The Russian wheat aphid is a small, greenish insect that feeds on plant phloem tissue (figure 2.1). The basic symptoms displayed by susceptible cultivars are leaf rolling, chlorotic streaking on the leaves, head trapping and even death of the plant in cases of severe infestation (Miller et al. 2001).

Figure 2.3:

Russian wheat aphid during feeding on a wheat leaf (Photograph by Leon van Eck and Paulette Bloomer).

Work carried out by Burd and Burton (1992) showed the leaf rolling symptom was generally caused by the failure of newly formed leaves to unroll, and that rolling of already expanded leaves was not observed. They concluded that prevention of leaf unfolding and the observed reduction in leaf size after aphid feeding, was due to decreased leaf turgor below the levels required for elongation of the cell and for cell wall extensibility. The white chlorotic streaking occurs as a consequence of photo-oxidation (Burd and Burton, 1992). The occurrence of these symptoms is mainly because of the phytotoxin(s) injected into the plant by the aphid during feeding (Smith et al. 1991).

2.5.2 Aphid feeding and survival

Aphids feed on sap extracted from phloem sieve elements. The aphid probes the wheat plant substrate with a hollow feeding stylet, leading to the subsequent formation of a stylet sheath (Miles, 1999). This sheath is of salivary origin. It quickly turns into a gel-like structure around the feeding stylet after exposure to air or the aqueous cellular environment and forms a stylet feeding-track aiding in the feeding process. This track may initially not go deeper than the epidermis of the plant during an initial probe, but generally continues deeper until stopping before reaching the vascular tissue, ending in the intercellular spaces (Miles 1999). Various factors significantly influence the life cycle of the RWA. This includes a combination of the effects generated by temperature, moisture, and plant quality (Girma et al. 1990). At lower temperatures (of around 10° to 13°C) aphids seem to be capable of reproducing for an extended period of time and their expected life span also increases. Lower temperatures however, cause nymphal development (in days) of the aphid to be drastically delayed. As environmental temperatures increase (18° to 29°C), the time required for aphids to reach maturity decreases, but so does the reproductive period and life span (Girma et al. 1990).

The RWA is not a serious pest of wheat in certain countries, including Hungary. In Hungary wheat is sown under conditions of high plant density (200 – 220 kg/ha), whereas in the United States (where RWA is a serious pest) the sowing rate is generally not higher than 120 kg/ha. This difference could be explained by the high relative humidity occurring in fields where plant density is higher, which is not favourable conditions for the RWA (Basky, 2003). Basky (2003) therefore recommended that increasing the sowing rate and subsequent plant density could provide a potential means of cultural control of the RWA and thus merits further investigation.

2.5.3 Host resistance phenotypes

Although little is known about the mechanisms by which resistant wheat cultivars prevent aphid infestation, their mode of action has been characterized on a phenotypic level. Host resistance to *D. noxia* has therefore been classified into three categories: antibiosis, antixenosis and tolerance. Antibiosis may be described as the negative effect of the host plant on the biology of the pest (Basky, 2003). It is measured by parameters such as aphid fecundity and mean adult longevity (Smith et al. 1992). Fecundity of aphids is thus expected to be lower in resistant cultivars. Antixenosis indicates the non-preference of the aphid for the host, i.e. it starts to feed then subsequently leaves that plant in search of a more suitable host. This is measured by aphid numbers, plant height and weight, and the number of leaves present after infestation (Smith et al. 1992). Tolerance in turn, suggests that the plant is capable of withstanding aphid attack without sustaining substantial damage. Tolerance is measured by height and weight of infested plants, as compared to uninfested plants. Susceptible plants are therefore expected to suffer more significant losses in plant weight and height. Because the plant host has been shown to rapidly recover these parameters after the aphids have been removed, Burd and Burton (1992) suggested that the duration of infestation, rather than the level of infestation is likely to be of greater significance to host survival.

The mode of action for a number of resistance genes (*Dn* genes) has been determined previously. *Dn1* for example, seems to confer antibiosis, whereas *Dn2* confers tolerance (Smith et al. 1992). *Dn4* is also known to confer tolerance, and the mode of action of *Dn7* is antixenosis (Anderson et al. 2003). *Dn7* has been shown to provide plants with a superior level of resistance as compared to other resistance related genes (Anderson et al. 2003).

In terms of the breakdown of RWA resistance, the ability of certain RWA populations to feed on a host plant previously resistant to such infestation defines a new biotype (Botha et al. 2006; Puterka and Burton, 1990). A number of RWA biotypes virulent to a number of cultivars (carrying either *Dn1, Dn2, Dn4, Dn5* or *Dn6* resistance genes) have appeared in the USA alone (Haley et al. 2004). At the moment seven novel biotypes have been found in the USA, on various cereal crops and wild grasses (Li et al. Unpublished). The emergence of these new biotypes exemplifies the need to continually examine aphid populations for the emergence of new biotypes (Puterka et al. 1992) and clearly shows the need for continual research and improvement of existing resistant cultivars.

2.5.4 Wheat - Russian wheat aphid interactions

Despite the diversity and abundance of phloem feeding insects and considering that the RWA in particular has been a serious pest worldwide for more than two decades, there is much to learn about the molecular nature of the interactions between plants and aphids in general (Moran and Thompson, 2001). The problem becomes particularly evident when we consider that greater knowledge, specifically pertaining to resistant plant – aphid interactions, may make an invaluable contribution to the aphid infestation problem. The molecular response in the host plant (induced by the aphid) has been shown to have striking similarities to the responses generated following pathogen attack (Moran and Thompson, 2001) and includes the hypersensitive response as well as the long term systemic acquired response (Botha et al. 2006).

As with plant-pathogen interactions, the RWA has been shown to induce the expression of a number of defence related compounds in resistant wheat cultivars, including important pathogenesis-related compounds such as β-1,3 glucanases, chitinases and peroxidases (Van der Westhuizen et al. 1998a,b). This is accompanied by the expression of reactive oxygen species (ROS) such as hydrogen peroxide,

which has been implicated in the accumulation of an important signalling molecule – salicylic acid (Chamnongpol et al. 1998). In addition to its signalling role, ROS may contribute more directly to defence of the resistant plant, by directly or indirectly causing oxidative stress to the invading aphid (Botha et al. 2006). Increased oxidative activity furthermore contributes to cell wall thickening, potentially inhibiting aphid probing (Van der Westhuizen et al. 1998a). These defence responses seem to be the result of the two main signalling pathways, mediated by salicylic acid and jasmonic acid, respectively. These pathways in turn activate the expression of pathogen-associated defence genes as well as genes that respond to physical plant damage (mediated by jasmonic acid in particular) (Montesano et al. 2003). The potential involvement of salicylic in RWA defence has been illustrated by the differential expression of this hormone in resistant wheat challenged with RWA (Mohase and van der Westhuizen, 2002). The similar responses seen in phloem feeding aphids as compared to pathogens (during the hypersensitive and systemic responses), may in part be explained by the similar effect of the aphid's feeding stylet and fungal hyphae on the host during infestation (Fidantsef et al. 1999).

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Chapter 3

Applying SSH to identify differentially regulated transcripts following RWA infestation

Abstract

Infestation of wheat and other cereal crops by the Russian wheat aphid (RWA, *Diuraphis noxia*) leads to severe annual losses in crop yield, and moreover to substantial financial losses. In addition to this, the ever increasing demand for wheat products as food supply requires the continual development of strategies in order to control this pest. As a result, the study of the molecular interactions between the RWA and its cereal host, plays a fundamental role in the advancement of management approaches. Therefore, in order to elucidate the molecular responses activated by the resistant wheat line 94M370 (carrying the *Dn7* resistance) in response to infestation with different aphid biotypes, we aimed to isolate and identify transcripts related to defence during these specific interactions, by means of suppression subtractive hybridization (SSH) and subsequent sequencing. This method however was unable to produce the expected results, which inevitably lead to an investigation as to the cause of this. An investigation into each of the experimental steps during SSH was carried out in order to identify the problem. Although it was concluded from this analysis that the adaptor ligation steps were most likely to be responsible, it was consequently decided to implement different molecular approaches in order to achieve the original experimental goals.

3.1 Introduction

In all higher organisms the regulation of various metabolic processes, such as plant organ development and plant defence against various stresses, can be ascribed to complex changes in gene expression patterns. It is therefore clear, that in order to understand these complex biological processes, analyzing patterns of differential gene expression is invaluable (Diatchenko et al., 1996). Many experimental strategies have been developed to achieve this goal, which have greatly increased after the development of PCR technology. One such PCR-based technique was originally described by Diatchenko et al. (1996), and is termed Suppression Subtractive Hybridization (SSH). This technique allows for the PCR based amplification of transcripts, differentially expressed in one mRNA population, as compared to another.

During SSH, complementary DNA (cDNA) is generated from both populations to be compared. During the subtraction procedure, enzymatically digested tester cDNA (sample of interest) and driver (untreated control population) samples are allowed to hybridize. These resulting hybrid sequences (representing mRNA that is expressed in both the tester and driver populations) are then subsequently removed (Diatchenko et al., 1996). The remaining unhybridized cDNA represents genes that are differentially expressed in the population of interest (the tester), and these fragments can subsequently be amplified by PCR and identified by sequencing. Subtractive hybridization has successfully been applied in a number of different studies. Dos Santos and colleagues (2003) applied SSH in order to characterise *Arabidopsis thaliana* defence related genes following infection by the root parasite, *Orobanche ramosa.* Park et al. (2004) generated expressed sequence tags (ESTs) involved in the secondary metabolism in tea (*Camellia sinensis*), using SSH. This technique has successfully been used in the study of transcripts activated following RWA infestation in the past (Lacock and Botha, 2003; van Niekerk and Botha, 2003).

The purpose of this particular study, was to identify transcripts that are differentially expressed in a resistant wheat line, 94M370 (Gamtoos R, *Dn7*$^{+}$) as compared to its susceptible variety Gamtoos (*Dn7*$^{-}$), following infestation with different biotypes of the Russian Wheat Aphid (RWA, *Diuraphis noxia*). 94M370 carries one of the eleven known RWA resistance genes (termed *Dn* genes), which was transferred from chromosome 1RS of rye into this wheat line (Marais et al., 1994). By identifying genes that are differentially expressed in Gamtoos R (*Dn7*$^{+}$) as a result of RWA infestation, we hope to elucidate the underlying molecular mechanisms that mediate resistance in this plant.

3.2 Materials and methods

3.2.1 Plant materials and insect infestations

Two varieties of wheat were used during experimentation, with the resistant variety 94M370 (Gamtoos R) carrying the *Dn7* resistance gene (Marais et al. 1994), whereas its susceptible counterpart, Gamtoos, does not. Plants were grown under standard greenhouse conditions, in a 1:1 acid bark compost: red soil mix, at 20°C until it reached the 3 to 4 leaf growth stage. Plants were watered 2 to 3 times per week. Upon reaching the desired growth stage, both 94M370 (Gamtoos *Dn7*) and Gamtoos were infested separately with 10 Russian Wheat Aphids (RWA, *Diuraphis noxia*) of the South African biotype, for a period of 48 hours (Botha et al. 1998). Following infestation, aphids were carefully removed by light brushing as to prevent contamination of aphid nucleic acids during plant RNA extractions. Second and third leaves were immediately harvested (Botha et al. 2005) for total RNA extractions.

3.2.2 Treatment of glassware, plastic ware and solutions

All glassware to be used during RNA extractions was treated overnight with 0.1% (v/v) Diethyl pyrocarbonate (DEPC), autoclaved for 20 minutes at 120°C and subsequently baked for 2 hours at 200°C (Sambrook et al., 1989). Plastic ware, as well as solutions not containing Tris (2-Amino-2-

(hydroxymethyl)-1,3-propandiol), were treated with 0.1% (v/v) DEPC overnight and autoclaved at 120°C for 20 minutes.

3.2.3 Total RNA extractions

Total RNA was extracted using a modified Guanidium thiocyanate (GITC) extraction protocol originally described by Chomczynski and Sacchi (1987). All centrifugation steps were carried out at 4°C. Leaves harvested from infested 94M370 and Gamtoos plants were immediately frozen in liquid nitrogen and ground to a fine powder. To this, 1ml of RNA extraction buffer was added (4M GITC; 100mM Tris, pH 8; 25mM Sodium Citrate, pH 8 and 0.5% (w/v) N-lauryl sarcosine). 2-Mercapto-ethanol was added to each sample (to 1.4% (v/v) of total extraction volume), which was subsequently incubated at room temperature for 10 minutes. After this incubation step samples were centrifuged at 10 000rpm for 10 minutes. The resulting supernatant was carefully transferred to new sterile tubes, after which 50µl of 2M Sodium Acetate (pH 4) was added. This was followed by the addition of 500µl phenol:chloroform (1:1) and another incubation for 10 minutes at room temperature.

Samples were subsequently centrifuged at 10 000rpm for 10 minutes, the supernatant was recovered and 1 volume of isopropanol was added to the recovered supernatant. Precipitation of RNA was carried out by incubating the samples at -20°C for at least 1 hour. The resulting precipitate was recovered by centrifugation at 13 000rpm for 30 minutes. The supernatant was discarded and the pellet washed with 1ml of 75% (v/v) ethanol and centrifuged for 15 minutes at 10 000rpm. Following this the ethanol was again removed, and the RNA pellet was allowed to air dry before being resuspended in 50µl DEPC treated distilled water.

RNA samples were purified from contaminants (including DNA) using the RNeasy Mini kit according to instructions provided by the supplier (Qiagen, USA). RNA samples were analyzed for quality and quantity by running a small aliquot of purified samples on a 1% (w/v) agarose gel stained with ethidium bromide (0.005 mg/ml) and visualized under UV light. RNA concentration was subsequently determined by reading absorbances at 260nm (with the Nanodrop ND-1000). All samples were stored at -80°C until further use.

3.2.4 cDNA synthesis

Full length, double stranded cDNA (from 94M370 and Gamtoos) was synthesized from approximately 15µg of total cellular RNA using the cDNA Synthesis System (Roche Applied Science, Germany) according to manufacturer's instructions. Resulting cDNA was purified using the MinElute Reaction Clean-up Kit (Qiagen, USA) also according to manufacturer's specifications. Samples were eluted twice in a volume of 30µl. cDNA concentrations were determined by the use of a spectrophotometer (Nanodrop ND-1000), and samples were subsequently stored at -20°C.

3.2.5 Suppression Subtractive Hybridization (SSH)

SSH was carried out using reagents supplied in the PCR-Select^{TM} cDNA Subtraction Kit (Clontech, Mountain View, CA), together with a modified protocol.

3.2.5.1 *Rsa*I digestion

The cDNA synthesized from 94M370 (tester) and Gamtoos (driver) were digested for 3 hours at 37°C with 15 units *Rsa*I restriction enzyme (Roche) and 1x restriction enzyme buffer [100mM Bis-Tris Propane-HCl (pH 7.0), 100mM $MgCl_2$, 1mM DTT] in a 15µl reaction volume. Both tester and driver

samples were purified after digestion using the MinElute reaction clean-up kit according to specifications supplied by the manufacturer (Qiagen, USA). Samples were eluted in 30µl sterile water (SABAX, Port Elizabeth, South Africa).

3.2.5.2 Ligation of adaptors

Adaptors were ligated to tester samples only. Tester samples were split into two separate aliquots and diluted 1:4 with sterile water. Each of the two aliquots were ligated to different adaptors. In one reaction 2µl of the diluted cDNA was ligated to 20pmol adaptor I (5' - CTA ATA CGA CTC ACT ATA GGG CTC GAG CGG CCG CCC GGG CAG GT - 3' ; 3' – GGC CCC GTC CA – 5') (cDNA subtraction kit user manual, Clontech) in a 10µl reaction containing 1x DNA ligation buffer (250mM Tris-HCl (pH 7.8), 50mM $MgCl_2$, 10mM DTT, 0.25mg/ml BSA) and 400 units T4 DNA ligase (Clontech). This sample was labelled sample 1-1. In a similar reaction 2µl of the diluted cDNA was ligated to 20pmol adaptor IIR (5' - CTA ATA CGA CTC ACT ATA GGG CAG CGT GGT CGC GGC CGA GGT - 3' ; 3' – GCC GGC TCC A – 5') (cDNA subtraction kit user manual, Clontech) and labelled sample 1-2. Using these samples, 2µl of sample 1-1 and 2µl from sample 1-2 were mixed, and labelled as sample 1-3, which served as a control during further experimentation. All ligation reactions were carried out at 14°C overnight, after which samples were heated to 65°C for 15 minutes in order to inactivate the DNA ligase.

In a separate experiment, ligation reactions were set up as above, with incubation temperature reduced to 4°C overnight, and T4 DNA ligase concentrations varied between 0.5 and 20 units. These experiments were carried out with the addition of 5% (w/v) Polyethylene Glycol (PEG 4000) to each reaction.

3.2.5.3 Test of adaptor ligations

Test PCR reactions were performed in order to confirm that adaptor ligation indeed take place. Sample 1-3 (containing a mix of adaptor 1 and adaptor IIR) was diluted 1:4 in sterile water, following ligation. From this, 1.5µl was used in a PCR reaction containing 1x PCR reaction buffer, 2.5mM $MgCl_2$, 10pmol PCR primer 1 (5' – CTA ATA CGA CTC ACT ATA GGG C – 3')(cDNA subtraction kit user manual, Clontech), 1 unit Taq DNA polymerase and 10mM dNTPs in a 25µl reaction. Cycling conditions commenced at 75°C for 5 minutes to extend the short strands of the adaptors. This was followed by one cycle at 94°C for 1 min, and 27 cycles at 94°C for 30 seconds; 66°C for 30 seconds and 72°C for 90 seconds. The PCR was terminated with a final extension step at 72°C for 5 minutes. PCR products were analyzed on 2% (w/v) agarose gels stained with ethidium bromide (0.005mg/ml) and visualized under UV light.

In a number of separate experiments, PCR reactions were carried out as above, except that primer annealing temperatures were varied between 48°C and 66°C in order to determine optimal reaction conditions.

3.2.5.4 Primary hybridization

Prior to starting with the primary hybridization reaction (between adaptor ligated tester and non-ligated driver), tester samples 1-1 and 1-2 were further diluted to 1:5, 1:20 and 1:200 with sterile, distilled water. From each of these samples 1.5µl was removed and mixed with 1.5µl *Rsa*I digested driver, 1µl 1x hybridization buffer (Contech) and overlaid with approximately 10µl of sterile mineral oil. Samples were subsequently denatured at 98°C for 1 minute in a thermal cycler. Hybridization was carried out at 68°C for no less than 6 hours, but did not exceed 12 hours.

3.2.5.5 Secondary hybridization

Before commencing with secondary hybridization, a fresh sample of *Rsa*I digested driver (without adaptors) was diluted 1:2 with sterile distilled water, 1µl of 4x hybridization buffer (Clontech) was added, and the reaction was overlaid with a drop of mineral oil and denatured at 98°C for 90 seconds. This freshly denatured driver, as well as the primary hybridization mix (sample 1-2) was immediately added to the primary hybridization mix 1-1 at 68°C (from 2.5.4). This step was repeated for each of three primary hybridization dilutions, 1:5, 1:20 and 1:200 respectively. Samples were then incubated at 68°C overnight. Following this incubation step, 100µl dilution buffer [20mM HEPES (pH 6.6), 20mM NaCl, 0.2mM EDTA (pH 8.0)] (Clontech) was added to each sample and again incubated at 68°C for 7 minutes. Reactions were stored at -20°C until commencing with PCR amplification of subtracted samples.

3.2.5.6 PCR amplification of subtracted samples

PCR amplification was carried out with secondary hybridization mix as template in order to amplify differentially expressed transcripts. The PCR was carried out with 3µl secondary hybridization mix, 1x PCR amplification buffer, 10pmol PCR primer 1 (5' – CTA ATA CGA CTC ACT ATA GGG C – 3') (Clontech), 2.5mM $MgCl_2$, 10mM dNTP mix and 1 unit of Taq DNA polymerase in a 15µl reaction. The PCR reaction started at 75°C for 5 minutes followed by 94°C for 1 minute. After this, 30 cycles at 94°C for 30 seconds; 66°C for 30 seconds and 72°C for 90 seconds was carried out. The reaction was ended by a final extension step at 72°C for 5 minutes. Resulting PCR products were analyzed on a 2% (w/v) agarose gel stained with ethidium bromide (0.005mg/ml) under UV light.

3.2.6 Treatment of adaptors and cDNA/adaptor ligation test PCR

A small aliquot of the SSH adaptors was treated with 5 units of Antarctic phosphatase (New England Biolabs) in a 10µl reaction, containing 1x reaction buffer (New England Biolabs) and incubated at 37°C for 15 minutes. The reaction was terminated by heating at 65°C for 15 minutes. These samples were used in a repeat of the SSH experiment in order to optimise sample/adaptor ligation.

Further analysis of adaptor ligation was carried out by performing a test PCR using SSH PCR primer 1 (5' – CTA ATA CGA CTC ACT ATA GGG C – 3') (Clontech), as well as a set of poly-ubiquitin primers (PolyU Forward 5' – TCA GAC GAC TCC ACC TCA AG – 3'; Poly U Reverse 5' – GAG ATG GCA AAT CCG TAG GG – 3'). These primer combinations amplify products spanning the adaptor/cDNA junction. Adaptor ligated cDNA samples (samples 1-1 and 1-2) were used as template, with either the poly-ubiquitin primers, or SSH PCR primer I and poly-ubiquitin reverse primer. The PCR reaction contained 1.5µl of either sample (1-1 and 1-2), 1x PCR buffer, 10µM of each primer, 2.5mM $MgCl_2$, 10mM dNTP mix and 1 unit Taq DNA polymerase in a 20µl reaction volume. Samples were placed in a thermal cycler at 94°C for 1 min, followed by 27 cycles at 94°C for 30 seconds; 55°C for 30 seconds and 72°C for 90 seconds. The PCR was terminated with a final extension step at 72°C for 5 minutes. Products were analyzed on a 1% (w/v) agarose gel stained with ethidium bromide (0.005mg/ml) visualized under UV light.

3.2.7 cDNA-AFLP analysis

A cDNA-AFLP experiment was carried out in order to test the integrity of the cDNA samples. The cDNA-AFLP analysis was carried out as originally described by Bachem et al. (1996), using the AFLP Expression Analysis Kit (Li-Cor, Lincoln, NE) with some adjustments.

3.2.7.1 Restriction enzyme digestion

Approximately 50ng of cDNA template was digested with 1.25 units of *Taq*I restriction enzyme in 1x RL buffer (50mM Tris-HCl (pH 7.5), 50mM Mg-acetate, 250mM K-acetate) in a final reaction volume of 20µl. The reaction was incubated in a thermal cycler at a constant temperature of 65°C for 2 hours. Following this, 1.25 units of *Mse*I restriction enzyme was added to the *Taq*I enzyme mix, together with fresh 1x RL buffer in a reaction volume of 25µl. This was followed by an incubation step at 37°C for 2 hours. Prior to proceeding to the adaptor ligation step, all samples were incubated at 80°C for 20 minutes in order to inactivate both restriction enzymes.

3.2.7.2 Adaptor ligations

The *Taq*I/*Mse*I restriction mix was incubated with 4.5µl adapter ligation mix (AFLP Expression Analysis kit), 7.5 units T4 DNA ligase in a 30µl reaction volume at 20°C for 2 hours. This ligation mixture was diluted 1:10 in sterile water (SABAX, Port Elizabeth, South Africa) and stored at -20°C until further use.

3.2.7.3 Pre-amplification

During the first round of PCR amplification, 2.5µl of the adapter ligated cDNA template (from the 1:10 dilution) was mixed with 10µl pre-amplification primer mix, 1x amplification buffer (200mM

Tris-HCl (pH 8.4), 15mM $MgCl_2$, 500mM KCl) and 2.5 units of *Taq* DNA polymerase in a 15µl reaction. Cycling conditions consisted of 20 cycles at 94°C for 30 seconds, 56°C for 1 minute and 72°C for 1 minute, terminated by one cycle at 72°C for 5 minutes.

Prior to proceeding with the selective amplification step, the successful completion of pre-amplification was determined by gel electrophoresis on a 1% (w/v) agarose gel (stained with ethidium bromide at 0.005mg/ml) and visualized under UV light. Samples were subsequently diluted 1:300 in sterile water and again stored at -20°C until continuation of the experiment.

3.2.7.4 Selective amplification and Gel electrophoresis

Selective PCR amplifications were carried out with 5 *TaqI/Mse*I (+2/+2) primer combinations (MCT/TCA; MGT/TGA; MGA/TAC; MGA/TCA; MTG/TAC). Each PCR reaction contained 2µl of diluted pre-amplification DNA template, 2µl of the appropriate *Mse*I (+2) primer containing dNTPs, 0.5µl of the fluorescently labelled *Taq*I (+2) primer (IRDye 700, Li-Cor), 1x PCR amplification buffer (200mM Tris-HCl (pH 8.4), 15mM $MgCl_2$, 500mM KCl) and approximately 4 units of *Taq* DNA polymerase in a final reaction volume of 10µl. The PCR cycling conditions consisted of one cycle at 94°C for 30 seconds, 65°C for 30 seconds, and 72°C for 1 minute. This was followed by 12 cycles at 94°C for 30 seconds, with the annealing temperature (65°C) being lowered by 0.7°C per cycle, and 72°C for 1 minute. The PCR continued with 23 cycles at 94°C for 30 seconds, 56°C for 30 seconds, and 72°C for 1 minute.

Samples were then mixed with 5µl loading dye and resolved on 8% denaturing polyacrylamide gels [8% Long Ranger™ gel solution (BMA, Rockland, ME, USA), 7.0M urea and 0.8x TBE consisting of

71.2mM Tris, 71.2mM boric acid and 1.6mM EDTA] (Myburg et al. 2001) in the Li-Cor DNA analyzer 4200S (at 1500V and 45°C).

3.3 Results

3.3.1 RNA extractions and double stranded cDNA synthesis

Total RNA was extracted from both 94M370 (Gamtoos *Dn7*) and Gamtoos after a 48 hour RWA infestation period. Results from RNA extractions are presented in figure 3.1. The smearing pattern observed in each of the 8 lanes represent mRNA, which serves as template for cDNA synthesis. The distinct bands visible above the 5148 base pair size marker, represent genomic DNA that was also precipitated during the extraction procedure, whereas rRNA bands are visible at around 950bp. Figure 3.1 (b) illustrates the RNA samples after purification. The smearing pattern still visible suggests the presence of intact mRNA. As compared to figure 3.1 (a) (RNA prior to purification) it can be seen that purification has successfully removed most of the contaminating genomic DNA.

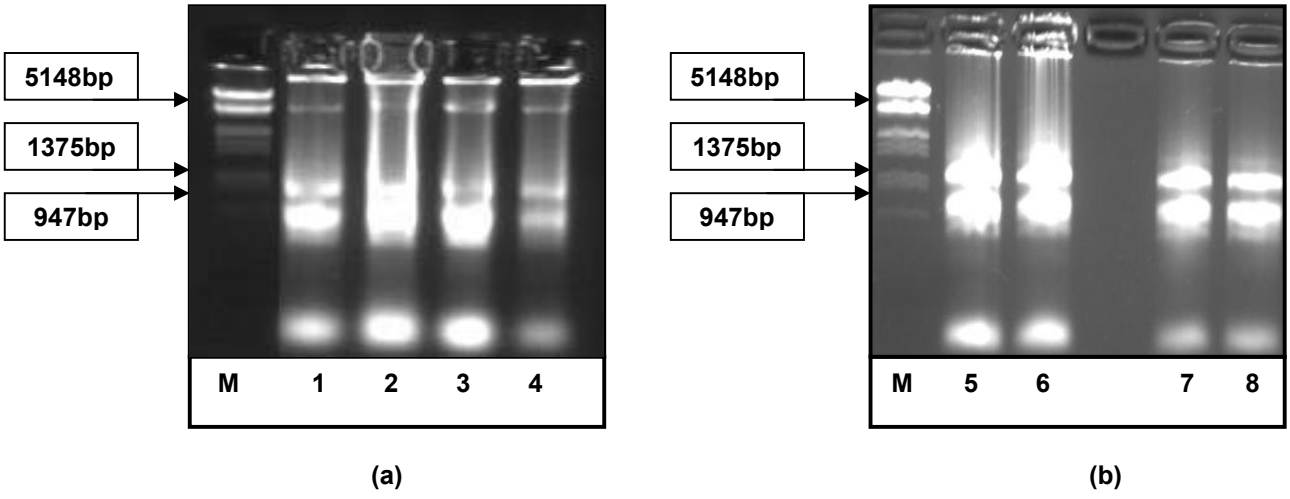

Figure 3.1:

A 1% (w/v) agarose gel stained with ethidium bromide, displaying products of total RNA extractions prior to (a) and following (b) purification with the MinElute reaction clean-up kit (Qiagen). Lane M = Molecular size marker. Lanes 1 to 4 = Total RNA extracted from 94M370 (Gamtoos *Dn7) and* Gamtoos. Lanes 5 to 8 = Total RNA extracted from 94M370 (Gamtoos *Dn7) and* Gamtoos following purification.

Following the purification of total RNA, full length double stranded cDNA was synthesized from the RNA, where 94M370 (Gamtoos *Dn7*) cDNA was designated tester samples and the Gamtoos cDNA was designated driver samples for the purpose of suppression subtractive hybridization. cDNA quality and quantity were spectrophotometrically measured (Table 3.1).

Table 3.1:

Concentration of cDNA samples measured after column purification.

cDNA Sample	Concentration (ng/µl)	260:280nm absorbance ratio
94M370 (SA biotype infested)	58.27	2.04
Gamtoos (SA biotype infested)	40.18	1.93

3.3.2 Suppression subtractive hybridization (SSH)

SSH was performed in order to isolate transcripts that are differentially expressed in 94M370 (*Dn7,* tester) as compared to its susceptible counterpart, Gamtoos (driver).

3.3.2.1 *Rsa*I digestions

Both tester and driver samples were first digested with *Rsa*I, a four base cutting restriction enzyme that yields blunt ends.

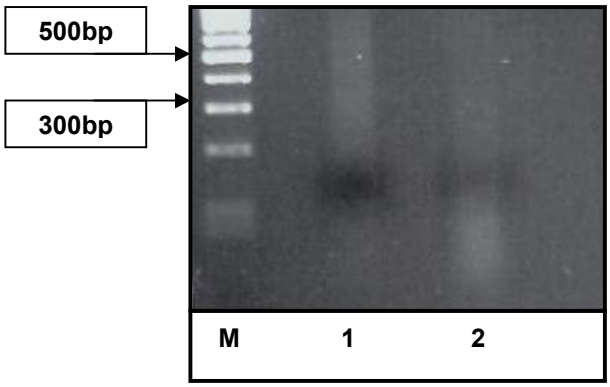

Figure 3.2:

A 2% (w/v) agarose gel image of 94M370 cDNA prior to (Lane 1) *Rsa*I digestion, and after (Lane 2) *Rsa*I digestion. Lane M = Size marker (Promega).

This allowed for subsequent adaptor ligation (Diatchenko et al., 1996). The complete restriction with *Rsa*I digestion was determined by agarose gel electrophoresis. A general downward shift in the smearing pattern of the cDNA after digestion, as compared to cDNA prior to digestion is expected (Figure 3.2).

3.3.2.2 Analysis of adaptor ligation and cDNA yield

A PCR based experiment was applied in order to confirm that at least 25% of cDNA samples had adaptors ligated on their ends – which is the minimum requirement for the application of SSH. In this experiment, primer combinations were used in a PCR reaction that span the cDNA/adaptor junctions of the tester samples. This entailed the use of primers that amplify a gene that is known to be constitutively expressed, poly-ubiquitin in this case. Because PCR products could not be amplified, we concluded that efficient adaptor ligation did not take place. Consequently, the PCR reaction carried out following secondary hybridization was also unsuccessful.

3.3.3 cDNA-AFLP analysis

A cDNA-AFLP experiment was carried out as a measure of cDNA integrity, in order to establish whether this could be a contributing factor to the failure of the subtraction procedure. The experiment was conducted with five primer combinations (2.7.4), after which a DNA fingerprint of 94M370 and its susceptible counterpart (Gamtoos) were achieved (Figure 3.3). A positive result in this regard suggested that the cDNA synthesis procedure was successful and that the samples are intact.

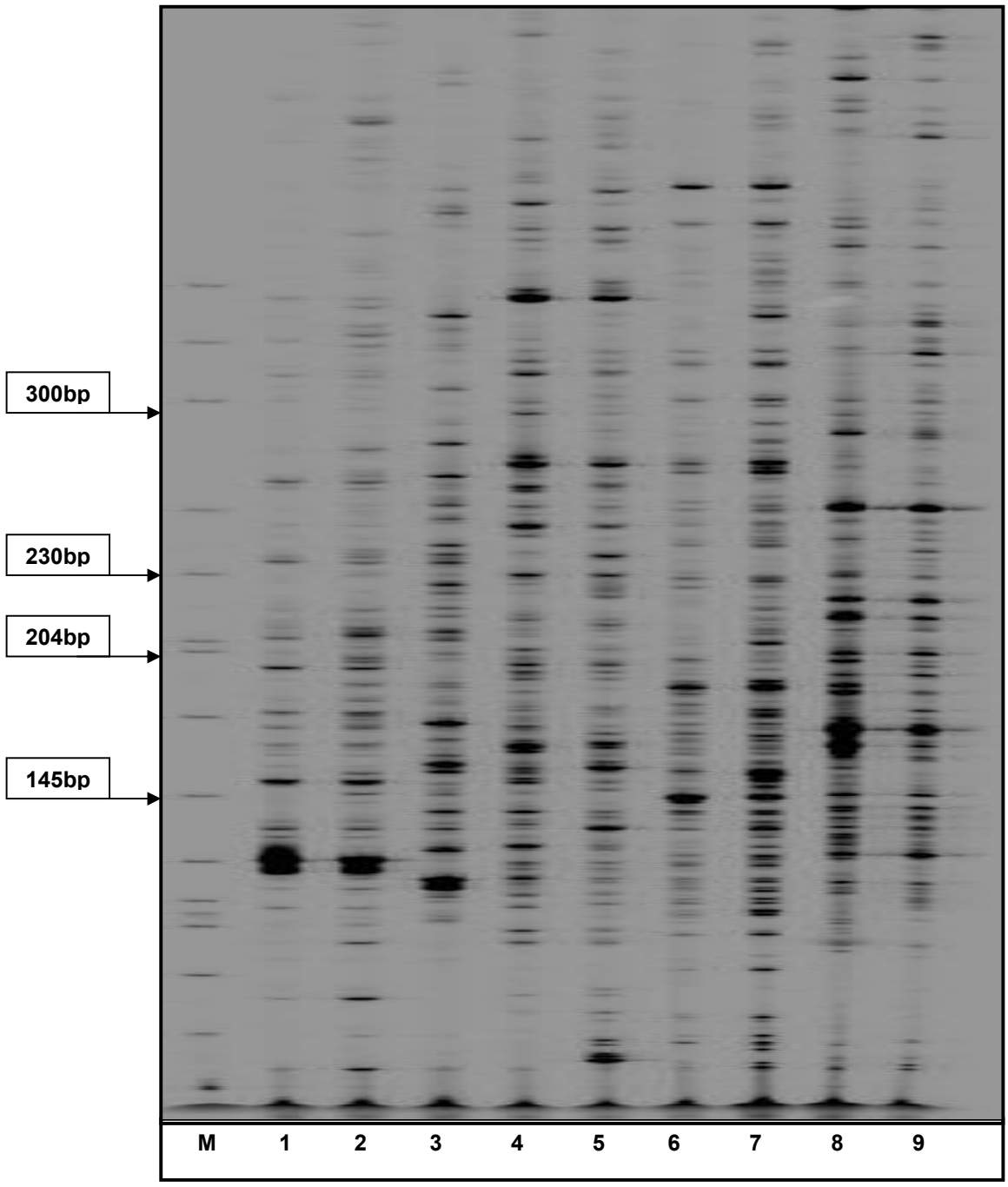

Figure 3.3:

cDNA-AFLP profile generated by the Li-Cor DNA analyzer. Lanes 1 to 5 = 94M370 (Gamtoos *Dn7*) cDNA. Lanes 6 to 9 = Gamtoos (susceptible) cDNA. Lane M = Size marker. Five different selective primer combinations were tested: Lanes 1 and 6 = MCT/TCA; lanes 2 and 7= MGT/TGA; lanes 3 and 8 = MGA/TAC; lanes 4 and 9 = MGA/TCA; lane 5 = MTG/TAC

3.4 Discussion

With this work, it was attempted to identify genes that are differentially expressed in a resistant wheat line, 94M370 (Gamtoos *Dn7*), following infestation with different biotypes of the Russian Wheat Aphid (RWA), by means of SSH - in order to elucidate the molecular responses activated in response to each aphid biotype. However, no transcripts could be amplified, leading to the assumption that one or a combination of reaction steps failed during the execution of the experiment.

Based on the premise that the following factors are the key elements in the successful execution of subtractive hybridization; (i) PCR reactions and components, (ii) restriction enzyme digestion, (iii) optimal cDNA/adaptor ligation and (iv) quantity and quality of the cDNA template as starting material, consequently lead to all of these being tested during experimentation in order to identify which step was contributing to the negative result.

Firstly, in order to confirm that all PCR reagents were functioning optimally, a random test PCR was conducted on a control fragment of DNA from another source - that is known to amplify successfully. The subsequent successful yield of PCR products (result not shown) confirmed that all reagents were suitable for PCR experimentation, which focused our attention on other PCR parameters. Therefore, during the adaptor ligation test PCRs (as in 3.2.5.3) optimal primer annealing temperatures as well as the use of different thermal cyclers at these temperatures were assessed. PCR cycling was carried out on the My-cycler (Biorad) thermal cycler, the I-cycler (Biorad) as well as the Perkin-Elmer GeneAmp 9600 (Applied Biosystems) using gradient of primer annealing temperatures varying from 48°C to 66°C. None of these experiments yielded PCR products (not shown), in the form of a smear as visualized on a ethidium bromide stained agarose gel. Such a result would represent ligation of adaptors to the cDNA samples. *Taq* DNA polymerases from different manufacturers, such as

Supertherm Taq (JMR Holdings) and Excel Taq polymerase (Roche) were tested, with neither yielding positive results.

Because varying PCR conditions and reagents did not generate any product, attention was turned to enzyme digestions as well as adaptor ligation conditions. *Rsa*I digestions were carried out as described in 2.5.1. The gel image from this (figure 3.3) suggests that complete digestion of the cDNA template did indeed occur, therefore the conclusion drawn from this was that incorrect restriction enzyme digestion conditions was unlikely to be responsible the absence of the desired amplification products during test PCR (3.2.5.3).

Since the SSH adaptors were designed without 5' phosphate groups (to ensure that the longer strand of the adaptors attach to the 5' end of the cDNA), long reaction incubation times together with a high amount of T4 DNA ligase was required (Diatchenko et al. 1996). Because test PCRs conducted after these ligations also failed; and since it was already established that this was not a result of failing PCR reagents, a test PCR reaction designed to amplify a fragment that spans adaptor-cDNA junctions was carried out to assess the ligation reaction (3.2.6). A negative result led to the conclusion that ligation did not occur at all, or did not occur sufficiently in order to produce a PCR product. Subsequently, ligation reaction conditions were varied considerably in an attempt to achieve maximum ligation efficiency (3.2.5.2). It was considered that higher ligation temperatures, although more suitable for the ligase enzyme, could result in unstable, unfavourable conditions, because only one 5' phosphate group was available to bind to one specific 3' hydroxyl group on the cDNA template. Conditions might therefore be unfavourable to support such a highly specific interaction between template and adaptor. Due to this possibility, the reaction temperature was reduced, and to compensate for reduced enzyme activity, incubation time was increased. Again, no products were visible during subsequent test PCRs. In addition to these variations, using different T4 DNA ligase concentrations together with

Polyethylene Glycol (PEG 4000) in order to increase reaction efficiency also proved unsuccessful (Pheiffer and Zimmerman, 1983).

Another plausible explanation that could potentially result in the failure to amplify transcripts, was the insufficient quality and purity of adaptors for such a demanding application. As a result, new adaptors with the same sequences (3.2.5.2) were acquired (Integrated DNA Technologies, Coralville, IA). Using these adaptors in ligation and subsequent PCR, again yielded no result. These adaptors were treated with phosphatases (3.2.6) in order to ensure that the 5' phosphate groups were removed, as required for SSH, since only one of the adaptor strands should ligate to the template sample. In addition, ligation of short oligonucleotides (such as the adaptors preferentially ligating to each other) may have occurred, causing inefficient adaptor-template ligation. Even these adjustments, failed to resolve the problem.

Finally, it was considered that the quality and quantity of the cDNA template itself was possibly responsible for the failure to amplify products. To test this, the cDNA was applied as template in a different PCR based profiling technique, namely cDNA-AFLPs (2.7) (Bachem et al. 1996). The successful generation of a DNA fingerprint (figure 3.3) showed that the cDNA template was of sufficient quality for PCR based applications.

In conclusion, a large number of variations were applied to the SSH experimental procedure in an attempt to resolve the problem. These experiments lead to the conclusion that the reason for this failure is likely to be due to failed or inefficient adaptor ligation. Experimentation could however, not pinpoint the exact reason for these failed ligations, and SSH could not be completed. It was therefore decided to

investigate the use of different molecular tools in order to achieve the experimental objective of transcriptional profiling of the resistant wheat line 94M370.

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Chapter 4

Investigating RWA biotype-specific responses in wheat by means of transcriptional profiling

Abstract

For sustainable solutions to the problem of insect infestation, the study of molecular plant-insect interactions is integral to resistance breeding strategies. This also holds true in the case of wheat (*Triticum aestivum*), where the Russian wheat aphid (*Diuraphis noxia,* Mordvilko, RWA) is responsible for significant crop losses in most major wheat producing countries around the world. Our study is focused on gaining a greater understanding of the resistance mechanisms activated by the RWA resistance gene *Dn7* by comparing responses following infestation with three different aphid biotypes (RWA-SA1, RWA-US1, RWA-US2). This consisted of analysing the resistant wheat line 94M370 (Gamtoos-*Dn7*) and its susceptible counterpart (Gamtoos) on a transcriptional level with complementary DNA amplified fragment length polymorphisms (cDNA-AFLPs) using 17 primer combinations, as well as quantitative Real-Time PCR of 15 differentially expressed transcripts. The results of this expression profile analysis suggest that *Dn7* activates similar responses against the two US aphid biotypes, which differ noticeably from the response following infestation with a South African aphid biotype. This is consistent with recent research showing limited molecular variation between the two US aphid biotypes (~0.12%), compared to a distinctly different South African biotype. We therefore conclude that *Dn7* recognises and interacts in a highly specific manner with the different aphid eliciting agents, which in turn activates specific defence pathways unique to that interaction.

4.1 Introduction

The importance of wheat as a major food source is illustrated in the fact that together with rice, wheat supplies us with almost two thirds of our daily calorie and protein intake (Gill et al. 2004). In order to meet the ever increasing human needs in the next 50 years, grain yield must increase by roughly 2% per annum on a land area that is not likely to expand beyond its current level (Gill et al. 2004). One economically important pest that greatly affects small grains such as wheat and barley is the Russian wheat aphid (RWA, *Diuraphis noxia,* Mordvilko).

The study of genetic resistance cultivars, and the breeding programmes thereof, has become an important endeavour in pursuit of finding an effective method of controlling the aphid (Van der Westhuizen et al. 1998). Ultimately, the most effective control of this pest will likely involve a combination of factors, such as genetic resistance, biological and chemical control. However, our limited understanding of the molecular interactions between the RWA and its wheat host, delays the development of sustainable strategies to control this pest (Botha et al. 2006). Further complications in this endeavour lies in the continual emergence of new RWA biotypes able to overcome existing resistant cultivars.

The RWA was introduced into the USA by 1986 (Burd and Burton, 1992), and was present in all major wheat producing countries worldwide (with the exception of Australia) four years later (Basky, 2003). The USA and South Africa have since then suffered the most severe effects of this pest (Botha et al. 2006). The ability of an insect species like the RWA, to feed on a host plant previously resistant to such infestation defines a new biotype, and these new biotypes clearly limit the usefulness of existing resistant cultivars (Botha et al. 2006). As an example of this eight different, RWA biotypes virulent to a number of cultivars (carrying either *Dn1, Dn2, Dn4, Dn5* or *Dn6*) have appeared in the USA alone

(Haley et al. 2004; Botha et al. 2006). At the moment seven novel biotypes have been found in the USA, on various cereal crops and wild grasses (Li et al. Unpublished). Data such as this warrants further investigation into the various different biotypes, and how they differently interact with various cereal hosts.

The introduction of amplified fragment length polymorphisms (AFLPs) technology as originally described by Vos et al. (1995), and the subsequent extension of this technique - which enables transcriptional profiling on complementary DNA (cDNA) level (Bachem et al. 1996) has greatly contributed to further our knowledge of the molecular interactions between pathogen or pest, and its potential plant host. In one such case, Ditt et al. (2001) applied complementary DNA amplified fragment length polymorphisms (cDNA-AFLPs) in order to elucidate the nature of plant responses on transcriptional level, following infection and transformation with *Agrobacterium tumefaciens*. Birch et al. (1999) employed both SSH and cDNA-AFLPs to identify transcripts activated in potato following infection with *Phytophtora infestans*. Their study focused on one key component of the plant defence response, the hypersensitive response (HR). The manner in which plants defend themselves against pathogens hinges on the concept of *Resistance* gene-*Avirulence* gene interactions (Keen 1990). This concept has been identified as a plausible explanation for plant-insect interactions as well, therefore illustrating the need for investigating these interactions on molecular level. In such a case, genes differentially regulated following the interaction between the *Cf-9* resistance gene in tomato, and the *Avr-9* gene in the fungal pathogen *Cladosporium vulvum* were identified by means of cDNA-AFLPs (Durrant et al. 2000).

In this particular study, the aim was to investigate molecular defence mechanisms in the resistant wheat line 94M370 (Gamtoos *Dn7*) (Marais et al. 1994), following infestation with three different Russian wheat aphid biotypes (one South African biotype, RWA-SA1, and two biotypes from the

United States, RWA-US1 and RWA-US2). This will contribute to our broader knowledge as to how the *Dn7* gene confers resistance, and how these responses elicited by different aphid biotypes compare. The results generated here will contribute to further research, eventually culminating in the development of new RWA resistance varieties, and the improvement of existing ones.

4.2 Materials and methods

4.2.1 Plant material and infestations

Plants were grown under the same conditions as described in Chapter 3 (section 3.2.1). Upon reaching the desired growth stage (3 to 4 leaf stage), both 94M370 (Gamtoos *Dn7*) and Gamtoos were infested individually with 10 Russian Wheat Aphids (RWA, *Diuraphis noxia*) of the South African biotype, for a period of 48 hours as cited by Botha et al. (1998). Similarly, 94M370 and Gamtoos were infested with either US Russian wheat aphid biotypes (RWA-US1 and RWA-US2) – at the Colorado State University, Fort Collins, USA. Following each infestation experiment, second and third leaves were immediately harvested (Botha et al. 2005) for the purpose of total RNA extractions.

4.2.2 Treatment of glassware, plastic ware and solutions

Laboratory equipment and solutions used during RNA extractions were pre-treated as outlined in Chapter 3 (section 3.2.2) and as described by Sambrook et al. (1989).

4.2.3 Total RNA extractions

Total RNA was extracted from harvested wheat leaves as described in section 3.2.3 (Chapter 3), according to a modified protocol of Chomczynski and Sacchi (1987). These included plants that were infested with three different RWA biotypes (RWA-SA1, RWA-US1, RWA-US2) as well as uninfested 94M370 (Gamtoos *Dn7*) and Gamtoos plants, to serve as controls. Extracted RNA was subsequently purified from contaminants by using the RNeasy Mini kit according to instructions supplied by the

manufacturer (Qiagen, USA). Samples were eluted twice in 30µl volumes. RNA samples were analyzed for quality and quantity by agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light. All samples were stored at -80°C until further experimentation.

4.2.4 cDNA synthesis

Synthesis of full length, double stranded cDNA was carried out on approximately 15µg of total cellular RNA using the cDNA Synthesis System (Roche Applied Science, Germany) according to supplied instructions. Resulting cDNA was purified using the MinElute Reaction Clean-up Kit (Qiagen, USA) also according to manufacturer's specifications. Samples were eluted twice in a total volume of 30µl. cDNA concentrations were determined using a spectrophotometer and then stored at -20°C.

4.2.5 cDNA-AFLP analysis

cDNA-AFLP analysis was carried out as described by Bachem et al. (1996), but using the AFLP Expression Analysis Kit (Li-Cor, Lincoln, NE). The supplied protocol was slightly modified in some cases, as described below.

4.2.5.1 Restriction enzyme digestion

Roughly 100ng of cDNA template was digested with 0.625 units of *Taq*I restriction enzyme in 1x RL buffer [50mM Tris-HCl (pH 7.5), 50mM Mg-acetate, 250mM K-acetate] in a total reaction volume of 20µl. The reaction was incubated at 65°C for 2 hours. Following this, 0.625 units of *Mse*I restriction enzyme was added to the *Taq*I enzyme mix, together with fresh 1x RL buffer in volume of 25µl. This digestion reaction was carried out for 2 hours at 37°C. Inactivation of both restriction enzymes was

carried out at 80°C for 20 minutes. Restriction products were stored at -20°C overnight, until continuation of the experiment.

4.2.5.2 Ligation of adaptors

The products of the *Taq*I/*Mse*I restriction reaction (4.2.5.1) was mixed with 4.5µl adapter ligation mix [*Taq*I and *Mse*I primers, 0.4mM ATP, 10mM Tris-HCl (pH 7.5) 10mM Mg-acetate, 50mM K-acetate], 7.5 units T4 DNA ligase and sterile water (SABAX) in a total reaction volume of 30µl. Ligation took place by incubating all samples at 20°C for 2 hours. The products of the ligation reaction were subsequently diluted 1:10 in sterile water (SABAX, Port Elizabeth, South Africa) and stored at -20°C until further use.

4.2.5.3 Pre-amplification

Pre-amplification was carried out in the same manner as described in 3.2.7.3 (Chapter 3). The success of pre-amplification was again determined by gel electrophoresis on a 1% (w/v) agarose gel [stained with ethidium bromide (0.005mg/ml)] and visualized under UV light.

4.2.5.4 Selective amplification and gel electrophoresis

Selective PCR amplifications were carried out on all DNA samples, with 17 of the respective *Taq*I/*Mse*I (+2/+2) primer combinations (MCT/TCA; MGT/TGA; MGA/TAC; MGA/TCA; MTG/TAC; MAG/TCA; MAG/TGT; MAC/TAC; MAC/TGA; MGT/TAC; MGT/TCA; MGT/TAG; MCT/TGT; MCT/TCA; MCT/TCT; MTG/TCA; MTG/TCT) as described in Chapter 3 (3.2.7.4). The PCR cycling conditions also were the same as those described in 3.2.7.4 (Chapter 3). On completion of the PCR, samples were mixed with 5µl loading dye and resolved on 8% denaturing polyacrylamide gels [8% Long Ranger™ gel solution (BMA, Rockland, ME, USA), 7.0M urea and 0.8x TBE

consisting of 71.2mM Tris, 71.2 mM boric acid and 1.6mM EDTA] (Myburg et al. 2001) in the Li-Cor DNA analyzer 4200S (at 1500V, 35mA, and 45°C).

4.2.6 Image analysis

Images generated on the Li-Cor DNA analyzers during electrophoresis were used to calculate band intensities of fragments judged to be differentially expressed, using the AFLP-QuantarPro software package (KeyGene Products B.V., Wageningen, Netherlands). Lane definitions, band scoring and sizing were carried out as described in the user's manual under default settings. Numerical values (band intensities) (generated by AFLP-QuantarPro), assigned to differentially expressed transcript derived fragments (TDFs) during the band scoring process were used in further analysis (generation of a normalized cluster image).

4.2.7 Clustering

The resulting band intensities were first normalized using the Cluster programme (Eisen et al. 1998), with mean-centering applying Spearman's rank correlation. A cluster image representing groups of differentially expressed fragments that share similar expression patterns, was generated from this normalized data with Java TreeView (Saldanha, 2004.)

4.2.8 Fragment isolation

A number of fragments from the original AFLP analysis, showing expression patterns of particular interest were identified and subsequently isolated for putative identification. Gels containing these fragments of interest were scanned on the Li-Cor Odyssey IR imager (LiCor, Lincoln, NE). These bands (PCR products) were subsequently excised from the polyacrylamide gels and stored in 50µl sterile water (SABAX, Port Elizabeth, South Africa). The DNA was eluted from these gel plugs by 5

to 8 cycles of freezing (-80°C) and thawing at 60°C. In cases where DNA could not be successfully eluted using this method, samples were stored at 4°C for 1 to 2 weeks, after which the freeze/thaw process was repeated. Re-amplification of PCR products was achieved by subjecting 5µl of the eluted samples to the selective amplification PCR as in 3.2.7.4 (Chapter 3). The sizes of these PCR products were determined by resolving them on 1% (w/v) agarose gels, visualized by ethidium bromide staining and UV light. These fragment sizes were compared to the corresponding fragment sizes on the original AFLP image, in order to confirm re-amplification of the correct product.

4.2.9 Cloning and identification of differentially expressed fragments

4.2.9.1 Preparation of competent cells

Competent cells were prepared by an adaptation of the rubidium chloride method originally described by Hanahan (1985) and published in the Sub-cloning Notebook (Promega, USA).

4.2.9.2 Ligation, transformation and sequencing

Differentially expressed fragments isolated during cDNA-AFLP analysis, were cloned into the pTZ57R/T vector of the InstaClone PCR cloning kit (Fermentas Life Sciences). A modified cloning protocol was however used.

PCR products to be identified were ligated into the pTZ57R/T cloning vector prior to transformation. The ligation reaction consisted of 2µl PCR product, 1.5µl vector, 1x ligation buffer (Fermentas Life Sciences) and 2.5 units of T4 DNA ligase in a 15µl final reaction volume. Samples were incubated at 4°C overnight. Ligation products were transformed into competent *Escherecia coli* (DH5α) cells as described by Ausubel et al. (1998). Transformants were screened by colony PCR (Gussow and Clackson 1989) to confirm the presence of inserts. The reaction contained 1 unit *Taq* DNA

polymerase, 1x PCR amplification buffer, 2.5mM $MgCl_2$, 5µM of each dNTP, 10pmol each of M13F (5'-GTA AAA CGA CGG CCA G-3') and M13R (5'-CGA GAA ACA GCT ATG AC-3') primers in a 20µl reaction. Thermal cycling commenced at 94°C for 3 minutes. This was followed by 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. The PCR was ended off with a final extention step at 72°C for 5 minutes. Samples successfully cloned (confirmed by colony PCR) were sequenced by the Macrogen Corporation (Rockville, MD). Thereafter, functional annotation of sequenced samples was carried out by subjecting them to translating BLAST (BLASTX) searches and nucleotide BLAST searches (BLASTN) (Altschul et al. 1990).

4.2.10 Quantitative Real-Time PCR of differentially expressed transcripts

To confirm the expression pattern of differentially expressed transcripts identified during cDNA-AFLP analysis, quantitative Real-Time PCR (qRT-PCR) was carried out on the iCycler IQ (Biorad) system. This involved an independent biological repeat of the experiment, therefore aphid infestations (4.3.2.1) and total RNA extractions (4.3.2.3) were repeated for the RWA-SA1 biotype infestations. RNA from a previous, independent experiment, extracted from plants following infestation with the two US biotypes was also used. Primer pairs were designed for each selected transcript to be assayed, based on the sequencing results obtained (4.3.2.9), using Primer designer 5 software (Scientific and Educational Software, Cary, NC). PCR reactions were performed in triplicate, with 0.5ng total RNA starting material, using the iScript One-Step RT-PCR kit with SYBR Green (Biorad), according to instructions supplied with the kit. Relative quantification of all samples was achieved by applying the method of Pfaffl (2001), using 16S rRNA as a reference standard.

4.2.11 Slot blot analysis

A final confirmation of cDNA-AFLP and qRT-PCR results was achieved through the use of Slot blot analysis. To start, 100ng of cDNA from each transcript to be analyzed was labelled with fluorescein-11-dUTPs with a random prime labelling method. This was achieved by using the Gene Images Random Prime Labelling Kit (Amersham Biosciences), according to instructions supplied by the manufacturer. Subsequent to this, 50ng of total RNA template (from each treatment condition) was heat denatured at 65°C for 3 minutes, and applied to Hybond N+ (Amersham Biosciences) nylon membrane using the Bio Dot SF apparatus (Biorad) according to manufacturer's usage instructions. The RNA templates were fixed to the membrane by means of UV cross-linking at 0.240 Joules. Overnight hybridisation with labelled cDNA probe and stringency washes, was also carried out according to the Gene Images Random Prime Labelling Kit (Amersham Biosciences).

Following overnight hybridisation and stringency washes, detection of bands were achieved by the use of the Gene Images DCP-Star Detection kit (Amersham Biosciences), according to manufacturers specifications. Treated membranes were placed in a film cassette and exposed to autoradiography Hyperfilm-MP (Amersham Biosciences) overnight and subsequently developed.

4.3. Results

4.3.1 cDNA-AFLP analysis

Total RNA was extracted from both the resistant and susceptible varieties of Gamtoos, and double stranded cDNA was subsequently synthesized for use in cDNA-AFLP analysis. The quality of this cDNA for use in a demanding application such as cDNA-AFLPs, has already been determined to be sufficient (Chapter 3, section 3.3.3). This technique allowed for the identification of differentially expressed transcripts, and the comparison of RWA feeding responses across eight different treatment conditions. Analysis over seventeen different primer combinations, yielded approximately 1697 transcript derived fragments (TDFs). Of these only an estimated 11.2% of fragments generated, exhibited no change in expression across all eight treatment conditions. Differential regulation of transcripts (relative up- and down-regulation as compared to the uninfested controls), following infestation with SA biotype aphids, represent 55.2% of all generated transcripts. Only 15.7% and 17.9% of fragments displayed differential regulation in plants infested with US biotypes 1 and 2, respectively. Figure 4.1 shows the cDNA-AFLP fingerprint constructed over five of the seventeen primer combinations. The expression profiles of 94M370 and Gamtoos infested with RWA-SA1 (lanes 3 and 4 of all primer combinations) can be seen to differ significantly from all other profiles. The profiles of plants infested with the two US biotypes (RWA-US1 and RWA-US2), show a more limited variation in expression (lanes 5 to 8 of all primer combinations) when compared to each other. This trend is evident across al seventeen primers combinations.

4.3.2 Cluster analysis

Approximately 195 transcripts judged to be differentially expressed, were subjected to band scoring (4.2.6) and subsequently clustered into groups of genes that share similar expression patterns (4.2.7). This cluster image, which consolidates all the cDNA-AFLP data together, is illustrated in figure 4.2.

Plants infested with the South African biotype of the Russian wheat aphid (RWA-SA1, lanes 3 and 4) show two distinct groups of expression - one up-regulated (red) and one down-regulated (green). Again when considering the two US biotypes RWA infested plants (lanes 5 to 8), clustering illustrates more subtle variations as compared to RWA-SA1 infested plants, indicative of diverse molecular responses.

Figure 4.3 shows the average expression levels of transcripts for each of six expression clusters, as indicated in figure 4.2. This is accompanied by graphs showing the expression levels of selected representative transcripts in each cluster. These graphs supply a clear view as to the trends observed in the cluster image (figure 4.2), and indicates the most significant changes in expression ratios to be present in plants infested with RWA-SA – whereas the differences in expression ratios of plants infested with the US biotype aphids are more subtle. Closer consideration of figure 4.3 shows a noticeable up-regulation of transcripts in response to RWA-SA infestation in cluster 1A, whereas relative down-regulation occurs across all other treatment conditions. A similar expression trend is observed in cluster 1B, whereas a relative down-regulation of transcripts in response to RWA-SA infestation as compared to US infested plants occurs in clusters 2A and 2B (figure 4.3). Transcripts that group into cluster 3A show more varying expression levels across untreated and RWA-SA infested plants, but with a decrease in expression levels of both RWA-US1 and 2 infested plants. The most prominent change evident in cluster 3B is the relative down-regulation of transcripts in response to RWA-SA infested plants, particularly the resistant variety. Each of the selected representative transcripts that group into each cluster (graphs A to F) show comparable expression levels for that cluster. The most important conclusion that can however be drawn from the cluster graphs in this figure, is the fact that striking differences in expression occur in RWA-SA infested plants as compared to RWA-US1 and 2 infested plants indicative of very diverse defence responses.

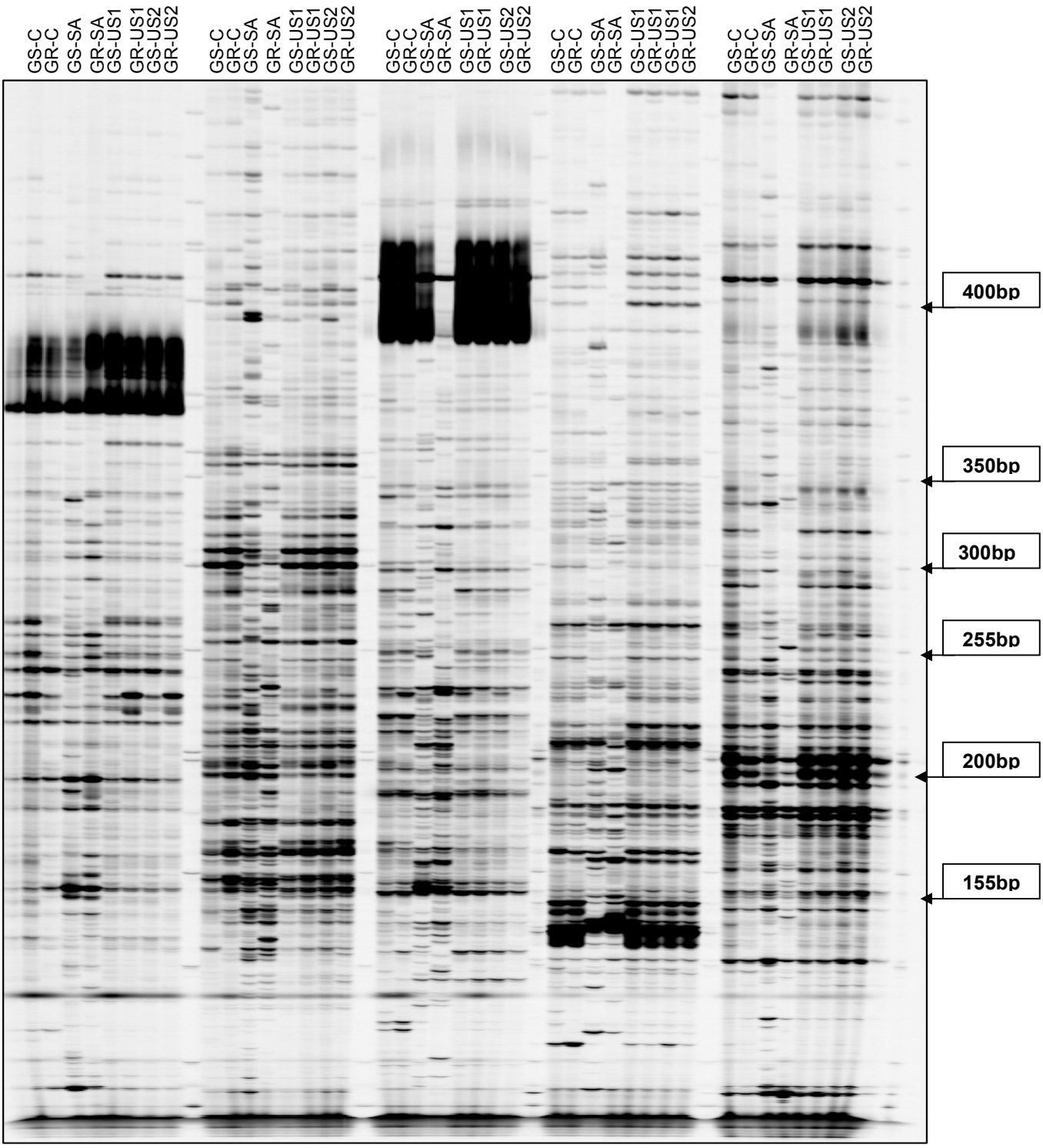

Figure 4.1:

cDNA-AFLP fingerprint constructed across five primer combinations. Treatment conditions for each primer combination are indicated above. Lanes in between each primer combination represent size markers (IRDye 700), with sizes indicated in base pairs (bp).

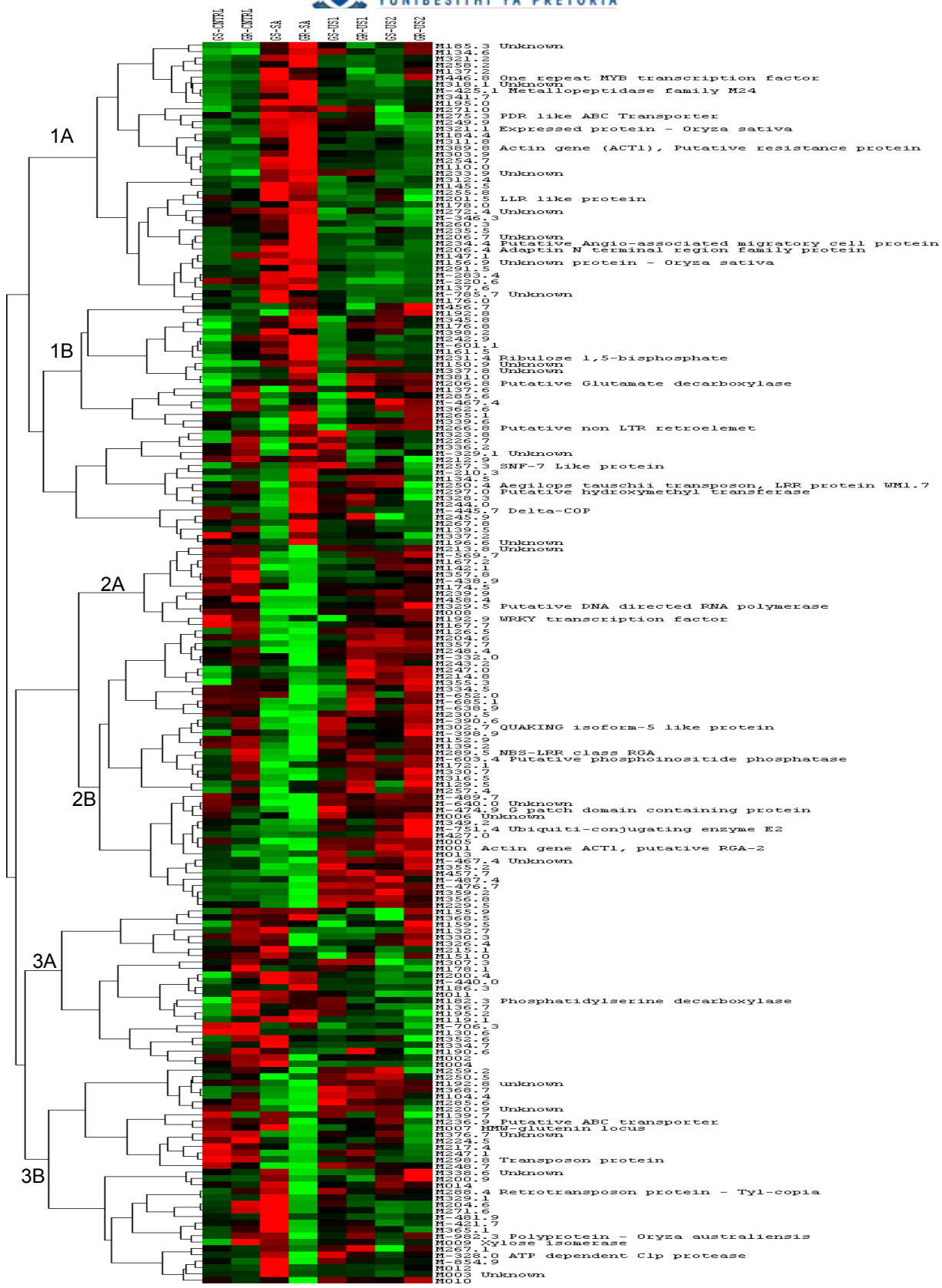

Figure 4.2:
Cluster image generated by Java Treeview. Red bands show relatively up-regulated transcripts, whereas green bands show down-regulated transcripts. Treatment conditions are indicated at the top of each column.

Expression clusters

Representative transcripts in each cluster

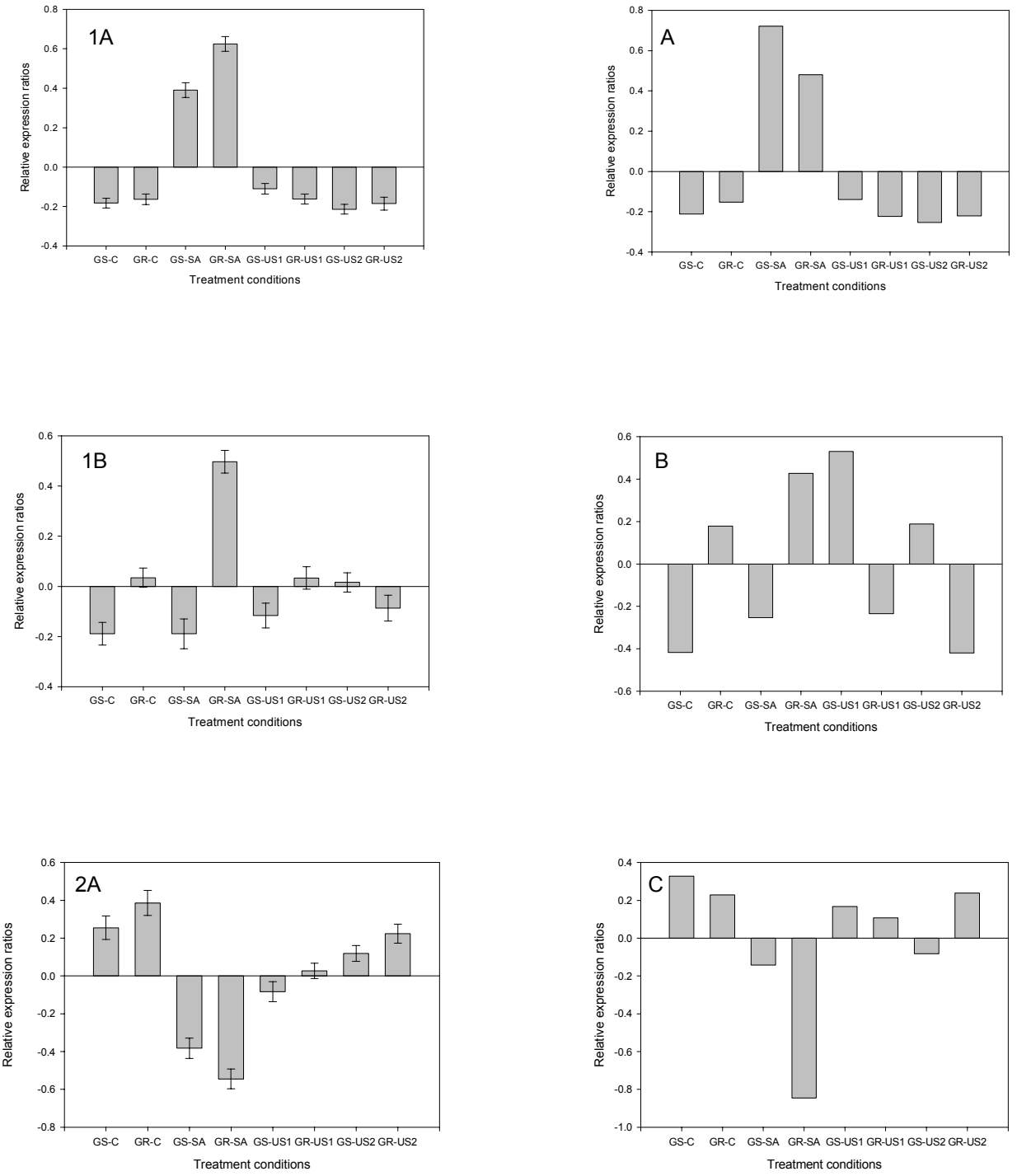

Figure 4.3:

Graphical representation of average expression levels for each cluster as indicated in figure 4.2. Cluster 1A, 1B and 2A are represented in graphs in the left column. Graphs in the right column show expression levels of one representative transcript that group into each cluster shown next to it. A= Unknown protein (cluster 1A); B= SNF-like protein (cluster 1B); C= Unknown transcript (cluster 2A).

Expression clusters

Representative transcripts in each cluster

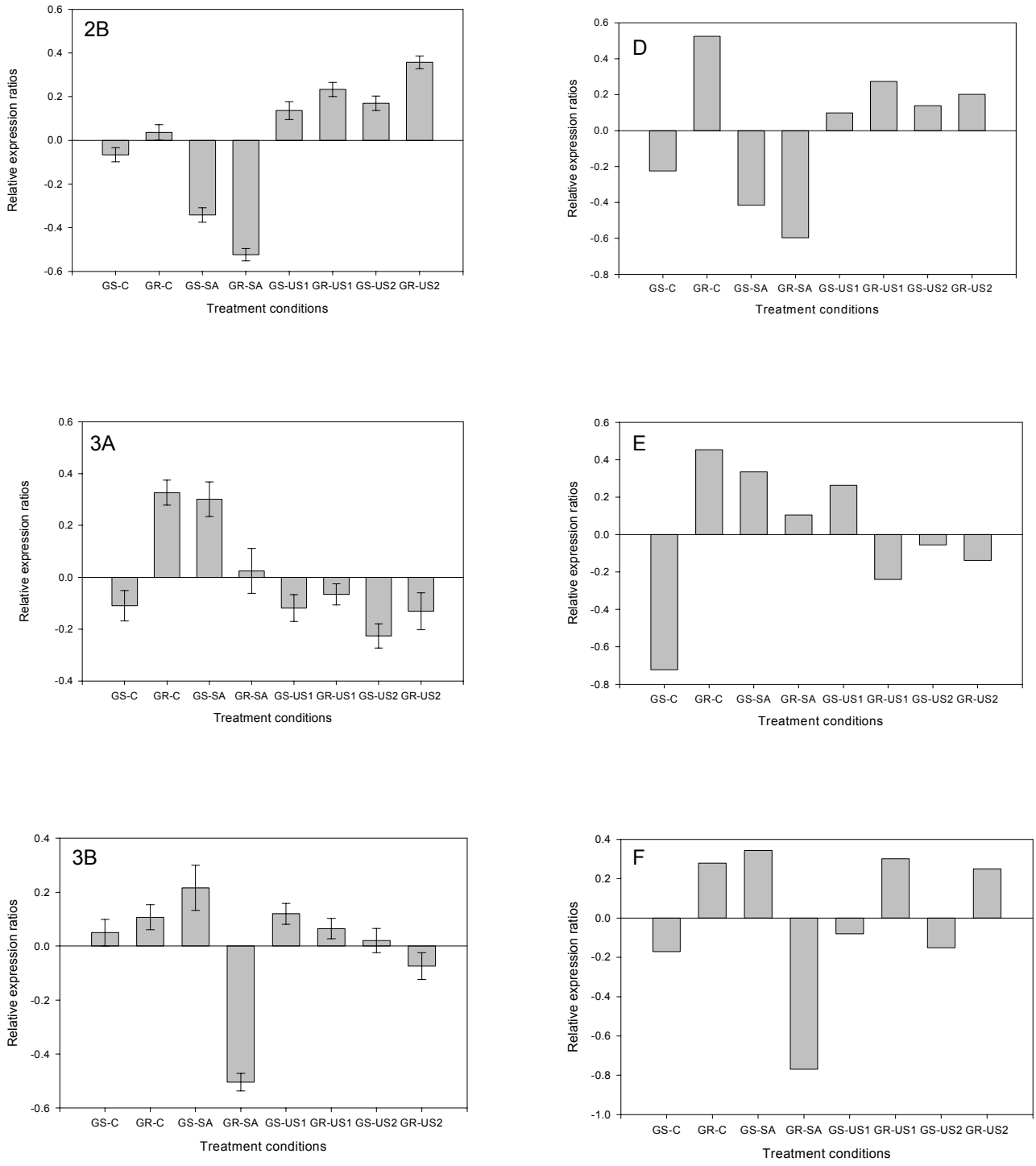

Figure 4.3 (continued):

Graphical representation of average expression levels for each cluster as indicated in figure 4.2. Cluster 2B, 3A and 3B are represented in graphs in the left column. Graphs in the right column show expression levels of one representative transcript that group into each cluster shown next to it. D= NBS-LRR class resitance gene analog (cluster 2B); E= Phosphatidyl serine decarboxylase (cluster 3A); F= Polyprotein (cluster 3B).

4.3.3 Fragment isolation and identification

After cluster analysis of cDNA-AFLP data, cDNA-AFLP bands representative of genes showing interesting expression patterns were excised from polyacrylamide gels and re-amplified with the selective-amplification PCR method (4.2.8). A gel scanned on the Odyssey infrared scanner (Li-Cor) prior to, and following fragment isolation is shown in figure 4.4. A total of 110 bands were recovered for potential further analysis, of which 60 were selected for re-amplification and sequencing. Of this, successful sequencing was completed for 53 transcripts, which was subjected to BLASTX as well as nucleotide blast (BLASTN) searches in order to assign putative identity to these transcripts. Table 4.1 shows the sequences to which putative function could be assigned. Transcripts were broadly grouped into 7 functional groups, based on gene-ontology identified on the Expasy (www.expasy.org) and Kegg (www.genome.jp/kegg) databases. These include four transcripts related to gene expression, such as the WRKY transcription factor, two retrotransposons, five transcripts putatively related to defence (like the LZ-NBS-LRR class resistance gene analog) and three nucleic acid binding proteins such as the G-patch domain containing protein. The same photosynthesis transcript (Ribulose-1,5-carboxylase/ oxygenase sub-unit) was identified twice in this study. Two expressed proteins identified in this study, have no known molecular function assigned to them yet and are indicated as expressed protein and unknown protein, respectively.

The majority of transcripts to which putative identity could be assigned, belonged to the protein modification/metabolism functional group. Among these nine transcripts, two ABC-transporters were identified, as well as a number of proteolytic enzymes. Seven transcripts were assigned as “others”, because they represent various different molecular functions that do not group well with the predefined functional classes. These include xylose isomerase, involved in carbohydrate metabolism, phosphatidylserine decarboxylase, involved in lipid metabolism and HMW-glutenin involved in

nutrient reservoir activity. Putative hydroxymethyl-transferase, identified twice in this study is involved in single carbon transfer reactions (such as amino-acid metabolism), and the adaptin N-terminal region family protein serves as a ligand in binding interactions. Finally, glutamate decarboxylase is known to be involved in glutamate metabolism and possesses lyase activity. Approximately 40% of genes show no significant similarity to any known gene, when subjected to these BLAST searches. These sequences were submitted to Genbank as expressed sequence tags (ESTs), with assigned accession numbers shown in table 4.2. Full sequence information of the ESTs submitted to Genbank is indicated in appendix A. Table 4.3 shows the identity of sequenced transcripts, grouped under each of the treatment conditions in which it was differentially expressed. The majority of identified transcripts were differentially expressed in RWA-SA infested plants (16 transcripts), with nine transcripts differentially expressed in RWA-US1 and 2 infested plants. Fewer transcripts were differentially expressed in RWA-US1 and 2 plants, respectively.

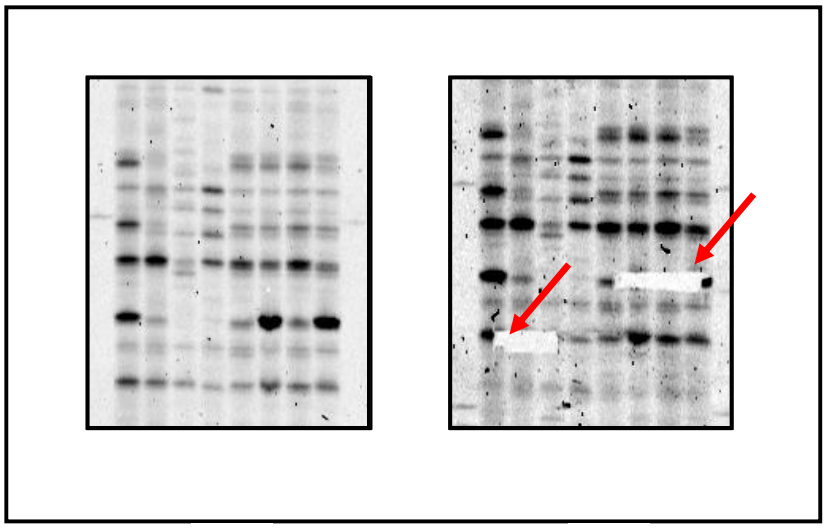

Figure 4.4:

Section of cDNA-AFLP gel image, scanned prior to band excision (a) and following band excision (b). The areas where gel plugs have been removed, containing the DNA of interest is indicated by the arrows.

Table 4.1:

Identities of sequenced transcripts to which putative identity could be assigned, following BLASTX searches. GenBank accession numbers, functional groups and E-values are also indicated. (Altschul et al. 1990).

Accession	*Functional Group and Putative Identity*	*E-value*
	Protein modification/metabolism	
XP482141	Putative PDR-like ABC transporter (*Oryza sativa*)	8E-38
AAP55198	ATP-dependent Clp protease proteolytic subunit (*Oryza sativa*)	6E-07
XP466538	SNF7-like protein (*Oryza sativa*)	2E-27
BAD35839	Putative Cyclophilin-40 (*Oryza sativa*)	1E-25
ABF93717	Ubiquitin conjugating enzyme (*Oryza sativa*)	2E-19
XP470554	Putative phosphoinositide phosphatase (*Oryza sativa*)	5E-29
AAF67098	Delta-COP (*Zea mays*)	1E-32
AAG49002	Putative ABC-transporter (*Hordeum volgare*)	1E-32
ABA98962	Metallopeptidase family M24 containing protein (*Oryza sativa*)	2E-24
	Gene expression related	
CAC86577	One repeat MYB transcription factor (*Zea mays*)	1E-16
ABC02814	WRKY Transcription factor 82 (*Oryza sativa*)	1E-26
XP469337	Putative angio-associated migratory cell protein (*Oryza sativa*)	4E-34
BAD68174	Putatitive DNA directed RNA polymerase II 23K chain (*Oryza sativa*)	2E-10
	Putative defence related genes	
AY534123	Complete LRR protein WM1.7 and LRR protein WM1.12 (*Aegilops tauschii*)	3E-19
AF446141	LZ-NBS-LRR class resistance gene analog (RGA), (*Aegilops tauschii*)	7E-48
AF326781	Putative resistance protein (RGA-2) (*Triticum monococcum*)	1E-13
AF326781	Putative resistance protein (RGA-2) (*Triticum monococcum*)	6E-157
AF497474	Leucine-rich like protein gene (*Aegilops tauchii*)	1E-26
	Nucleic acid binding	
BAD82121	QUAKING isoform 5-like (*Oryza sativa*)	2E-35
BAA22288	Polyprotein (*Oryza australiensis*)	2E-36
ABA99854	G-patch domain containing protein, expressed (*Oryza sativa*)	9E-77
	Retrotransposons	
ABG22008	Putative retrotransposon protein, Ty1-Copia subclass (*Oryza sativa*)	5E-13
AY672998	Soleil putative non-LTR retroelement (*Triticum aestivum*)	9E-78
	Photosynthesis	
AAV33293	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*Molinia caerulea*)	1E-39
AAV33293	Ribulose-1,5-bisphosphate carboxylase/oxygenase subunit (*Molinia caerulea*)	1E-39
	Unknown	
AAV67830	Unknown protein (*Oryza sativa*)	2E-09
ABF96767	Expressed protein (*Oryza sativa*)	2E-30

Accession	*Functional Group and Putative Identity*	*E-value*
	Others	
AY485644	Phosphatidylserine decarboxylase (*Triticum monococcum*)	2E-68
AY368673	HMW-glutenin locus (*Triticum turgidum*)	2E-32
CAA64545	Xylose isomerase (*Hordeum vulgare*)	2E-31
XP463512	Putative hydroxymethyltransferase (*Oryza sativa*)	3E-44
ABF93610	Adaptin N terminal region family protein, expressed (*Oryza sativa*)	2E-20
AAV65329	Putative glutamate decarboxylase (*Hordeum vulgare*)	1E-27
XP563512	Putative hydroxymethyl-transferase (*Oryza sativa*)	3E-44

Table 4.2:

Genbank accession numbers assigned to unknown transcripts, following submission to DBest database.

Accession	*Putative Identity*	*Clone ID*
EL763491	Unknown transcript	A1
EL763492	Unknown transcript	A4
EL763493	Unknown transcript	B1
EL763494	Unknown transcript	D1
EL763495	Unknown transcript	D2
EL763496	Unknown transcript	E1
EL763497	Unknown transcript	E7
EL763498	Unknown transcript	F1
EL763499	Unknown transcript	F7
EL763500	Unknown transcript	G3
EL763501	Unknown transcript	H2
EL763502	Unknown transcript	H5
EL763503	Unknown transcript	J2
EL763504	Unknown transcript	J4
EL763505	Unknown transcript	K7
EL763506	Unknown transcript	L1
EL763507	Unknown transcript	M3
EL763508	Unknown transcript	O5
EL763509	Unknown transcript	O6
EL763510	Unknown transcript	O8

Table 4.3

Identities of sequenced transcripts to which putative identity could be assigned, classified according to differential expression - in response to RWA-SA, RWA-US1 or RWA-US2 infestation.

Accession	*Transcripts differentially expressed in response to RWA-SA infestation*
XP482141	Putative PDR-like ABC transporter (*Oryza sativa*)
XP466538	SNF7-like protein (*Oryza sativa*)
BAD35839	Putative Cyclophilin-40 (*Oryza sativa*)
AAF67098	Delta-COP (*Zea mays*)
ABA98962	Metallopeptidase family M24 containing protein (*Oryza sativa*)
XP469337	Putative angio-associated migratory cell protein (*Oryza sativa*)
AF326781	Putative resistance protein (RGA-2) (*Triticum monococcum*)
AF497474	Leucine-rich like protein gene (*Aegilops tauchii*)
AAV33293	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*Molinia caerulea*)
AY672998	Soleil putative non-LTR retroelement (*Triticum aestivum*)
AAV67830	Unknown protein (*Oryza sativa*)
ABF96767	Expressed protein (*Oryza sativa*)
AY485644	Phosphatidylserine decarboxylase (*Triticum monococcum*)
CAA64545	Xylose isomerase (*Hordeum vulgare*)
XP463512	Putative hydroxymethyltransferase (*Oryza sativa*)
ABF93610	Adaptin N terminal region family protein, expressed (*Oryza sativa*)

Accession	*Transcripts differentially expressed in response to RWA-US 1 and 2 infestation*
AAP55198	ATP-dependent Clp protease proteolytic subunit (*Oryza sativa*)
ABF93717	Ubiquitin conjugating enzyme (*Oryza sativa*)
XP470554	Putative phosphoinositide phosphatase (*Oryza sativa*)
AAG49002	Putative ABC-transporter (*Hordeum volgare*)
BAA22288	Polyprotein (*Oryza australiensis*)
AF446141	LZ-NBS-LRR class resistance gene analog (RGA), (*Aegilops tauschii*)
AF326781	Putative resistance protein (RGA-2) (*Triticum monococcum*)
ABG22008	Putative retrotransposon protein, Ty1-Copia subclass (*Oryza sativa*)
AY368673	HMW-glutenin locus (*Triticum turgidum*)

Accession	*Transcripts differentially expressed in response to RWA-US1 infestation*
AY534123	Complete LRR protein WM1.7 and LRR protein WM1.12 (*Aegilops tauschii*)
ABA99854	G-patch domain containing protein, expressed (*Oryza sativa*)
AAV65329	Putative glutamate decarboxylase (*Hordeum vulgare*)

Accession	*Transcripts differentially expressed in response to RWA-US2 infestation*
BAD68174	Putatitive DNA directed RNA polymerase II 23K chain (*Oryza sativa*)
CAC86577	One repeat MYB transcription factor (*Zea mays*)
ABC02814	WRKY Transcription factor 82 (*Oryza sativa*)
BAD82121	QUAKING isoform 5-like (*Oryza sativa*)

4.3.4 Quantitative Real-Time PCR and Slot blot analysis

The initial results obtained during the cDNA-AFLP experiment were validated by an independent biological repeat of the experiment, by employing quantitative Real-Time PCR (qRT-PCR) (4.2.10) and Slot blot analysis (4.2.11). Fragments that represent a number of varying expression patterns during cDNA-AFLPs, were chosen to be analyzed, including a putative ABC transporter (figure 4.4a) ATP-dependent Clp protease (figure 4.4b), WRKY transcription factor (figure 4.4c) and a Ribulose-1,5-bisphosphate carboxylase/oxygenase subunit (figure 4.4d). In figure 4.4a qRT-PCR indicates relative up-regulation, primarly in the susceptible varieties, with exception of the resistant variety infested with RWA-US2. Such a trend is evident when comparing this to the slot-blot for the same gene. With analysis of the putative ATP-dependent Clp protease (figure 4.4b), a general corresponding trend of increased expression in the resistant varieties were observed, in this case with the exception of the RWA-US1 infested plants.

Good correlation between qRT-PCR results for the putative WRKY-transcription factor and the cDNA-AFLP fingerprint was achieved, with a significant increase in expression observed in the resistant plants infested with RWA-SA (figure 4.4c). Good correlation for the expression pattern displayed by ribulose-1,5-bisphosphate carboxylase/ oxygenase was achieved during all 3 experiments on this particular gene (figure 4.4d). Compared to uninfested controls, all treatments displayed significant down-regulation, except for the RWA-US2 infested plants which showed no change in expression level. Positive correlation in the general trends of expression patterns observed across all different experiments, support conclusions drawn from the initial cDNA-AFLP fingerprinting experiment.

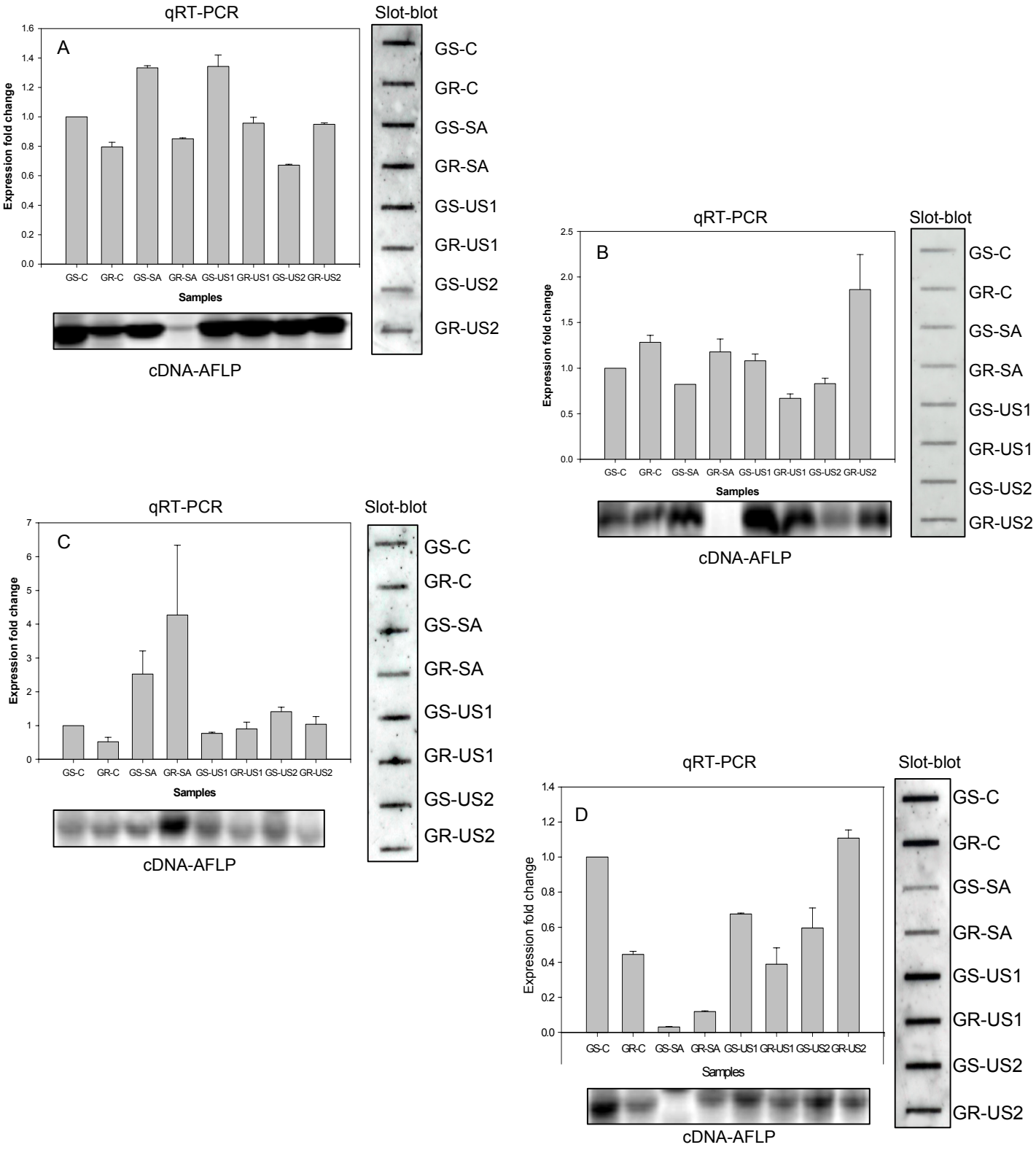

Figure 4.5:

Comparisons between qRT-PCR, cDNA-AFLP and slot blot results. Bar graphs (qRT-PCR data) and corresponding cDNA-AFLP fingerprints (below each graph) are indicated with treatment conditions. Corresponding slot blots are shown to the right of each graph with corresponding treatment conditions. A= Putative ABC-transporter; B= ATP-dependent Clp protease – proteolytic sub-unit; C= WRKY transcription factor; D= Ribulose-1,5-bisphosphate carboxylase/oxygenase large sub-unit.

4.4 Discussion

Our limited understanding of the interaction between the RWA and its cereal host, particularly on the molecular level, severely hampers our ability to develop sustainable strategies for the control of this pest. Consequently, the goal of this experiment was to contribute to the larger endeavour of expanding our understanding of RWA-wheat interactions in particular, by identifying the molecular mechanisms by which the RWA resistance gene *Dn7*, activates resistance responses. The focus was on those mechanisms activated following infestation with three different RWA biotypes. This approach also addresses the issue of how conserved or specific the response activated by *Dn7* is in reaction to different aphid elicitors.

When applying cDNA-AFLP technology as the method of choice for analyzing the transcriptome of any given organism, Ranik et al. (2006) raised two important issues to consider, being that of genomic DNA and rRNA contamination. Both these molecules are likely to produce false positive results, and it is therefore important that they be removed from the cDNA samples prior to continuation of the experiment (Ranik et al. 2006). One main advantage of this technique on the other hand, is that expression profiles of any gene can be identified, with the main criterion being the presence of suitable restriction sites in such transcripts to be analyzed (Durrant et al. 2000). This advantage negates the necessity of having existing sequence information at one's disposal, where the application of micro-arrays for example, are limited to that of previously cloned genes (Kehoe et al. 1999).

4.4.1 Potential roles of differentially expressed transcripts in RWA defence

In addition to cluster analysis, a number of transcripts showing differential expression were selected for identification by sequencing, in order to gain a more comprehensive understanding of the specific mechanisms involved in the *Dn7* mediated response. One particular gene identified twice in this study, is the ABC transporter (table 4.1). Despite genome sequence initiatives, like that of the *Arabidopsis* genome, the function of ABC transporters in plants is not clearly understood (Martinoia et al. 2002), with most thought to be involved in the intracellular binding of cytotoxins (Jasinkski et al. 2001). However PDR-like ABC transporters have been implicated in plant pathogen defence, particularly the PDR5-like family, where its increased expression has been linked to the increase of anti-fungal protein expression. It is likely that members of this family of ABC transporters play a role in this defence response, by being responsible for the excretion of secondary compounds from cells (Jasinski et al. 2001).

Another group of transcripts identified in this study that are of particular interest to defence, are those related to gene expression. Stress responses such as those induced by pathogen attack or wounding for example, have been shown to up-regulate a particular class of transcription factors, containing the WRKY domain (Chen et al. 2002). The function of MYB class transcription factors in plants is thought to be very diverse, amongst which is also that of pathogen defence (Yang and Klessig, 1996). Both of these classes of transcription factors have been identified in this study (table 4.1), supporting existing evidence for their involvement in plant defence. Evidence is continually accumulating for the presence of retrotransposons in all plant genomes, in some instances forming a large part thereof (Grandbastien, 1998). Identifying retro-elements as part of a defence response is also not uncommon. Research has shown that because retrotransposons have an RNA intermediate, they are subject to regulation – and are responsive to various biotic and abiotic stresses (Grandbastien 1998). The expression of well

studied retro-elements, such as *Tnt1A*, showed induction by numerous biotic stresses that can activate plant defence responses (Grandbastien 1998). For example, in a paper by Grandbastien et al. (1997), *Tnt1A* expression was shown to correlate strongly with the activation of early defence signalling cascades. Therefore, in studies where the identification of defence related genes is a key priority, the detection of certain retrotransposons may serve as a molecular indicator to further corroborate evidence for the activation of plant defence responses.

Proteins that have the Leucine-rich-repeat (LRR) motif have been well represented in this investigation. Although this structure is recognized to be involved in various forms of protein-protein interactions, all characterized resistance (*R*) genes important in plant defence are known to carry this motif (Jones and Takemoto 2004). Furthermore, Swanepoel et al. (2003) demonstrated close linkage between an NBS-LRR sequence and a RWA resistance gene. It is therefore possible that such genes activated in response to Russian wheat aphid infestation, may play a role in direct recognition of the aphid's eliciting agents (direct *R* gene – *avr* gene interaction) and thereby activate signalling cascades ultimately leading to resistance. Alternatively, such genes may mediate protein interactions on another level and play a more important role in downstream events, thereby contributing to resistance.

Various protein processing genes are expected to also be important during the programming of cellular responses, in order for the cellular environment to adapt to the eliciting signal originating from the Russian wheat aphid. Such genes may include various peptidases, protein transporters, proteases and phosphatases, all of which have been identified here. For instance, δ-COP (table 4.1) is a sub-unit coat protein that forms an integral part of protein transport vesicles, responsible for mediating such transport from the endoplasmic reticulum and Golgi network (Stenbeck et al. 1993). Hydroxymethyl-transferases are catalysts involved in a number of single carbon transfer reactions – like amino acid metabolism (Kastanos et al. 1997). Phosphoinositide phosphatases alternatively, are integral to lipid-

based signalling mechanisms (Schorr et al. 2001). The potential role of this enzyme during defence should not be underestimated, as signal transduction plays a very central role in plant defence. The identification of transcripts related to photosynthesis (Ribulose-1,5-bisphosphate carboxylase/ oxygenase, Rubisco), also highlights the importance of the photosynthetic machinery in defence against the aphid. As discussed by Botha et al. (2005), it is an important trait of resistant wheat varieties, to be able to overcome infestation by preserving its photosynthetic processes.

4.4.2 Comparison of expression patterns following different RWA biotype infestation

When considering the expression profile in figure 4.1, it is evident that the transcripts activated by *Dn7* in response to infestation with the South African biotype are distinctly different from those infested with either of the US biotypes. This trend is also observed in the cluster image (figure 4.2), which consolidates the data of all transcripts analysed. Figure 4.3 gives a graphical representation of the expression trends observed in the cluster image, and more clearly shows that the plants infested with the South African biotype aphid underwent the most significant changes in gene transcription. This is clear across all 6 clusters, as defined in figure 4.2. For example, figure 4.3 a and b illustrates the increase of transcript expression levels in the resistant plant infested with the SA biotype aphid relative to other treatment conditions. In different clusters (as illustrated in figures 4.3 c, g, and i) the same treatment condition shows a substantial decrease in expression levels relative to other treatments. The sum of all these observed trends therefore suggest that the mechanisms related to plant defence activated or induced by *Dn7*, is distinctly different for the South African biotype infested plants when compared to the two US biotype infested plants.

After comparing the US biotype infested plants to each other (RWA-US1 and RWA-US2), it is apparent that the expression profiles, and accordingly the defence responses to these biotypes, differ

substantially less. This is again evident in figure 4.3, where the observed expression trends across all US infested plants showed very little variation between each other. This result addresses the question of how conserved this gene's activated defences are against different response eliciting agents. From this, it is evident that *Dn7* interacts in a highly specific manner with each RWA biotype, which in turn leads to the activation of the appropriate defence pathways, unique to that particular interaction. The limited response variation detected between the two different US aphid biotype infested plants may contradict this statement, but the possible reason for this conservancy may be interpreted in favour thereof. Li et al. (unpublished results) illustrated very little molecular variation between the eight known US biotype aphids, and it is therefore conceivable that the conserved responses to the US biotypes are indeed due to their relatedness. Such conclusions are supported by the finding that 55.2% of differentially expressed transcripts identified in this study belong to plants infested with the South African aphid biotype.

From the presentation of data in table 4.3, the majority of differentially expressed transcripts identified here, show up-regulation in RWA-SA infested plants – demonstrating the extensive cellular reprogramming taking place in RWA defence. Genes activated in response to this treatment condition, mainly belong to the protein metabolism/modification class, as well as the group of transcripts related to gene expression (see table 4.1). A number of transcripts also related to protein modification have been identified as differentially expressed in RWA-US1 and RWA-US2 infested plants (table 4.3). The increased expression of different transcripts between RWA-SA and RWA-US infested plants, belonging to the same functional group, may suggest the activation of related defence strategies, but via divergent pathways. Further differences in defence strategies between RWA-SA and RWA-US 1 and 2 plants become evident when considering the transcripts uniquely expressed in response to each biotype. Transcripts identified in response to RWA-US2 infestation for example, mainly relate to

transcription, signifying an important role for gene expression in the activation of defence to this biotype.

The use of qRT-PCR and slot blot analysis basically served as a confirmation of these cDNA-AFLP results and thus the conclusions drawn from it. The sensitivity of qRT-PCR is well illustrated in figure 4.5 (a), where subtle differences are identified in the qRT-PCR results, which could not be identified in the cDNA-AFLP fingerprint. Considering figure 4.5 in general, similar trends between the different methods employed do emerge, confirming the validity of the cDNA-AFLP data. This is noticeable when considering figure 4.5 b and d, where very good correlation can be seen between qRT-PCR, cDNA-AFLP fingerprints and slot blots. Small discrepancies do however exist, such as the absence of bands in the cDNA-AFLP fingerprint (figure 4.5 a and b). This can possibly be the result of failed PCRs for that particular product. Such cases support the importance of performing additional analyses on samples, in order to gain a better picture of the changes that do occur on the level of gene expression.

4.4.3 Conclusions

In conclusion, findings show that the *Dn7* resistance gene responds in a highly specific manner to infestation with different RWA biotypes. Thus, the recognition of each biotype by *Dn7*, and the activation of subsequent defence responses are events unique to each interaction. This is exemplified in a study where it was determined that different RWA biotypes (from SA and USA), in fact have different eliciting agents. These differing eliciting agents are therefore expected to induce defence pathways unique to those elicitors (van Zyl, 2007). The sequencing of a number of transcripts gives us a closer view as to the exact molecular pathways likely to be involved. The fact that *Dn7* has shown to confer resistance to all three biotypes, conforms to existing hypotheses for the methods by which

recognition of the aphid elicitor takes place. The gene-for-gene model of recognition may be applicable to *Dn7*, where the binding of different aphid elicitors take place on multiple binding sites of the *Dn7* protein. It is however also possible that *Dn7* conforms to the guard hypothesis. Thereby, *Dn7* potentially acts as a guard protein, monitoring interactions between elicitors and bait proteins. Detection of these interactions prompts *Dn7* to activate the defence response (A-M. Botha, N.L.V. Lapitan; Pers. Comm.). Finally, conclusions can also be drawn regarding the different RWA biotypes. Because of the distinctive responses activated by *Dn7* during infestation with the South African biotype of RWA, it suggests that this biotype is molecularly distinct from the two US biotypes – or at the very least, that the response eliciting compounds are very different between different aphid biotypes. This theory is supported by Li et al. (unpublished results) where the differences between eight US aphid biotypes were found to be very small (~ 0.13%), whereas the South African biotypes seem to differ from US biotypes by 2 to 7%. Considering this in terms of total genome size, this difference could be quite substantial on DNA and amino acid level. Potential avenues to be pursued in future would be an investigation as to what genes are indispensable to *Dn7* mediated resistance. Such experiments could be conducted with the aid of RNA interference (RNAi) based methods. Long term research goals on this resistance gene in particular, would include mapping and subsequent map based cloning. Sequence information of this gene will definitely aid in clarifying its mode of action, which is currently only theorised. Research of this nature could contribute to the further enhancement and development of RWA resistant cultivars.

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Chapter 5

Summary

The Russian wheat aphid (*Diuraphis noxia*, RWA), is a serious pest in most wheat producing countries around the world. Infestation of wheat fields by this pest has a severe economic impact, as a result of heavy losses in crop yield. Because of the importance of wheat as a food source and its ever growing supply demand, the study of wheat-Russian wheat aphid interactions on the molecular level are integral to the development of management strategies. This is highlighted by the fact that new RWA biotypes that overcome resistance in a number of wheat varieties, continually emerge. Therefore, this study aims to contribute to this endeavour, by elucidating the molecular mechanisms by which the RWA resistance gene *Dn7* confers resistance to three different RWA biotypes (one from SA, and two from the USA).

Firstly, suppression subtractive hybridization (SSH) was applied in order to isolate transcripts differentially expressed in the RWA resistant wheat line, 94M370, carrying the *Dn7* gene. There are two main advantages to this technique. One is that the relative representation of rare transcripts is increased in the subsequent cDNA population, and it is these low abundance transcripts that are arguably the ones of particular interest. Secondly, this method allows for the isolation of unknown transcripts, without the need for existing sequence information. Experiments with this method however, failed, leading to an investigation as to probable causes. The various steps involved in the SSH procedure were individually assessed in an attempt to identify and correct the problem. Various adjustments were made to PCR procedures, template enzyme digestions and ligation reactions, without success. After creating a basic cDNA-AFLP fingerprint from the existing cDNA template, in order to confirm that the template is not responsible for experimental difficulties – it was decided to apply a different strategy in order to meet research objectives.

Consequently, the study on *Dn7* mediated defence responses was continued with cDNA-AFLP. In addition to studying the response by *Dn7* to South African biotype RWA infestation, its responses to

infestation by two United States RWA biotypes was also explored. This allowed us to gain a greater comprehension of the methods by which *Dn7* activates defences against different aphid eliciting agents. Findings suggest that this gene activates responses that are unique to each of the different aphid interactions. Although the interactions between *Dn7* and the two US biotypes were very similar, this can possibly be explained by the fact that the differences between these two biotypes on molecular level are minuscule. *Dn7* responds to the South African biotype of the RWA in a completely different manner, as judged by the very dissimilar expression patterns obtained during cDNA-AFLP analysis. Reasons for this phenomenon could include molecular differences between the South African and US RWA biotypes, differences in response generating elicitor molecules (which has indeed been shown to be the case between South African and US aphid biotypes), or a combination of both. The sequencing of fragments displaying differential expression patterns during cDNA-AFLP fingerprinting, provides us with additional information as to the exact mechanisms potentially involved. As expected, various compounds related to plant defence were identified, such as a number of Leucine rich repeat (LRR) domain containing proteins, genes related to cell signalling and genes involved in protein processing (proteases, peptidases). Finally, these results are consistent with theories that *Dn7* may recognise and interact with its distinct aphid elicitors either directly, by the presence of multiple bindings sites on the same protein, or indirectly. In that case, in accordance with the guard hypothesis, *Dn7* may simply monitor interactions between aphid elicitors and other recognition factors- after which a response cascade is activated. Useful potential research would focus on *Dn7* itself, including mapping, isolation as well as structural and functional characterization.

Appendix A

Sequences submitted to Genbank (dbEST)

```
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DEFINITION  CG2007_018 cDNA-AFLP of wheat 94M370 in response to Diuraphis noxia
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VERSION     EL763491.1  GI:129309976
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
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            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 472)
  AUTHORS   Zaayman,D. and Botha-Oberholster,A.M.
  TITLE     Elucidating Diuraphis noxia biotype specific responses in Triticum
            aestivum (94M370, Dn7)
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
            Institute
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            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3984
            Fax: 27 12 420 3947
            Email: anna.oberholster@up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
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      241 agggtcagta atgagctaga aactaactct aaactctagc taagttgagt atccaaacat
      301 gtctgtgttg tttttgttgt tgctgctgct gctcttctac gtatatctag acatcattag
      361 aacatttatg taacaccctt ccatgttgct atctcaccta gtattgcata tttagacatt
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  ORGANISM  Triticum aestivum
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            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 142)
  AUTHORS   Zaayman,D. and Botha-Oberholster,A.M.
  TITLE     Elucidating Diuraphis noxia biotype specific responses in Triticum
            aestivum (94M370, Dn7)
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
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            Faculty of Agriculture and Natural Science, University of Pretoria
            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3945
            Fax: 27 12 420 3947
            Email: ambothao@postino.up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
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REFERENCE   1  (bases 1 to 431)
  AUTHORS   Zaayman,D. and Botha-Oberholster,A.M.
  TITLE     Elucidating Diuraphis noxia biotype specific responses in Triticum
            aestivum (94M370, Dn7)
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
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            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3945
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            Email: ambothao@postino.up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
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REFERENCE   1  (bases 1 to 294)
  AUTHORS   Zaayman,D. and Botha-Oberholster,A.M.
  TITLE     Elucidating Diuraphis noxia biotype specific responses in Triticum
            aestivum (94M370, Dn7)
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
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            Faculty of Agriculture and Natural Science, University of Pretoria
            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3945
            Fax: 27 12 420 3947
            Email: ambothao@postino.up.ac.za
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  AUTHORS   Zaayman,D. and Botha-Oberholster,A.M.
  TITLE     Elucidating Diuraphis noxia biotype specific responses in Triticum
            aestivum (94M370, Dn7)
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
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            Faculty of Agriculture and Natural Science, University of Pretoria
            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3945
            Fax: 27 12 420 3947
            Email: ambothao@postino.up.ac.za
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                     aphid (Diuraphis noxia)"
ORIGIN
        1 gaagaacatc accacaccac acacacaaga gaatttacac atggagcgta cacaaagcac
       61 acacatacac ccatcactcg ctagcatgca gcgagtagac cgaggcaaga aaagaaagtt
      121 acaagcacta gtcgataagt agtacgaagc tcaagacaaa ctacatgcag cctcagacag
      181 caacgtacgc tatgcaggat tagataacca aaagagcagc aaacatggcg gcgtcgcaga
      241 acttcctgtc
```

```
LOCUS       EL763496                447 bp    mRNA    linear   EST 12-MAR-2007
DEFINITION  CG2007_023 cDNA-AFLP of wheat 94M370 in response to Diuraphis noxia
            Triticum aestivum cDNA, mRNA sequence.
ACCESSION   EL763496
VERSION     EL763496.1  GI:129309986
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 447)
  AUTHORS   Zaayman,D. and Botha-Oberholster,A.M.
  TITLE     Elucidating Diuraphis noxia biotype specific responses in Triticum
            aestivum (94M370, Dn7)
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
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            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3984
            Fax: 27 12 420 3947
            Email: anna.oberholster@up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
FEATURES             Location/Qualifiers
     source          1..447
                     /organism="Triticum aestivum"
                     /mol_type="mRNA"
                     /cultivar="94M370"
                     /db_xref="taxon:4565"
                     /dev_stage="2-3 leaf stage"
                     /lab_host="Eschericia coli (DH5a)"
                     /clone_lib="cDNA-AFLP of wheat 94M370 in response to
                     Diuraphis noxia"
                     /note="Organ: leaf; Vector: pTZ57R/T; cDNA-AFLP of wheat
                     mRNA in response to infestation with the Russian wheat
                     aphid (Diuraphis noxia)"
ORIGIN
        1 tgacaagatg aatacaagat ttatacacaa catgaataca aaatgaatac aagatgaata
       61 caagatttat acacatggat gaatagacaa ctcaacaaat gaaccaacca tcatcctatg
      121 aacaaaactc aacatacaac aaatggacac cttttgtcga acaacggagg gaggatcatg
      181 gaccaactcc acgaagttgc aacaccacaa cctgtaattc caccgattgt aaatttttta
      241 ggcaaaaccc aagtatagta gcactcgcat ggcaaaaaag ctagtaaaac ctagtaaaca
      301 taagtatatg ctagtaaagt ataccaatca ctttctacaa tagacctaat gattgacacg
      361 gcaaaaaaac aacattacta agtttgccac aagattacta aagtttgcca caactttgcc
      421 atataaggtc aaaagtttgc cacaaca
```

```
LOCUS       EL763497                175 bp    mRNA    linear   EST 12-MAR-2007
DEFINITION  CG2007_024 cDNA-AFLP of wheat 94M370 in response to Diuraphis noxia
            Triticum aestivum cDNA, mRNA sequence.
ACCESSION   EL763497
VERSION     EL763497.1  GI:129309988
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 175)
  AUTHORS   Zaayman,D. and Botha-Oberholster,A.M.
  TITLE     Elucidating Diuraphis noxia biotype specific responses in Triticum
            aestivum (94M370, Dn7)
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
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            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3984
            Fax: 27 12 420 3947
            Email: anna.oberholster@up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
FEATURES             Location/Qualifiers
     source          1..175
                     /organism="Triticum aestivum"
                     /mol_type="mRNA"
                     /cultivar="94M370"
                     /db_xref="taxon:4565"
                     /dev_stage="2-3 leaf stage"
                     /lab_host="Eschericia coli (DH5a)"
                     /clone_lib="cDNA-AFLP of wheat 94M370 in response to
                     Diuraphis noxia"
                     /note="Organ: leaf; Vector: pTZ57R/T; cDNA-AFLP of wheat
                     mRNA in response to infestation with the Russian wheat
                     aphid (Diuraphis noxia)"
ORIGIN
        1 tgcaatagga agaggttgan caagatacta tggcagaaga aagtatggaa caagaacagg
       61 aggaaatggt ggaggaggga gagcaagcca tgtttataac tgcccatgca ataggtcaac
      121 aaattgcagt tccaactcca atctgtgaca attcacatta cctcaggact catca
```

```
LOCUS       EL763498                 332 bp    mRNA    linear   EST 12-MAR-2007
DEFINITION  CG2007_025 cDNA-AFLP of wheat 94M370 in response to Diuraphis noxia
            Triticum aestivum cDNA, mRNA sequence.
ACCESSION   EL763498
VERSION     EL763498.1  GI:129309990
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 332)
  AUTHORS   Zaayman,D. and Botha-Oberholster,A.M.
  TITLE     Elucidating Diuraphis noxia biotype specific responses in Triticum
            aestivum (94M370, Dn7)
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
            Institute
            Faculty of Agriculture and Natural Science, University of Pretoria
            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3984
            Fax: 27 12 420 3947
            Email: anna.oberholster@up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
FEATURES             Location/Qualifiers
     source          1..332
                     /organism="Triticum aestivum"
                     /mol_type="mRNA"
                     /cultivar="94M370"
                     /db_xref="taxon:4565"
                     /dev_stage="2-3 leaf stage"
                     /lab_host="Eschericia coli (DH5a)"
                     /clone_lib="cDNA-AFLP of wheat 94M370 in response to
                     Diuraphis noxia"
                     /note="Organ: leaf; Vector: pTZ57R/T; cDNA-AFLP of wheat
                     mRNA in response to infestation with the Russian wheat
                     aphid (Diuraphis noxia)"
ORIGIN
        1 ttggatgagt cctggagnta aaggtataag actatgcctt ttttgcttgt gcaaataaag
       61 gaaagaaata tatgcccagn ttggtgtcgc ctagctaatt gcctatgtat ccactctctt
      121 agctggaaca atcttgccta tgttcacact caagtctatc ccccaatgat ttatttttgag
      181 ataaattgtt gcctttataa ccaagcctta tatttcaaag cttccctact ttttataga
      241 atttgtattt ccagcattcc agtgctcttg aaacaatggg ctgattgttt tgggggtttt
      301 gcccattcta catttgccca taaatgtaac ct
```

```
LOCUS       EL763499                 173 bp    mRNA    linear   EST 12-MAR-2007
DEFINITION  CG2007_026 cDNA-AFLP of wheat 94M370 in response to Diuraphis noxia
            Triticum aestivum cDNA, mRNA sequence.
ACCESSION   EL763499
VERSION     EL763499.1  GI:129309992
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 173)
  AUTHORS   Zaayman,D. and Botha-Oberholster,A.M.
  TITLE     Elucidating Diuraphis noxia biotype specific responses in Triticum
            aestivum (94M370, Dn7)
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
            Institute
            Faculty of Agriculture and Natural Science, University of Pretoria
            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3945
            Fax: 27 12 420 3947
            Email: ambothao@postino.up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
FEATURES             Location/Qualifiers
     source          1..173
                     /organism="Triticum aestivum"
                     /mol_type="mRNA"
                     /cultivar="94M370"
                     /db_xref="taxon:4565"
                     /dev_stage="2-3 leaf stage"
                     /lab_host="Eschericia coli (DH5a)"
                     /clone_lib="cDNA-AFLP of wheat 94M370 in response to
                     Diuraphis noxia"
                     /note="Organ: leaf; Vector: pTZ57R/T; cDNA-AFLP of wheat
                     mRNA in response to infestation with the Russian wheat
                     aphid (Diuraphis noxia)"
ORIGIN
        1 tgcaatagga agaggttgaa caagatacta tggcagaaga aagtatggaa caagaacagg
       61 aggaaatggt ggaggaggga gagcaagcca tgtttataac tgcccatgca ataggtcaac
      121 aaattgcagt tccaactcca actgtgacaa ttcacattac tcaggactca tca
```

```
LOCUS       EL763500                352 bp    mRNA    linear   EST 12-MAR-2007
DEFINITION  CG2007_027 cDNA-AFLP of wheat 94M370 in response to Diuraphis noxia
            Triticum aestivum cDNA, mRNA sequence.
ACCESSION   EL763500
VERSION     EL763500.1  GI:129309994
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 352)
  AUTHORS   Zaayman,D. and Botha-Oberholster,A.M.
  TITLE     Elucidating Diuraphis noxia biotype specific responses in Triticum
            aestivum (94M370, Dn7)
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
            Institute
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            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3984
            Fax: 27 12 420 3947
            Email: anna.oberholster@up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
FEATURES             Location/Qualifiers
     source          1..352
                     /organism="Triticum aestivum"
                     /mol_type="mRNA"
                     /cultivar="94M370"
                     /db_xref="taxon:4565"
                     /dev_stage="2-3 leaf stage"
                     /lab_host="Eschericia coli (DH5a)"
                     /clone_lib="cDNA-AFLP of wheat 94M370 in response to
                     Diuraphis noxia"
                     /note="Organ: leaf; Vector: pTZ57R/T; cDNA-AFLP of wheat
                     mRNA in response to infestation with the Russian wheat
                     aphid (Diuraphis noxia)"
ORIGIN
        1 aggttgtgat ttttgtattt cacagtgata gattttacac tatactgtta cctgaattta
       61 cagtgctaca ttttttacta gaagaactat ttgaaggaga ttgtgagagt actgacttac
      121 agtttgtctt cttggtgtga ctgtagtttc ctgattgtgt agctattctg tccccttctt
      181 cctccatcct ctttcttgtt cttatctgat cataaagcac acattgttac tggttgtctc
      241 tccttatttt tactgaagtt ccagtatctt tgttgtgatc tggttgttac tgtctggaat
      301 tttagtaaaa tgaacactgt aaaattcatc aaagctgaat gtgtgataca ct
```

```
LOCUS       EL763501                290 bp    mRNA    linear   EST 12-MAR-2007
DEFINITION  CG2007_028 cDNA-AFLP of wheat 94M370 in response to Diuraphis noxia
            Triticum aestivum cDNA, mRNA sequence.
ACCESSION   EL763501
VERSION     EL763501.1  GI:129309996
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 290)
  AUTHORS   Zaayman,D. and Botha-Oberholster,A.M.
  TITLE     Elucidating Diuraphis noxia biotype specific responses in Triticum
            aestivum (94M370, Dn7)
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
            Institute
            Faculty of Agriculture and Natural Science, University of Pretoria
            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3945
            Fax: 27 12 420 3947
            Email: ambothao@postino.up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
FEATURES             Location/Qualifiers
     source          1..290
                     /organism="Triticum aestivum"
                     /mol_type="mRNA"
                     /cultivar="94M370"
                     /db_xref="taxon:4565"
                     /dev_stage="2-3 leaf stage"
                     /lab_host="Eschericia coli (DH5a)"
                     /clone_lib="cDNA-AFLP of wheat 94M370 in response to
                     Diuraphis noxia"
                     /note="Organ: leaf; Vector: pTZ57R/T; cDNA-AFLP of wheat
                     mRNA in response to infestation with the Russian wheat
                     aphid (Diuraphis noxia)"
ORIGIN
        1 acgaaagtat gaaactactg aactattgaa ataccaaaca cactgtccaa ctaaatcatg
       61 taaagactaa aaattgaaaa aagcataaat tagactaact aacaaagtgg aaaagtctga
      121 tctatgaaag agataaacta tcaagaatca taggggtcta ttgagatgga ctaaaatact
      181 ggcaaatgat gcaagcacaa tgactaaata aaggaacaaa aaactgaagg aacgaagagg
      241 aaggaacaaa aactaaacca aatcaataaa ctgaatgtga catgagtggt
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LOCUS       EL763502                 173 bp    mRNA    linear   EST 12-MAR-2007
DEFINITION  CG2007_029 cDNA-AFLP of wheat 94M370 in response to Diuraphis noxia
            Triticum aestivum cDNA, mRNA sequence.
ACCESSION   EL763502
VERSION     EL763502.1  GI:129309998
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 173)
  AUTHORS   Zaayman,D. and Botha-Oberholster,A.M.
  TITLE     Elucidating Diuraphis noxia biotype specific responses in Triticum
            aestivum (94M370, Dn7)
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
            Institute
            Faculty of Agriculture and Natural Science, University of Pretoria
            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3984
            Fax: 27 12 420 3947
            Email: anna.oberholster@up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
FEATURES             Location/Qualifiers
     source          1..173
                     /organism="Triticum aestivum"
                     /mol_type="mRNA"
                     /cultivar="94M370"
                     /db_xref="taxon:4565"
                     /dev_stage="2-3 leaf stage"
                     /lab_host="Eschericia coli (DH5a)"
                     /clone_lib="cDNA-AFLP of wheat 94M370 in response to
                     Diuraphis noxia"
                     /note="Organ: leaf; Vector: pTZ57R/T; cDNA-AFLP of wheat
                     mRNA in response to infestation with the Russian wheat
                     aphid (Diuraphis noxia)"
ORIGIN
        1 acatcagcca tcacacggtg tgtgtgcgtg tgtgtgtgtg tgtgtgtgaa taatctaggt
       61 aaactacaaa attagccggg taaatgtagt ggaatacatg cttgtaaaca aacttgtcat
      121 aatttgtgat ctaaggttat tatttgacat tagcaatctg ggtaatttcc agt
```

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LOCUS       EL763503                206 bp    mRNA    linear   EST 12-MAR-2007
DEFINITION  CG2007_030 cDNA-AFLP of wheat 94M370 in response to Diuraphis noxia
            Triticum aestivum cDNA, mRNA sequence.
ACCESSION   EL763503
VERSION     EL763503.1  GI:129310000
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 206)
  AUTHORS   Zaayman,D. and Botha-Oberholster,A.M.
  TITLE     Elucidating Diuraphis noxia biotype specific responses in Triticum
            aestivum (94M370, Dn7)
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
            Institute
            Faculty of Agriculture and Natural Science, University of Pretoria
            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3984
            Fax: 27 12 420 3947
            Email: anna.oberholster@up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
FEATURES             Location/Qualifiers
     source          1..206
                     /organism="Triticum aestivum"
                     /mol_type="mRNA"
                     /cultivar="94M370"
                     /db_xref="taxon:4565"
                     /dev_stage="2-3 leaf stage"
                     /lab_host="Eschericia coli (DH5a)"
                     /clone_lib="cDNA-AFLP of wheat 94M370 in response to
                     Diuraphis noxia"
                     /note="Organ: leaf; Vector: pTZ57R/T; cDNA-AFLP of wheat
                     mRNA in response to infestation with the Russian wheat
                     aphid (Diuraphis noxia)"
ORIGIN
        1 gtagactgcg taccgactat ggcgcagatt cgtctaacta gatgtcacca cgatcaatca
       61 agtcatcagg catttgctac caacttcaca ggcttgacca tccatccctt gtgataaata
      121 cacactgaga aacacgactg tgggggaaca ctgagaggtg cttaggtaca gttttttta
      181 gtttcacgga catccgacgt cggcag
```

```
LOCUS       EL763504                 213 bp    mRNA    linear   EST 12-MAR-2007
DEFINITION  CG2007_031 cDNA-AFLP of wheat 94M370 in response to Diuraphis noxia
            Triticum aestivum cDNA, mRNA sequence.
ACCESSION   EL763504
VERSION     EL763504.1  GI:129310002
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 213)
  AUTHORS   Zaayman,D. and Botha-Oberholster,A.M.
  TITLE     Elucidating Diuraphis noxia biotype specific responses in Triticum
            aestivum (94M370, Dn7)
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
            Institute
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            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3945
            Fax: 27 12 420 3947
            Email: ambothao@postino.up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
FEATURES             Location/Qualifiers
     source          1..213
                     /organism="Triticum aestivum"
                     /mol_type="mRNA"
                     /cultivar="94M370"
                     /db_xref="taxon:4565"
                     /dev_stage="2-3 leaf stage"
                     /lab_host="Eschericia coli (DH5a)"
                     /clone_lib="cDNA-AFLP of wheat 94M370 in response to
                     Diuraphis noxia"
                     /note="Organ: leaf; Vector: pTZ57R/T; cDNA-AFLP of wheat
                     mRNA in response to infestation with the Russian wheat
                     aphid (Diuraphis noxia)"
ORIGIN
        1 ctgttatgtc cattcataaa gtacttatta gattatgtga ataccagttt gggaaactag
       61 aacttgtcaa ctatgtcact ggcagggtcg cgcagcaatt agttcatgtc atataaaaca
      121 attgactaac tgtgacttag aaatttcaat acctaagaat attttggtca tggtatgttg
      181 gttagagttg aattgcatcg gtacgcagtc taa
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```
LOCUS       EL763505                207 bp    mRNA    linear   EST 12-MAR-2007
DEFINITION  CG2007_032 cDNA-AFLP of wheat 94M370 in response to Diuraphis noxia
            Triticum aestivum cDNA, mRNA sequence.
ACCESSION   EL763505
VERSION     EL763505.1  GI:129310004
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 207)
  AUTHORS   Zaayman,D. and Botha-Oberholster,A.M.
  TITLE     Elucidating Diuraphis noxia biotype specific responses in Triticum
            aestivum (94M370, Dn7)
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
            Institute
            Faculty of Agriculture and Natural Science, University of Pretoria
            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3945
            Fax: 27 12 420 3947
            Email: ambothao@postino.up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
FEATURES             Location/Qualifiers
     source          1..207
                     /organism="Triticum aestivum"
                     /mol_type="mRNA"
                     /cultivar="94M370"
                     /db_xref="taxon:4565"
                     /dev_stage="2-3 leaf stage"
                     /lab_host="Eschericia coli (DH5a)"
                     /clone_lib="cDNA-AFLP of wheat 94M370 in response to
                     Diuraphis noxia"
                     /note="Organ: leaf; Vector: pTZ57R/T; cDNA-AFLP of wheat
                     mRNA in response to infestation with the Russian wheat
                     aphid (Diuraphis noxia)"
ORIGIN
        1 cttgtctaaa gtgtgcatca ccttgaatca catcaaccag atcattcaag agattaggtt
       61 atactacgtg tcacctattt ctccacgcaa attgatatgg ctgtgcttag gtaggttact
      121 agacgcgttt cagattcttc ctggcaagtc tgtgttcaac aacaagcaag atggactttt
      181 ttttctagaa ctgaagaaca gcttcag
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```
LOCUS       EL763506                271 bp    mRNA    linear   EST 12-MAR-2007
DEFINITION  CG2007_033 cDNA-AFLP of wheat 94M370 in response to Diuraphis noxia
            Triticum aestivum cDNA, mRNA sequence.
ACCESSION   EL763506
VERSION     EL763506.1  GI:129310006
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 271)
  AUTHORS   Zaayman,D. and Botha-Oberholster,A.M.
  TITLE     Elucidating Diuraphis noxia biotype specific responses in Triticum
            aestivum (94M370, Dn7)
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
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            Tel: 27 12 420 3984
            Fax: 27 12 420 3947
            Email: anna.oberholster@up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
FEATURES             Location/Qualifiers
     source          1..271
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                     /mol_type="mRNA"
                     /cultivar="94M370"
                     /db_xref="taxon:4565"
                     /dev_stage="2-3 leaf stage"
                     /lab_host="Eschericia coli (DH5a)"
                     /clone_lib="cDNA-AFLP of wheat 94M370 in response to
                     Diuraphis noxia"
                     /note="Organ: leaf; Vector: pTZ57R/T; cDNA-AFLP of wheat
                     mRNA in response to infestation with the Russian wheat
                     aphid (Diuraphis noxia)"
ORIGIN
        1 tgtagcgcaa gtgcataccc ccacaccaat agcaacagat gcatgcacga ttcagaagtc
       61 atacaggagg aaagattacc ctcagagcaa agagggattt acagcaacaa agccaacagg
      121 caacattcaa gtcagcgtga acatccttgc agcaactttt gaacacgatt agctttcaca
      181 taatgctgga actgaaatca gttcaaccca acagctgaga cgcgacaaag agcctcagcc
      241 tgacaccatt ttcacaacaa tgataagaac a
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LOCUS       EL763507                 178 bp    mRNA    linear   EST 12-MAR-2007
DEFINITION  CG2007_034 cDNA-AFLP of wheat 94M370 in response to Diuraphis noxia
            Triticum aestivum cDNA, mRNA sequence.
ACCESSION   EL763507
VERSION     EL763507.1  GI:129310008
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 178)
  AUTHORS   Zaayman,D. and Botha-Oberholster,A.M.
  TITLE     Elucidating Diuraphis noxia biotype specific responses in Triticum
            aestivum (94M370, Dn7)
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
            Institute
            Faculty of Agriculture and Natural Science, University of Pretoria
            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3945
            Fax: 27 12 420 3947
            Email: ambothao@postino.up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
FEATURES             Location/Qualifiers
     source          1..178
                     /organism="Triticum aestivum"
                     /mol_type="mRNA"
                     /cultivar="94M370"
                     /db_xref="taxon:4565"
                     /dev_stage="2-3 leaf stage"
                     /lab_host="Eschericia coli (DH5a)"
                     /clone_lib="cDNA-AFLP of wheat 94M370 in response to
                     Diuraphis noxia"
                     /note="Organ: leaf; Vector: pTZ57R/T; cDNA-AFLP of wheat
                     mRNA in response to infestation with the Russian wheat
                     aphid (Diuraphis noxia)"
ORIGIN
        1 gtattactac aaaaatacaa aaatgaatgt tctaaagaaa ccagctcaag gaaaaataca
       61 tcatcctgct atcaggccta tcacttaccc tgtgttgggt ggaaagggaa agaaatatca
      121 acaaagtgct ttacaaccaa cagaacagga ctaggcagca acttactcag gactcatc
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LOCUS       EL763508                362 bp    mRNA    linear   EST 12-MAR-2007
DEFINITION  CG2007_035 cDNA-AFLP of wheat 94M370 in response to Diuraphis noxia
            Triticum aestivum cDNA, mRNA sequence.
ACCESSION   EL763508
VERSION     EL763508.1  GI:129310010
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 362)
  AUTHORS   Zaayman,D. and Botha-Oberholster,A.M.
  TITLE     Elucidating Diuraphis noxia biotype specific responses in Triticum
            aestivum (94M370, Dn7)
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
            Institute
            Faculty of Agriculture and Natural Science, University of Pretoria
            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3984
            Fax: 27 12 420 3947
            Email: anna.oberholster@up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
FEATURES             Location/Qualifiers
     source          1..362
                     /organism="Triticum aestivum"
                     /mol_type="mRNA"
                     /cultivar="94M370"
                     /db_xref="taxon:4565"
                     /dev_stage="2-3 leaf stage"
                     /lab_host="Eschericia coli (DH5a)"
                     /clone_lib="cDNA-AFLP of wheat 94M370 in response to
                     Diuraphis noxia"
                     /note="Organ: leaf; Vector: pTZ57R/T; cDNA-AFLP of wheat
                     mRNA in response to infestation with the Russian wheat
                     aphid (Diuraphis noxia)"
ORIGIN
        1 gtgttttttc tatagcaact aaatattgtg aaagtaaccc tgaaacatac acacatctac
       61 taaatgcaaa attgtttctc tgttggtacg aacaatcatg tagattactg catgtagttt
      121 cgttttgcac acaacatact ctccagtagt gccgaaatta gaatctcgat ccctgtccat
      181 tatacaagat gcaaaagagt tcagaataag atattcatca tcatattcac gcattttccg
      241 ttgtaatata tattttgtat ttgtgtacag gttgacacat tacacataca tacagtatgt
      301 ctcacttatg cagaagaaaa gggaccctta tataaattat ttgcatgctc atagttatga
      361 ac
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LOCUS       EL763509                211 bp    mRNA    linear   EST 12-MAR-2007
DEFINITION  CG2007_036 cDNA-AFLP of wheat 94M370 in response to Diuraphis noxia
            Triticum aestivum cDNA, mRNA sequence.
ACCESSION   EL763509
VERSION     EL763509.1  GI:129310012
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 211)
  AUTHORS   Zaayman,D. and Botha-Oberholster,A.M.
  TITLE     Elucidating Diuraphis noxia biotype specific responses in Triticum
aestivum (94M370, Dn7)

  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
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            Institute
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            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3984
            Fax: 27 12 420 3947
            Email: anna.oberholster@up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
FEATURES             Location/Qualifiers
     source          1..211
                     /organism="Triticum aestivum"
                     /mol_type="mRNA"
                     /cultivar="94M370"
                     /db_xref="taxon:4565"
                     /dev_stage="2-3 leaf stage"
                     /lab_host="Eschericia coli (DH5a)"
                     /clone_lib="cDNA-AFLP of wheat 94M370 in response to
                     Diuraphis noxia"
                     /note="Organ: leaf; Vector: pTZ57R/T; cDNA-AFLP of wheat
                     mRNA in response to infestation with the Russian wheat
                     aphid (Diuraphis noxia)"
ORIGIN
        1 gttttcacta gtatctcaag tatgcaatgc ataggctgcc ttgtacatgt ttataagttg
       61 catgccagtg ctcttatcct cgtgaacttt tttgtctgta tcttagatcc tatgtcttgt
      121 cgttttccgg ttattccttg ctatgacacc aaagtacttc tgctctgatc tagccaacgc
      181 catgatcaat tatgtaataa atagtttgga c
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LOCUS       EL763510                311 bp    mRNA    linear   EST 12-MAR-2007
DEFINITION  CG2007_037 cDNA-AFLP of wheat 94M370 in response to Diuraphis noxia
            Triticum aestivum cDNA, mRNA sequence.
ACCESSION   EL763510
VERSION     EL763510.1  GI:129310014
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 311)
  AUTHORS   Zaayman,D. and Botha-Oberholster,A.M.
  TITLE     Elucidating Diuraphis noxia biotype specific responses in Triticum
            aestivum (94M370, Dn7)
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
            Institute
            Faculty of Agriculture and Natural Science, University of Pretoria
            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3945
            Fax: 27 12 420 3947
            Email: ambothao@postino.up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
FEATURES             Location/Qualifiers
     source          1..311
                     /organism="Triticum aestivum"
                     /mol_type="mRNA"
                     /cultivar="94M370"
                     /db_xref="taxon:4565"
                     /dev_stage="2-3 leaf stage"
                     /lab_host="Eschericia coli (DH5a)"
                     /clone_lib="cDNA-AFLP of wheat 94M370 in response to
                     Diuraphis noxia"
                     /note="Organ: leaf; Vector: pTZ57R/T; cDNA-AFLP of wheat
                     mRNA in response to infestation with the Russian wheat
                     aphid (Diuraphis noxia)"
ORIGIN
        1 gtatgtgtag tatgcttcag atcagatcca aaatcaggat acatgacagc tgcaaagcaa
       61 atatatcaaa gttgctcggg tggatctgtt gtggttcaag ctcctcaagt ggatctaatc
      121 aagactactt accgaccatc ttccatttga actgcagcaa tgtggggtgg cgcatgtgga
      181 gtaggagggt agaagagaag atggagaagg ttattctggc cttacgttga gtggtgagtc
      241 tggggtatac gaaaatactt gagttgaaga atacccggta ctggatataa atgattgagg
      301 agacgcatga c
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