

**Cloning of genes involved in the early response of
wheat towards Russian wheat aphid infestation**

By

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"In three words I can sum up everything I've learned about life: it goes on."

-Robert Frost

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List of Abbreviations

A

| | |
|-----|---------------------------------------|
| ACC | 1-aminocyclopropane-1-carboxylic acid |
| APS | Ammonium peroxodisulfate |
| Avr | Avirulence genes |
| ATP | Adenosine triphosphate |

B

| | |
|-----|----------------------|
| BSA | Bovine serum albumin |
| BTH | Benzothiadiazole |

C

| | |
|-----|------------------------------|
| CRP | Conservation reserve program |
|-----|------------------------------|

D

| | |
|-----------|--|
| DD RT-PCR | Differential display reverse transcription PCR |
| DTE | Dithioerythritol |
| DTT | Dithiothreitol |
| DMSO | Dimethylsulfoxide |
| DMPC | Dimethyl pyrocarbonate |
| DN | <i>Diuraphis noxia</i> |
| dCTP | Deoxycytidine triphosphate |
| dNTP | Deoxynucleotide triphosphate |

E

| | |
|------|-----------------------------------|
| EGF | Epidermal growth factor |
| EDTA | Ethylenedinitrilotetraacetic acid |
| ET | Ethylene |
| EtBr | Ethidium bromide |

H

| | |
|-------------------------------|-------------------------|
| H ₂ O ₂ | Hydrogen peroxide |
| h.p.i. | Hours post infection |
| HR | Hypersensitive response |

I

| | |
|------|-------------------------------------|
| IGL | Indole-3-glycerol phosphate lyase |
| INA | 2,2-dichloroisonicotinic acid |
| IPTG | Isopropyl β-D-thiogalactopyranoside |

J

| | |
|----|---------------|
| JA | Jasmonic acid |
|----|---------------|

L

| | |
|-----|-------------------------------|
| LAR | Localized acquired resistance |
| LRR | Leucine-rich repeat |

M

| | |
|--------|--|
| MAPK | Mitogen-activated protein kinase |
| MAPKK | Mitogen-activated protein kinase kinase |
| MAPKKK | Mitogen-activated protein kinase kinase kinase |
| MeJA | Methyl jasmonate |

N

| | |
|-------------|---------------------------------------|
| NBS | Nucleotide binding site |
| Nonidet P40 | Octylphenolpoly (ethyleneglycolether) |

O

| | |
|------------------------------|--------------------|
| O ₂ ^{·-} | Hydroxyl radical |
| OH [·] | Superoxide radical |
| ORF | Open reading frame |

P

| | |
|----------|---------------------------|
| PCR | Polymerase chain reaction |
| PEG 4000 | Polyethylene glycol 4000 |
| PR | Pathogenesis-related |
| PVP | Polyvinylpyrrolidone |

R

| | |
|----------------|----------------------------------|
| RACE | Rapid amplification of cDNA ends |
| RLK | Receptor-like protein kinase |
| RPK | Receptor protein kinase |
| RT | Reverse transcription |
| RT-PCR | Reverse transcription PCR |
| ROI | Reactive oxygen intermediates |
| ROS | Reactive oxygen species |
| <i>R</i> genes | Resistance genes |
| RWA | Russian wheat aphid |

S

| | |
|-----|--|
| SA | Salicylic acid |
| SAR | Systemic acquired resistance |
| SDS | Sodium dodecyl sulfate |
| SLG | Self-incompatibility-locus glycoproteins |
| SH2 | Src homology 2 |

T

| | |
|-----------|---|
| TEMED | N, N, N', N'-tetramethylethylenediamine |
| Tris | Tris (hydroxymethyl)-aminomethane |
| Tween™ 20 | Polyoxyethylene sorbitan monolaurate |
| TNFR | Tumor necrosis factor receptor |

U

| | |
|----|-------------|
| UV | Ultraviolet |
|----|-------------|

W

WAKs Wall-associated receptor kinases

WIPK Wound-induced protein kinase

X

X-Gal 5-bromo-4-chloro-3-indolyl β -D-galactoside

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Chapter

1

Introduction

1.1. Introduction

Wheat is one of the most extensively grown economic important small grain crop and is believed to have originated from South Western Asia (Feldman, 2001). The first wheat was planted in South Africa by Jan van Riebeeck in 1652 and by 1684 wheat production was well established (van Niekerk, 2001).

Pests are responsible for major yield losses in wheat production world wide (Narayanan, 2004). The Russian wheat aphid is a major pest of wheat (Du Toit and Walters, 1984). The aphid injects saliva containing a possible toxin into the host plant while feeding (Miles, 1999) and consequently the leaves develop streaks and the plant wilts (Du Toit, 1986; Walters *et al.*, 1980; Miles, 1999).

Plants under attack by different pathogens and pests activate a range of mechanisms to combat them. These include physical barriers (León *et al.*, 2001) and biochemical defences (Pieterse *et al.*, 2001). The most common resistance response of plants towards invading pathogens is the induction of the hypersensitive response (Keen, 1990). This response is characterized by rapid localized cell death at the site of infection that is mediated by elevated levels of reactive oxygen species (Lam, 2001). In addition, various signalling molecules such as salicylic acid, jasmonic acid and ethylene are also produced.

As part of the hypersensitive response, the expression of several defence-related genes is activated (Hammond-Kosack and Jones, 1996). This elevated expression leads the establishment of an effective defence response that includes the accumulation of pathogenesis-related (PR) proteins, phenolics (including salicylic acid) and the involvement of glycoproteins in the eliciting events in the case of Russian wheat aphid infested resistant wheat (van der Westhuizen and Pretorius, 1996; Botha *et al.*, 1998; van der Westhuizen *et al.*, 1998a; van der Westhuizen *et al.*, 1998b; Mohase and van der Westhuizen, 2002a). Russian wheat aphid PR

proteins are also expressed systemically which is indicative of systemic acquired resistance (van der Westhuizen *et al.*, 1998a; van der Westhuizen *et al.*, 1998b)

The success of the plant's ability to overcome an invader depends on the timely activation of the resistance response (Sessa and Martin, 2000). This activation is mediated through an appropriate signal transduction event initiated by receptor proteins located on the exterior of the plant cell. Two broad types of defence systems are present within plants. The first is a basal defence mechanism whereby general elicitors produced by invading organisms are recognised by a diverse array of receptors which then activates the defence response. This leads to the production of signalling compounds that activate the induced expression of defence genes (Johal *et al.*, 1995).

A second more specific activation of the defence response is mediated by the gene-for-gene interaction (Flor, 1971). The recognition of the invader by the plant is dependent on two genes. The first is a disease resistance gene located within the plant, while the second is a pathogen-borne avirulence gene (Flor, 1971). The resistance gene product acts as a receptor that recognises and binds a ligand or elicitor that is either the avirulence gene product itself, or a molecule that was produced directly or indirectly by the avirulence protein (Baker *et al.*, 1997). Once this elicitor is recognised and bound by the receptor, the defence reaction will be activated (Vanoosthuyse, 2003).

Several classes of disease resistance genes were identified in plants. Included within this group are several types of protein kinases, including the receptor-like protein kinases (Torii, 2000). These proteins are located on the plasma membrane and have the ability to phosphorylate both serine and threonine amino acids (Walker, 1994). In addition to the receptor-like protein kinases, other protein kinases such as mitogen activated protein kinases also play key roles in the transfer of the signal from the cell membrane to the nucleus (Jouannic *et al.*, 2000).

It is thus clear that protein kinases are key components of the activation of the plant defence reaction, since they are implicated to play a major role in the very critical recognition and signal transduction event immediately following the pathogen attack. Plants lacking this ability will be susceptible to the pathogen and will succumb.

The aim of this study was therefore to identify protein kinase genes that are expressed very early after the infestation of wheat by the Russian wheat aphid. The encoded proteins of such genes are thought to play key roles in the adaptation of the plant towards infestation, as well as the activation of the defence reaction.

Chapter

2

Literature

2.1. Introduction

Plants are a major source of food for humans but are constantly exposed to various pathogens, insects and fungi (van't Slood and Knogge, 2002; Hammond-Kosack and Parker, 2003). These organisms severely decrease the annual production of important crops. As the human population increases, the demand for food and the need to improve crop yield is also on the increase. Although the use of pesticides controls disease, their continued usage has a detrimental effect on the environment (Baker *et al.*, 1997), forcing researchers to look for alternative ways to combat disease.

2.2. Plant defence

Over the millennia, plants have developed several mechanisms to combat and prevent disease. These include pre-existing physical barriers that limit infection damage, such as the cuticle and hardened, woody covers that may successfully withstand the attack of small herbivores. For larger herbivores, plants developed trichomes, thorns and other specialised organs that restrict access of herbivores to important parts of the plant (Kerstiens *et al.*, 1996; Sieber *et al.*, 2000; León *et al.*, 2001).

Plants have developed complicated biochemical defence strategies. These defences are involved in the healing of damaged tissues, the prevention of further damage (León *et al.*, 2001) as well as the deterrence of either the pest or the pathogen (Walling, 2000). These defences are inducible and are activated after an appropriate defence signal is generated.

2.3. Gene-for-gene concept

As a whole, plants are naturally resistant to most pathogens (Dangl and Jones, 2001). Pathogens can however overcome these natural defences of the plant, making the plant susceptible to the particular organism

(Bergelson *et al.*, 2001). Two genes are involved in this co-evolution of resistance and susceptibility, namely a plant-borne disease resistance (*R*) gene and a pathogenic avirulence (*avr*) gene.

Resistance genes play an important role in the gene-for-gene interaction which confers resistance to pathogens carrying the corresponding avirulence genes (Flor, 1971). In 1956, Flor defined the gene-for-gene concept as being that, for every incompatible reaction, the infected plant contains an *R* gene while the complementary *avr* gene occurs in the invading pathogen. Specific pathogen recognition is dependent on the genetic interaction between the encoded products of the *R* and *avr* genes.

If the *R* or the *avr* gene is lacking in either host or pathogen, respectively, disease will occur (Fig 2.1). If matching *R* and *avr* genes are present, resistance of the host to the pathogen will occur (Flor, 1956; Dangl and Holub, 1997) resulting in the rapid localized death of host cells at the site of infection. This forms part of the hypersensitive response (HR) (Richter, 2000). Following HR, plant defence is also activated in distal uninfected regions. This is called systemic acquired resistance (SAR) (Ryals *et al.*, 1996). When SAR is activated, plants become resistant to a large variety of other pathogens for an extended period of time (Boller and Keen, 1999).

2.4. Plants and disease

The success of a plant's defence against pathogens depends on the resistance mechanism and the pathogen's ability to overcome it. Advances in technology have enabled researchers to shed more light on the basic mechanisms that allow pathogens to penetrate and damage plants. It has also given a clearer picture of the system plants use to combat pathogens (Keen, 1999).

Different aspects of plant defence have been revealed by genetic, genomic and biochemical analyses. There are several degrees of resistance, namely 1) non-host resistance, 2) race non-specific resistance, 3) race

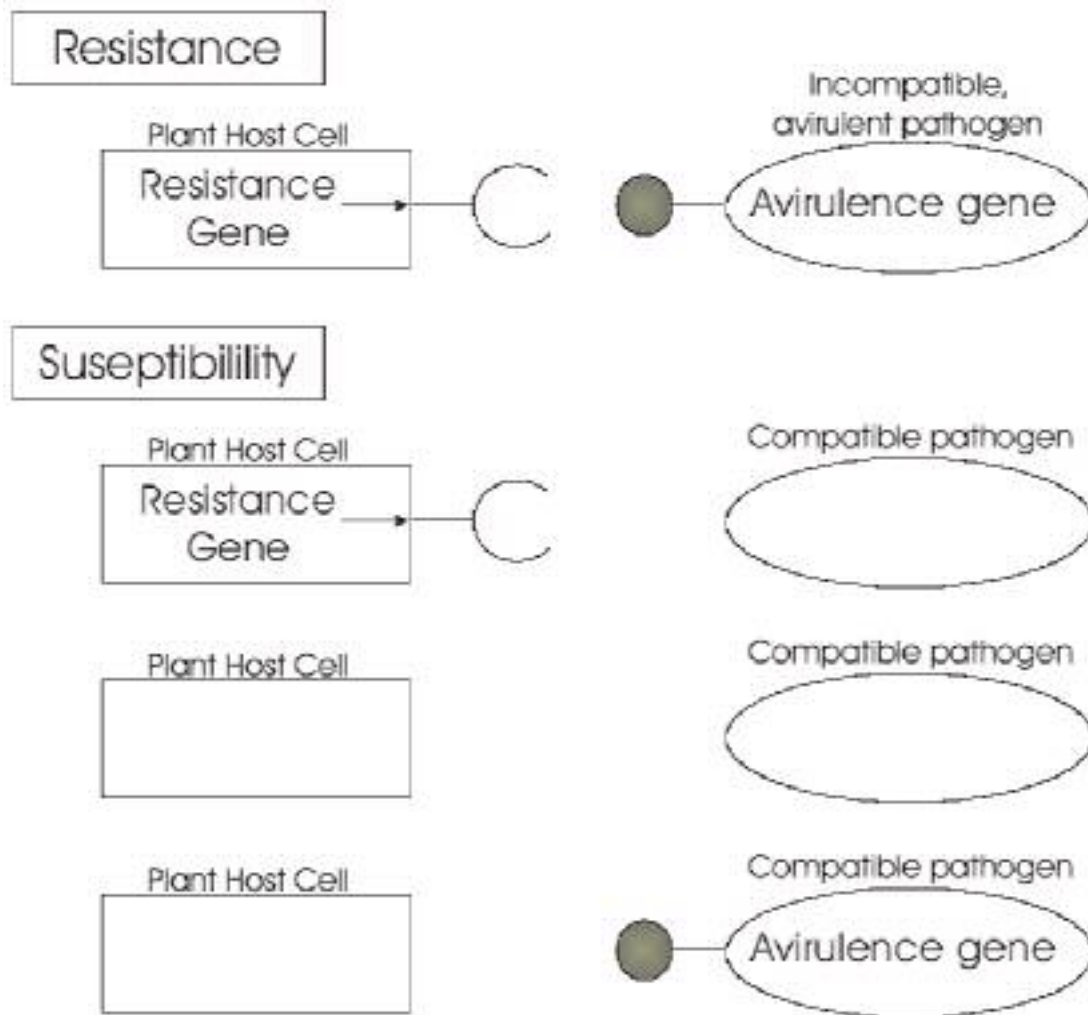


Figure 2.1. Molecular model of the gene-for gene interaction in plants (Staskawicz *et al.*, 1995).

specific resistance and 4) basal defence (Hammond-Kosack and Parker, 2003).

Non-host resistance occurs when pathogens pass between species with the resistance being effective against all known isolates of the pathogen. The outcome of this is that no disease symptoms are visible, thereby rendering the plant resistant. Race non-specific resistance occurs when disease resistance operates within a species and is effective against all known isolates of the pathogen, but is *R*-protein-mediated. The effect of this type of resistance is that only some plant genotypes are fully resistant. Race-specific resistance occurs when disease resistance varies within species. In the event of this, each plant genotype exhibits differential disease resistance and susceptibility to a single isolate. Finally, basal defence is only effective in plants with R proteins that correspond to elicitors produced by specific isolates of the pathogen. Basal defence is activated in susceptible genotypes of a host plant species. The outcome of this defence is that disease severity varies between susceptible plant genotypes (Hammond-Kosack and Parker, 2003).

The key to the activation of an effective plant defence against an invading pathogen is an appropriate, effective and timely signal transduction event. Resistant plants have the ability to recognise a pathogen invasion because they are molecularly equipped with an alert signalling system (Sessa and Martin, 2000).

Several components are involved in this signalling event. The first is a unique receptor protein that is located either at the outer limits of the plant cell or within the cytosol. Other components include proteins that are responsible to transduce the signal to the nucleus where the induced expression of defence genes is activated (Vanoosthuyse *et al.*, 2003). Included within this group is the so-called mitogen-activated protein kinase (MAPK) signalling cascades (Jouannic *et al.*, 2000).

2.5. The protein receptors

All *R*-genes thus far described are receptor proteins involved in the binding of an appropriate elicitor (Hahn, 1996). In several cases, the elicitor was proven to be the *avr* gene product (Greenberg, 1997; Boller and Keen, 1999; Keller *et al.*, 2000). *R* proteins can be placed into five different categories based on their structural characteristics: 1) intracellular protein kinases (e.g. Pto from tomato (Martin *et al.*, 1993)), 2) trans membrane receptor-like proteins with an extracellular leucine-rich repeat (LRR) domain and cytoplasmic protein kinase domain (e.g. Xa21 from rice (Song *et al.*, 1995)), 3) intracellular receptor-like proteins with LRR domains and nucleotide binding sites (NBS) (e.g. RPS2 and RPM1 from *Arabidopsis* (Bent *et al.*, 1996; Grant *et al.*, 1995) and *Prf* from tomato (Salmeron *et al.*, 1996)), 4) intracellular receptor-like proteins with LRR and NBS domains and a region of homology to the *Drosophila* Toll and the mammalian interleukin-1 receptors (e.g. RPP5 from *Arabidopsis* (Parker *et al.*, 1997)) and 5) transmembrane receptor-like proteins with extracellular LRR domains (e.g. Cf-9 from tomato (Jones *et al.*, 1994)).

2.6. Receptor-like protein kinases

There has been great interest in protein kinases that may play a role in signal transduction pathways involved in plant-pathogen interactions (Walker, 1994). Receptor protein kinases (RPKs) play an essential role in signal perception in animal systems since they mediate the response to various growth factors and hormones (Fantl *et al.*, 1993). These receptors have a large extracellular domain with a transmembrane domain spanning the plasma membrane. Ligands bound by this extracellular domain stimulates receptor autophosphorylation on tyrosine residues within the cytoplasmic protein kinase domain. The binding of the ligand to the extracellular domain causes receptor dimerization thereby activating the cytoplasmic kinase domain by intermolecular phosphorylation and transduction of the signal to the downstream effectors (Song *et al.*, 1995).

The first plant receptor-like protein kinases (RLKs) gene was cloned from maize and was identified by Walker and Zhang (1990). Plant RLKs are similar to RPKs except that autophosphorylation is serine and/or threonine specific (Walker, 1994). Only one plant RLK was found to be a tyrosine specific protein kinase, namely PRK1 (Mu *et al.*, 1994).

Table 2.1 is a summary of the RLKs identified in plants (Stein *et al.*, 1991; Chang *et al.*, 1992; Kohorn *et al.*, 1992; Walker, 1993). A database has therefore been set up to regulate the information on RLKs.

2.6.1. Classification of RLKs

Plant RLKs share common features, such as an extracellular ligand binding domain, a transmembrane domain and an intracellular kinase domain. The structure of the extracellular domain aids in the classification of RLKs into different classes (Table 2.1). Several classes have thus far been identified:

- S-domain class: These RLKs possess an extracellular domain that is homologous to the self-incompatibility-locus glycoproteins (SLG) of *Brassica oleracea* (Nasrallah and Nasrallah, 1993). Characteristic of the S-domain is 12 conserved cysteine residues within the following consensus WQSFDXPTDTΦL sequence (X = nonconserved amino acid, Φ = aliphatic amino acid) (Walker, 1994). SLG is proposed to function during the self-incompatibility recognition between pollen and the stigma (Walker, 1993; Torii and Clark, 2000);
- LRR class: Currently the LRR class is the largest class of RLKs. The extracellular region of these proteins contains 24 amino acid tandem repeats consisting of conserved leucines with gaps and insertions between the repeats (Walker, 1994);
- TNFR class: The gene product of *CR4* (*CRINKLY4*) from maize is the only member to date included in this class. The tumour necrosis factor (TNFR)-like repeat motif has a 6 cysteine-conserved

Table 2.1. Plant RLKs with their functions (modified and updated from Torii and Clark, 2000; Becraft, 2002). Highlighted fields are RLKs involved in disease resistance.

| RLK/Class | Plant species | Biological function (if not known, expression pattern) | Reference |
|--|--|--|--|
| S-domain class | | | |
| <i>SRK</i> | <i>Brassica oleracea</i> | Self-incompatibility recognition | Stein <i>et al.</i> , 1991 |
| <i>SFR2</i> | <i>Brassica oleracea</i> | Defence response signalling | Pastuglia <i>et al.</i> , 1997 |
| <i>ARK1</i> <i>ARK2</i> | <i>Arabidopsis thaliana</i> | (Leaf cell expansion) (Cotyledon, leaf, sepal) | Tobias <i>et al.</i> , 1992 Dwyer <i>et al.</i> , 1994 |
| <i>ARK3</i> <i>RLK1</i> | | (Flower pedicles) (Rosettes) | Dwyer <i>et al.</i> , 1994 Walker, 1993 |
| <i>RLK4</i> <i>ZmPK1</i> | <i>Zea mays</i> | (Root-hypocotyl boundary, base of lateral root, base of the petiole) (Seedling roots, shoots and silks) | Coello <i>et al.</i> , 1999 Walker and Zhang, 1990 |
| <i>KIK1</i> <i>OsPK10</i> | <i>Oryza sativa</i> | (Husks, etiolated shoots) (Upregulated by light) | Braun <i>et al.</i> , 1997 Zhao <i>et al.</i> , 1994 |
| LRR class | | | |
| <i>BR1</i> | <i>Arabidopsis thaliana</i> | BR perception | Li and Chory, 1997 |
| <i>CLAVATA1</i> <i>ERECTA</i> | | Meristem and flower development Organ elongation | Clark <i>et al.</i> , 1997 Torii <i>et al.</i> , 1996 |
| <i>PRK1</i> <i>SERK</i> | <i>Petunia inflata</i> <i>Daucus carota</i> | Pollen development Correlation with embryogenic potential | Lee <i>et al.</i> , 1996 Schmidt <i>et al.</i> , 1997 |
| <i>Xa21</i> | <i>Oryza sativa</i> | Resistance to <i>Oryza sativa</i> | Song <i>et al.</i> , 1995 |
| <i>LePRK1, 2</i> | <i>Lycopersicon esculentum</i> | (pollen-pistil interaction) | Muschietti <i>et al.</i> , 1998 |
| <i>RKF1</i> <i>RPK1</i> | <i>Arabidopsis thaliana</i> | (anther specific) (osmotic-stress induced) | Takahashi <i>et al.</i> , 1998 Hong <i>et al.</i> , 1997 |
| <i>LRRPK</i> <i>TMK1</i> | | (Light-repressed) (Abscisic acid-, dehydration-, high salt- and cold-induced) | Deeken and Kaldenhoff, 1997 Chang <i>et al.</i> , 1992; Hong <i>et al.</i> , 1997 |
| <i>RLK5/HAESA</i> <i>LTK1, 2, 3</i> | <i>Zea mays</i> | Floral abscission (endosperm specific) | Jinn <i>et al.</i> , 2000 Li and Wurtzel, 1998 |
| <i>OsTMK1</i> | <i>Oryza sativa</i> | Gibberellin-induced cell division and elongation | van der Knaap <i>et al.</i> , 1999 |
| <i>EILP</i> | <i>Nicotiana tabacum</i> | Non-host disease resistance | Takemoto <i>et al.</i> , 2000 |
| <i>OsLRK1</i> <i>SARK</i> | <i>Oryza sativa</i> <i>Phaseolus vulgaris</i> | Floral meristem activity Senescence induced | Kim <i>et al.</i> , 2000 Hajouj <i>et al.</i> , 2000 |
| <i>SbRLK1</i> <i>LRPKm1</i> | <i>Sorghum bicolor</i> <i>Malus x domestica</i> | (mesophyll cells) Disease resistance | Annen and Stockhaus, 1999 Komjanc <i>et al.</i> , 1999 |
| TNFR class | | | |
| <i>CRINKLY 4 (CR4)</i> | <i>Zea mays</i> | Epidermal cell specification | Becraft <i>et al.</i> , 1996 |
| EGF class | | | |
| <i>WAK1, 2, 3, 4</i> | <i>Arabidopsis thaliana</i> | Cell expansion and disease response | He <i>et al.</i> , 1996; Wagner and Kohorn, 2001 |
| PR5 class | | | |
| <i>PR5K</i> | <i>Arabidopsis thaliana</i> | Disease/stress response | Wang <i>et al.</i> , 1996 |
| Lectin class | | | |
| <i>LecRK1</i> | <i>Arabidopsis thaliana</i> | Development and adaptation | Riou <i>et al.</i> , 2002 |
| Other class | | | |
| <i>CrRLK1</i> | <i>Catharanthus roseus</i> | | Schulze-Muth <i>et al.</i> , 1996 |
| <i>RKF2, 3</i> <i>Lrk10</i> | <i>Arabidopsis thaliana</i> <i>Triticum aestivum</i> | (Ubiquitous) Leaf rust resistance | Takahashi <i>et al.</i> , 1998 Feuillet <i>et al.</i> , 1997 |
| <i>At-RLK3</i> <i>PvRK20-1</i> | <i>Arabidopsis thaliana</i> <i>Phaseolus vulgaris</i> | (induced by oxidative stress, pathogen attack) (plant-microbe interaction) | Czernic <i>et al.</i> , 1999 Lange <i>et al.</i> , 1999 |

arrangement. The CR4 protein plays a role in epidermal cell fate (Becraft *et al.*, 1996; Torii and Clark, 2000);

- EGF class: The cell wall-associated receptor kinases (WAKs) in *Arabidopsis* represent the epidermal growth factor (EGF) class (Walker, 1994). The RLKs included in this class have a 6 cysteine consensus sequence located on the extracellular domain. WAK1 is associated with both the cell wall and the plasma membrane (He *et al.*, 1996);
- PR class: The *Arabidopsis* PR5K (PR5-like receptor kinase) is currently the only known member of this class. The extracellular domain of PR5K is similar to PR5 (pathogenesis related protein 5), whose expression is induced during pathogen attack (Wang *et al.*, 1996);
- Lectin class: The LecRK1 gene from *Arabidopsis* encodes a protein possessing an extracellular domain similar to carbohydrate-binding proteins. The function of LecRK1 is not known but the structure suggests that it may be involved in oligosaccharide-mediated signal transduction (Riou *et al.*, 2002); and
- Others: Other RLKs possess extracellular domains that do not share homology to any known motifs. These include LRK10 from wheat (Feuillet *et al.*, 1997), CnRLK1 of *Catharanthus roseus* (Schulze-Muth *et al.*, 1996) and LRRPK and RKF3 from *Arabidopsis* (Deeken and Kaldenhoff, 1997; Torii and Clark, 2000).

The synthesis of protein kinases involved in plant signalling is regulated on both transcriptional and post-translational levels (Xing *et al.*, 2002). This regulation and the overall response contributing to each level, might differ.

2.6.2. RLKs specifically involved in defence response

Several plant RLKs are involved in the defence response of plants. *Wak1* expression is induced upon pathogen attack and the application of chemical activators such as salicylic acid (SA) and 2,2-dichloroisonicotinic acid (INA) (He *et al.*, 1998).

Xa21 confers resistance to bacterial leaf blight in rice (Song *et al.*, 1995). Xa21 carries both a LRR motif and a Ser/Thr kinase domain suggesting that it may play a role in cell surface recognition of a pathogenic ligand.

PR5K from *Arabidopsis thaliana* shows similarity to antifungal PR proteins (Wang *et al.*, 1996). *PR5K* and *PR5* are structurally similar, suggesting that *PR5K* is involved in pathogenesis.

SFR2 from *Brassica oleracea* is believed to play a role in the signal transduction pathway leading to the activation of the plant defence response, including the synthesis of PR proteins and enzymes involved in phenylpropanoid metabolism (Pastuglia *et al.*, 1997). *SFR2* is induced upon wounding, pathogen infection and application of SA.

At-RLK3 from *Arabidopsis thaliana* is activated upon oxidative stress, salicylic acid treatment and pathogen infection and was detected in the root, stem, leaf and flower (Czernic *et al.*, 1999).

FLAGELLIN INSENSITIVE 2 from *Arabidopsis* encodes *FLS2* which is a LRR-RLK responsible for the detection of the flagellin peptide (Gómez-Gómez *et al.*, 2001). *FLS2* expression is induced after *Arabidopsis* plants were treated with flagellin.

Plants wounded or infected by the fungus, *Sclerotinia sclerotiorum* showed increased transcript levels of *PERK1* (Silva and Goring, 2002). *PERK1* may be involved in the early perception and response to a wound and/or pathogen stimulus by recognising physical changes in the cell wall caused by pathogens or herbivory. *PERK1* is localized on the plasma membrane.

Another RLK isolated from wheat is *Lrk10* that forms part of the *wlrk* family of plant RLKs that was mapped to the *Lr10* disease resistance locus (Feuillet *et al.*, 1997). *Lr10* confers resistance to leaf rust. *LRK10* bears an extracellular domain to which no other protein showed homology to.

2.6.3. Other receptor protein kinases involved in defence responses

Pto is a protein that acts as a R protein and gives tomatoes resistance against *Pseudomonas syringae* pv (Martin *et al.*, 1993). Although plasma membrane associated, it is not an integral protein. Susceptible plants transformed with the gene gained resistance against the pathogen. Upon binding of the avirulence protein (avrPto), the defence is activated when Pto autophosphorylates (Martin *et al.*, 1993).

2.7. Mitogen-activated protein kinases

MAPKs are encoded by a large gene family in eukaryotic genomes. Individual members combine to form signalling networks where a selection of upstream signals is integrated into an efficient signal transduction cascade. Also involved are G proteins that often serve directly as coupling agents between plasma membrane located sensors of extracellular stimuli and the cytoplasmic MAPK modules (Sopory and Munshi, 1998; Hirt, 2000).

The MAP kinase cascade generally involves three functionally linked protein kinases, a MAP kinase kinase kinase (MAPKKK), a MAP kinase kinase (MAPKK) and a MAPK (Hirt, 1997). MAPKKK will activate MAPKK in response to external stimuli via phosphorylation of Ser and Ser/Thr residues within the SXXXS/T motif (X = any amino acid). MAPKK then activates MAPK by phosphorylating the Thr and Tyr residues within the TXY motif. MAPK then finally phosphorylates specific effector proteins leading to the activation of cellular responses.

Activation of the downstream end of the cytoplasmic MAPK module often induces the translocation of the MAPK into the nucleus where the kinase activates the expression of certain sets of genes through the phosphorylation of specific transcriptional factors (Hirt, 2000). Figure 2.2 shows an example of the MAPK cascade activated during hypo-osmotic stress or a mechanical stimulus (Taylor *et al.*, 2001). A hypo-osmotic or mechanical stimulus activates the MAPKK which in this case activates a MAPK. The MAPK activates the internal Ca²⁺ store by opening the anion

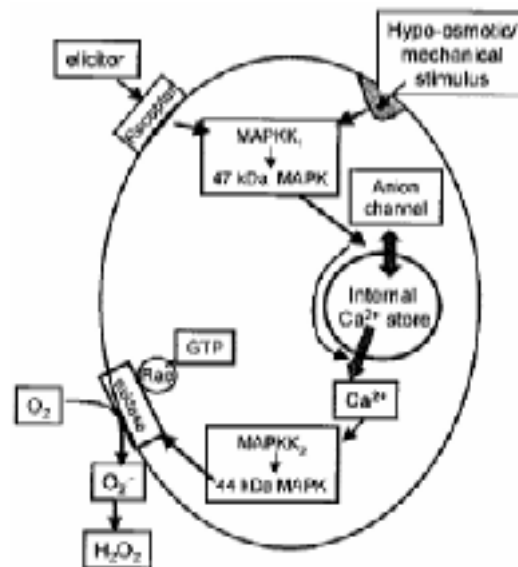


Figure 2.2. Schematic diagram of the signal-transduction pathway leading to the oxidative burst (Taylor *et al.*, 2001).

channel regulating the Ca^{2+} concentration in the cell. The change in Ca^{2+} concentration activates another MAPKK and MAPK which will then activate NADPH oxidase to convert O_2 to superoxide to form H_2O_2 .

A number of MAPKs have been cloned and characterized in plants (Mizoguchi *et al.*, 1997). These MAPKs are activated by several factors including abiotic and biotic stress conditions, high salt concentrations, heavy metals, radiation, extreme pH, heat, wounding, drought and pathogen attack (Suzuki and Shinshi, 1995; Usami *et al.*, 1995; Börge *et al.*, 1996, 1997; Sheen, 1996; Shinozaki and Yamaguchi-Shinozaki, 1996; Hirt, 1997; Mizoguchi *et al.*, 1997).

Various MAP kinases have also been found to play a role during the plant defence response. MPK6 in *Arabidopsis* (Menke *et al.*, 2004) plays a role in the basal resistance of the plant against a virulent bacterial pathogen. When MPK6 is silenced, plants showed increased susceptibility. p48 SIP kinase in tobacco belongs to the MAP kinase family (Zhang and Klessig, 1997) and is activated by SA treatment. Because p48 SIP kinase is activated by SA which plays an important role in signalling the defence response, it was suggested that p48 SIP kinase is also involved in the activation of defence responses.

Wound-induced protein kinase (WIPK) from tobacco is a MAPK induced upon wounding (Seo *et al.*, 1995) that is involved with jasmonic acid (JA) and methyl jasmonate (MeJA) biosynthesis. Plants transformed with the antisense WIPK gene showed decreased production of JA and other wound-induced gene transcripts. On the other hand, the levels of SA and transcripts for pathogen-inducible, acidic PR proteins were increased upon wounding, indicating that WIPK is part of the initial response of higher plants to mechanical wounding.

OsBIMK1 shows similarity to previously reported MAPK genes (Song and Goodman, 2002). The expression of *OsBIMK1* is activated upon treatment of plants with benzothiadiazole (BTH) as well as INA,

probenazole, JA, MeJA, *Pseudomonas syringae* pv. *syringae* or wounding. This suggests that *OsBIMK1* plays an important role in rice disease resistance.

Further study of the role of MAP kinase signalling pathways will enable the better understanding of the molecular mechanisms controlling plant development and plant environmental responses. Therefore, in addition to MAPK, RLKs and a variety of other protein kinases partake in the defence response of plants against pathogens. This clearly indicates the importance of phosphorylation in this activation of plant defences.

2.8. Biochemical defences

Plants have developed detailed inducible defence responses following elicitor treatment, mechanical damage and/or pathogen attack. Various signalling pathways are induced upon pathogen attack (Fig 2.3) in which signalling molecules like SA, JA and ethylene (ET) play important roles in the primary defence of plants against pathogens (Pieterse *et al.*, 2001).

The defence response includes the synthesis of various chemicals and enzymes that allow the plant to survive the attack. Included are anti-microbial phytoalexins (Keen, 1999), protease inhibitors (Dangl, 1998), lytic enzymes such as chitinases and glucanases which attack the pathogen cell wall (Lamb *et al.*, 1989; Ryan and Jagendorf, 1995) and other chemicals such as cyanogenic glycosides and glucosinolates (Osborn, 1996). The latter occurs as inactive precursors of secondary metabolites that have antifungal activity that are produced in response to tissue damage or pathogenic attack. Various genes coding for proteins involved in metabolic processes are also induced upon pathogen infection (Lu *et al.*, 2004).

Induced resistance is a state of enhanced defensive ability developed by a plant when appropriately stimulated (Kuc, 1982). This includes the

activation of latent resistance mechanisms that are expressed upon repetitive inoculation with a pathogen (van Loon, 1997). Induced resistance occurs naturally due to limited infection by a pathogen, especially when the plant develops a HR. It can also be induced by certain chemicals, non-pathogens, avirulent forms of pathogens, incompatible races of pathogens or by virulent pathogens under circumstances where infection is delayed due to environmental conditions (Ryals *et al.*, 1994; Hahn, 1996; van Loon *et al.*, 1998). The HR can also be induced by elicitors, chemicals that have the ability to activate a signalling cascade that could lead to the activation of SAR (Ryals *et al.*, 1996).

In general, the effect of the induced resistance is systemic, because the defensive capabilities do not only occur in the cells at the primary site of pathogen infection but also in uninfected parts of the plant (Ward *et al.*, 1991; Ryals *et al.*, 1996; Sticher *et al.*, 1997).

When induced resistance is not expressed systemically, it is known as localized acquired resistance (LAR). LAR occurs when only the tissue exposed to the pathogen or chemical becomes resistant (Ross, 1961). SAR and LAR are similar in that they are effective against a range of pathogens. They differ in that the signal that distributes the enhanced defensive ability throughout the plant in SAR, seems to be lacking in LAR (van Loon *et al.*, 1998).

2.9. Hypersensitive Response

The HR is a widely occurring active defence response system which occurs in higher plants responding to all known groups of plant pathogens. HR is characterised by rapid and localised cell death at the point of pathogen attack (Keen, 1990). The activation of the HR requires mechanisms that transmit signals via signal transduction pathways (Braun *et al.*, 1997).

The HR consists of three different phases namely, the induction phase, the latent phase and the presentation or collapse phase. In the induction phase, *avr* gene expression is activated in the pathogen and the *avr* products are transported into the host cell. This phase involves a rapid reaction to close the wound thereby protecting the plant from losing cellular components and restricting micro-organisms from entering the plant tissue. The latent phase does not need living bacteria and macroscopic symptoms occur during this phase. Membrane damage associated with HR also occurs during this phase. During this phase, photosynthetic protein synthesis is inhibited by arresting the translation of nuclear encoded photosynthetic genes. In the final phase, the host cells will collapse (Jabs and Slusarenko, 2000).

The effective activation of the HR is dependent on several different factors. This includes newly synthesized enzymes, hormones and other molecules. A brief description of some of these will now be given.

2.9.1. Reactive oxygen species

Reactive oxygen species (ROS) play an important role in the early signalling of biotic and abiotic stresses (Mittler, 2002). ROS that are detected in the plant-pathogen interaction are superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-) (Wojtaszek, 1997). Enzymes generating ROS during the defence response are NADPH oxidase (Lamb and Dixon, 1997), peroxidase (Benhamou, 1996), oxalate oxidase and amine oxidase (Bolwell and Wojtaszek, 1997). The production of ROS is pH-dependent and shows optimal production at a neutral to basic pH (Bolwell and Wojtaszek, 1997). H_2O_2 also controls the influx of Ca^{2+} in the cell. The increase of Ca^{2+} is shown to be an important factor in the development of reactive oxygen intermediate (ROI) mediated cell death (Levine *et al.*, 1996).

2.9.2. Salicylic acid

SA is a phenolic acid and plays a role during signalling in the primary defence against pathogens. Application of SA to plants induces SAR genes (Sticher *et al.*, 1997). The role of SA in signalling during the defence response was elucidated by using *NahG* transformants (Delaney *et al.*, 1994). Plants over-expressing the *NahG* gene coding for salicylate hydroxylase, which converts SA to an inactive catechol, showed enhanced susceptibility to pathogen attack (Ryals *et al.*, 1995). Other mutants, including *sid1*, *sid2* and *pad4* affecting SA signalling, also showed susceptibility to pathogen infection. These mutants are defective in SA accumulation during the response to pathogen infection (Nawrath and Métraux, 1999; Zhou *et al.*, 1998). This confirms that SA is important for basic resistance against different types of pathogens (Pieterse *et al.*, 2001).

2.9.3. Jasmonic acid

The role of JA signalling in defence was shown using *Arabidopsis* mutants affected in the biosynthesis or perception of JA. A JA-response mutant, *coi1*, displaying susceptibility to the necrotrophic fungi *Alternaria brassicicola* and *Botrytis cinerea* (Thomma *et al.*, 1998), was used to confirm the role of JA in defence. Mutants which were deficient in the biosynthesis of the JA precursor linolenic acid, *jar1* (Staswick *et al.*, 1992) and a *fad3 fad7 fad8* triple mutant from *Arabidopsis*, also showed susceptibility to normally non-pathogenic soil-borne *Pythium* spp, indicating that JA plays a role in non-host resistance against pathogens. This also shows that JA-dependent defences contribute to basic resistance against different microbial pathogens and confirms that JA is important in the basic resistance against herbivorous insects (Staswick *et al.*, 1998; Vijayan *et al.*, 1998; Pieterse *et al.*, 2001).

2.9.4. Systemin

Systemin is an 18-amino acid peptide that was isolated from tomato that is involved in systemic signalling after insect attack (Schaller and Ryan,

1994). Leaves wounded by herbivory or mechanical damage showed a local and rapid transcriptional activation of proteinase inhibitor genes but also in the distal unwounded leaves (Schaller and Ryan, 1994; Zhou and Thornburg, 1999). Systemin must be proteolytically processed from prosystemin to the active systemin peptide (Zhou and Thornburg, 1999). Plants transformed with an antisense copy of prosystemin cDNA showed dramatic inhibition of proteinase inhibitor expression in the leaves of the transgenic plants (McGurl *et al.*, 1992). Over-expression of prosystemin cDNA in tomato plants resulted in a constitutive expression of proteinase inhibitor proteins in leaves (McGurl *et al.*, 1994). Systemin has the ability to induce other plant defensive proteins, including polyphenol oxidase (Constabel *et al.*, 1995), demonstrating that systemin plays a role in the induced expression of plant defensive genes other than proteinase inhibitors.

2.9.5. Ethylene

Ethylene is a gaseous plant hormone that plays a role in various developmentally processes (Zhou and Thornburg, 1999). Ethylene is synthesized from *S*-adenosyl-L-methionine via 1-aminocyclopropane-1-carboxylic acid (ACC) and plays an important role in various plant disease resistance pathways (Zhou and Thornburg, 1999; Guo and Ecker, 2004). Plants deficient in ethylene signalling show either increased susceptibility or increased resistance (Wang *et al.*, 2002). Soybean mutants with reduced ethylene sensitivity produce less severe chlorotic symptoms when challenged with the virulent *Pseudomonas syringae* pv *glycinea* and *Phytophthora sojae* strains (Hoffman *et al.*, 1999), whereas virulent strains of the fungi *Septoria glycines* and *Rhizoctonia solani* cause more severe symptoms (Hoffman *et al.*, 1999).

2.9.6. Pathogenesis-related proteins

Both pathogen- and SA-induced resistance are associated with the induced expression of several families of PR protein encoding genes during the HR (Table 2.2). The induction of PR gene expression is regularly linked

Table 2.2. The families of pathogenesis-related proteins (modified and updated from van Loon and van Strien, 1999).

| Family | Type Member | Properties | Gene Symbols |
|--------------|-------------------------------------|---|---|
| <i>PR-1</i> | Tobacco PR-1a | antifungal, 14-17kD | <i>Ypr1</i> |
| <i>PR-2</i> | Tobacco PR-2 | class I, II, and III endo-beta-1,3-glucanases, 25-35kD | <i>Ypr2</i> , [<i>Gns2</i> (' <i>Glb</i> ')] |
| <i>PR-3</i> | Tobacco P, Q | class I, II, IV, V, VI, and VII endochitinases, about 30kD | <i>Ypr3</i> , <i>Chia</i> |
| <i>PR-4</i> | Tobacco R | antifungal, <i>win</i> -like proteins, endochitinase activity, similar to prohevein C-terminal domain, 13-19kD | <i>Ypr4</i> , <i>Chid</i> |
| <i>PR-5</i> | Tobacco S | antifungal, thaumatin-like proteins, osmotins, zeamotins, permeatins, similar to alpha-amylase/trypsin inhibitors | <i>Ypr5</i> |
| <i>PR-6</i> | Tomato Inhibitor I | protease inhibitors, 6-13kD | <i>Ypr6</i> , <i>Pis</i> (' <i>Pin</i> ') |
| <i>PR-7</i> | Tomato P ₆₉ | endoproteinase | <i>Ypr7</i> |
| <i>PR-8</i> | Cucumber chitinase | class III chitinases, chitinase/lysozyme | <i>Ypr8</i> , <i>Chib</i> |
| <i>PR-9</i> | Tobacco 'lignin-forming peroxidase' | peroxidases, peroxidase-like proteins | <i>Ypr9</i> , <i>Prx</i> |
| <i>PR-10</i> | Parsley 'PR1' | ribonucleases, Bet v 1-related proteins | <i>Ypr10</i> |
| <i>PR-11</i> | Tobacco class V chitinase | endochitinase activity, type I | <i>Ypr11</i> , <i>Chic</i> |
| <i>PR-12</i> | Radish Rs-AFP3 | plant defensins | <i>Ypr12</i> |
| <i>PR-13</i> | <i>Arabidopsis</i> THI2.1 | thionin | <i>Ypr13</i> , <i>Thi</i> |
| <i>PR-14</i> | Barley LTP4 | Non-specific lipid transfer proteins (ns-LTPs) | <i>Ypr14</i> , <i>Ltp</i> |
| <i>PR-15</i> | barley OxOa (germin) | oxalate oxidase | |
| <i>PR-16</i> | barley OxOLP | oxalate-oxidase-like proteins | |
| <i>PR-17</i> | tobacco PRp27 | unknown | |

to necrotizing infections giving rise to SAR, and has been used as a marker of the induced defensive state (Ward *et al.*, 1991; Uknes *et al.*, 1992). PR proteins play a major role in the defence response in many plants under stress and are detected in plants after exposure to insects (Bronner *et al.*, 1991; van der Westhuizen and Pretorius, 1995; Broderick *et al.*, 1997; van der Westhuizen *et al.*, 1998b). The accumulation of PR proteins during the onset and maintenance of SAR is thought to be responsible for the enhanced resistance of the uninfected plant tissues so that they are referred to as SAR proteins. The *PR4* gene in wheat is an example of a gene that is expressed when the plant is exposed to chemical activators of SAR and wounding (Bertini *et al.*, 2003). Two other important PR proteins are β -1,3-glucanases and chitinases.

2.9.7. β -1,3-glucanases

β -1,3-glucanases form part of the PR-2 protein family that is able to catalyse endo-type hydrolytic cleavage of the 1,3- β -D-glucosidic bonds in β -1,3-glucans (Leubner-Metzger and Meins, 1999). It is suggested to play a role in the response of plants to wounding and pathogen attack (Leubner-Metzger and Meins, 1999). β -1,3-glucanases are divided into four classes. Class I is produced as a pre-protein with an N-terminal hydrophobic signal peptide which is co-translationally removed and a C-terminal extension that is N-glycosylated at a single site. The proteins in this class are localized in the cell vacuole. Class II, III and IV are acidic proteins lacking the C-terminal extension present in the class I enzymes and are secreted into the extracellular space (van Loon and van Strien, 1999).

2.9.8. Chitinases

Plant chitinases are suggested to be involved in plant disease resistance during pathogen infection (Cheng *et al.*, 2002). Chitinases catalyses the hydrolysis of chitin which is a linear polymer of β -1,4-linked *N*-acetylglucosamine residues (Khan and Shih, 2004). Chitinases have been

found to be activated by fungal infection and plant activators such as INA and BTH which induce SAR (Busam *et al.*, 1997).

2.9.9. Programmed cell death

Programmed cell death is the active process of cell death occurring in response to environmental stresses and pathogen infection (Jabs and Slusarenko, 2000). Programmed cell death involves chromatin aggregation, cytoplasmic and nuclear condensation and fragmentation of the cytoplasm and nucleus into membrane-bound vesicles (Jabs and Slusarenko, 2000). The role of programmed cell death during pathogenesis is that of limiting the spread of disease after the induction of HR at the site of infection (Greenberg, 1996; Lam *et al.*, 2001). There are two different mechanisms involved in cell death occurring during the compatible and incompatible interactions respectively (Greenberg, 1997). The mechanism by which cell death occurs in susceptible plants is not fully understood but it is thought that a toxin produced by the pathogen may directly kill the plant cells. In the resistant interaction, HR is induced and rapid cell death occurs (Greenberg, 1997).

2.10. Systemic Acquired Resistance

SAR is a secondary response characterized by the accumulation of SA and PR proteins (Ward *et al.*, 1991; Uknes *et al.*, 1992; Ryals *et al.*, 1996; Sticher *et al.*, 1997). Various compounds activate SAR, including JA, ethylene, SA and systemin (Sticher *et al.*, 1997; van Loon, 2000). SA accumulation occurs both locally and, at lower levels, systemically parallel with the development of SAR. Exogenous application of SA also induces SAR in several plants species (Gaffney *et al.*, 1993; Chen *et al.*, 1995; Ryals *et al.*, 1996). SAR will in the end lead to the whole plant being resistant to a broad spectrum of pathogens (Sticher *et al.*, 1997).

The infestation of wheat by the Russian wheat aphid (RWA) has received a lot of attention, since it is an economically important pest. A description of the interaction between wheat and the RWA will follow.

2.11. Wheat and the Russian wheat aphid

2.11.1. Wheat

Wheat (*Triticum aestivum* L.), a cereal of the genus *Triticum* of the family Gramineae, is an important economic crop in South Africa and around the world. Wheat was originally a wild grass native to the arid countries of western Asia. Altogether, there are approximately 600 genera of grasses that have since evolved. Amongst them are assorted forms of the genus *Triticum* of which *aestivum* (vulgare) is more commonly known as the 'Common wheat' (Cornell and Hoveling, 1998).

The ancestry of the common races of wheat grown today remains a mystery, but evidence exists that cultivated einkorn was developed from a type of wild grass native to the arid pasture lands of south eastern Europe and Asia Minor (Schellenberger, 1969).

Wheat was one of the first grains domesticated by humans. Its cultivation began in the Neolithic period. Bread wheat was grown in the Nile valley by 5000 B.C. and it is apparent later cultivation in other regions (e.g., the Indus and Euphrates valleys by 4000 B.C., China by 2500 B.C., and England by 2000 B.C.) indicates that it spread from Mediterranean centres of domestication. The civilizations of Western Asia and Europe have been largely based on wheat, while rice has been more important in Eastern Asia (Feldman, 2001).

Since agriculture began, wheat has been the chief source of bread for Europe and the Middle East. It was introduced into Mexico by the Spaniards (c.1520) and into Virginia by English colonists early in the 17th century (Feldman, 2001; Gibson and Benson, 2002).

The main source of wheat to Europe was from Anatolia to Greece. From there it branched into Italy, southern France and Spain where the first regions for cultivation were the southern plains bordering the coast. Wheat cultivation spread to Africa via several routes. The earliest route was to Egypt. From there it spread southwards to Sudan and Ethiopia and westwards to Libya. There were also routes across the Mediterranean from Greece to Crete and to Libya. The spread to Asia was through Iran and was at a similar rate as in Europe at one kilometre per year (Feldman, 2001).

Pests are the source of major crop yield losses in all areas of wheat production world wide. In most parts of the world, losses due to pests can be higher than that of diseases (Narayanan, 2004). The main pests for wheat include birds, insects, mammals, mites, molluscs and nematodes. Insects may also be carriers of viral diseases which can be transmitted to wheat. Most of the time, pests can be controlled by pesticides. However, pests have managed to develop resistance to the commercial pesticides.

2.11.2. The Russian wheat aphid

Aphids are soft-bodied, plant sucking insects (Dixon, 1985). The RWA is less than 0.25 cm in length and greenish to grayish-green. Several characteristics are important for identification (Fig 2.4). The shape of the insect is distinctive. The RWA is more elongate (spindle-shaped) than other aphids which are teardrop-shaped. Its antennae are short and less than half the length of the body. The cornicles, which are obvious on most other aphids, are very short on the RWA. From the side, the cauda and the supracaudal process appear to make up a "double tail." This double tail is not noticeable on winged RWA. All of these combined characteristics distinguish the RWA from other common aphids found in small grains (Hein *et al.*, 1998).

The aphid is indigenous to Southern Russia and countries bordering the Mediterranean Sea, Iran and Afganistan (Walters *et al.*, 1980). It was also reported in Yemen, in Mexico and as early as 1986 from the cereal

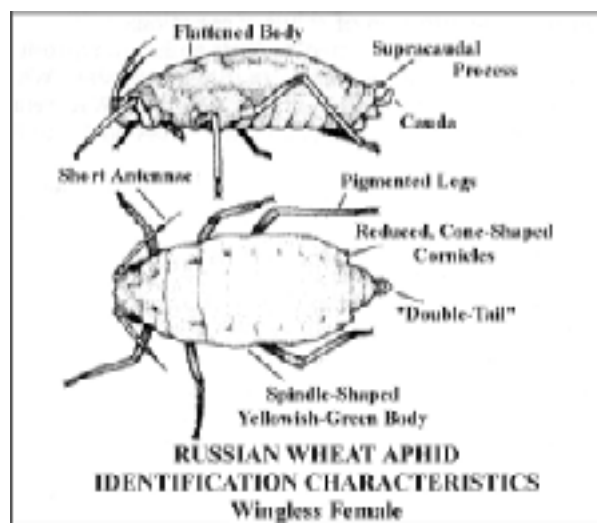


Figure 2.4. Russian wheat aphid identification characteristics (Hein *et al.*, 1998).

producing areas of the south-western USA (Du Toit and Walters, 1984). It was first detected in South Africa in 1978. During March 1979, it was noticed in wheat fields and in some areas, control measures were necessary by May. By the beginning of 1979 season, it was a pest only in the Eastern Free State but by September had spread throughout most of the Western Free State and Lesotho. Infestations were isolated in some areas of Gauteng, North West Province and KwaZulu-Natal (Walters *et al.*, 1980).

The RWA is a major pest of wheat and other cereals (Du Toit and Walters, 1984). Limited problems have also been noted in triticale, oats and rye. Other grass crops such as corn, sorghum and proso millet have so far been proven not to be hosts for the RWA (Hein *et al.*, 1998). The first known South African cultivars to show resistance to *D. noxia* were SA 2199 and SA 1684 reported by F. du Toit in 1987. Currently there are five resistance genes available, namely *DN1* (PI 137739), *DN2* (PI 262660), *DN3*, *DN5* (PI 294994) and *DN7*.

The RWA have the ability to reproduce sexually and asexually, allowing aphids to colonise the host plant rapidly (parthenogenesis). While some species of aphids deposit eggs, the RWA retain their eggs inside the female and she gives birth to living young. In many species of aphids, males are present only in the fall when a sexual generation of aphids results in the production of eggs that are able to over-winter (Hein *et al.*, 1998).

A major concern in managing the RWA is how the aphid bridges the gap between one harvest and the planting of new winter wheat. The most likely way it passes the summer is on alternate hosts, primarily volunteer wheat (Hein *et al.*, 1998).

Many of the plants in the Conservation Reserve Program (CRP) and grassland areas provide additional hosts for successful overwintering. Alternate host plants include most of the wheatgrasses, downy brome, jointed goatgrass, wild rye and several other mostly cool season grasses (Hein *et al.*, 1998).

2.11.3. The interaction between wheat and the RWA

The interaction between the RWA and wheat is complex with toxins in the saliva being the major and other salivary components the minor stimuli of the defence response (Ryan, 1990). Possible functions of the various salivary components in the aphid-plant interaction are shown in Figure 2.5.

On the right it shows the different components of aphid saliva and the different effects these components have on the plant. Mechanical damage of the plant by the aphid will cause the plant to produce polysaccharide messengers that will initiate the defence response. This will lead to the activation of various pathways leading to the activation of HR causing necrosis at the site of infestation which will lead to the deprivation of nutrients to the aphid and decrease or inhibit feeding activity. Deployment of allelochemicals during the defence response also has an inhibitory effect on feeding activity (Miles, 1999).

The RWA inject toxic saliva into the leaves of wheat plants and feed on the phloem fluid. This causes the plant to wilt causing major yield losses (Miles, 1999). RWA initiates feeding at the base of the leaves near the top of the plant. As the colony develops, the leaf edges begin to roll inward, enclosing the aphids in a tubular, protective structure (Dixon, 1973). This protection makes the RWA less accessible to natural enemies and insecticidal sprays (Hein *et al.*, 1998).

Resulting from the salivary toxins injected as the aphids feed, the plants become purplish and leaves develop longitudinal yellow and white streaks (Du Toit, 1986; Walters *et al.*, 1980). Plants that are heavily infested with RWA are killed. Severely infested plants will have reduced vigour, will be less able to compete with weeds and will be more susceptible to environmental stresses (Hein *et al.*, 1998).

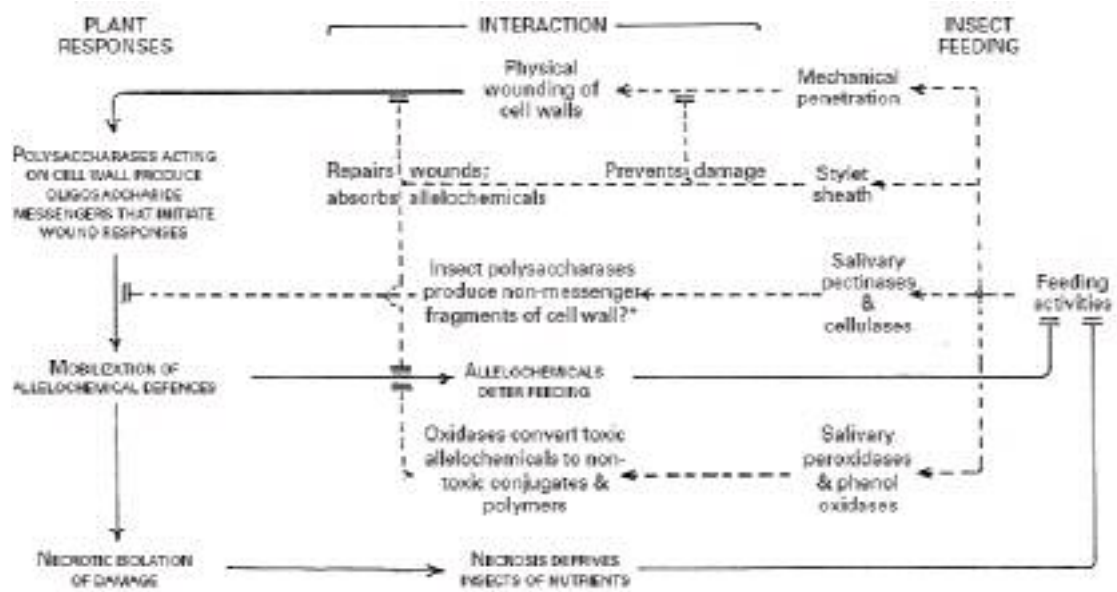


Figure 2.5. Suggested interactions between the feeding process of aphids in general (lower case lettering, dashed lines) and the defensive reactions of plants (upper case lettering, unbroken lines). Arrows represent potentiation and bars represent inhibition. *Untested suggestion (Miles, 1999).

The most significant late-spring yield losses occur when RWA damages and curls the flag leaf. If the heads are able to emerge, the aphids move up into the heads and continue feeding. This late feeding on the heads may result in reduced grain quality. Under heavy infestations, severe yield reductions of up to 100 percent can result. Grain test weights can be reduced to only 20% of normal (Hein *et al.*, 1998).

A lot of work has been done on the biochemical defence response of wheat upon RWA infestation (Botha *et al.*, 1998; van der Westhuizen *et al.*, 1998b; Mohase and van der Westhuizen, 2002a). Following infestation, susceptible and resistant wheat showed the accumulation of β -1,3-glucanases, chitinases and glycoproteins (Botha *et al.*, 1998; van der Westhuizen *et al.*, 1998b; Mohase and van der Westhuizen, 2002a). The susceptible plants showed lower levels of induction of glycoproteins, β -1,3-glucanases and chitinases. Infested resistant plants showed an induction of β -1,3-glucanases within 48 h (van der Westhuizen *et al.*, 1998b) and accumulated where tissues were affected most by feeding aphids (van der Westhuizen *et al.*, 2002). Chitinases was shown to be induced in plants infested with RWA (Botha *et al.*, 1998). SA was also shown to be involved in the resistance response of wheat against RWA (Mohase and van der Westhuizen *et al.*, 2002b). RWA also causes leakage of the contents of the chloroplasts, therefore decreasing levels of CO₂ assimilation (Ryan *et al.*, 1990).

2.12. Aim

Since protein kinases, and the implicated phosphorylation of proteins, were shown to be involved in several other plant-pathogen interactions, the focus of this study fell on the cloning of protein kinase genes, as well as other genes encoding proteins whose activity is modulated by phosphorylation during the interaction between wheat and the RWA.

Chapter

3

Materials and Methods

3.1. Biological Material

Wheat (*Triticum aestivum*) seed of the susceptible cultivar Tugela and the near isogenic resistant Tugela cultivar containing the Dn1 (PI 137739) resistance gene, was obtained from the Small Grain Institute at Bethlehem. The plants were grown in a 2:1 sand/soil mixture in pots in the greenhouse at a day temperature of 24°C ($\pm 2^\circ\text{C}$) and night temperature of 17°C ($\pm 2^\circ\text{C}$). The Russian wheat aphid (*Diuraphis noxia* (Mordvilko)) population was propagated on young susceptible Tugela wheat plants.

3.2. Methods

3.2.1. Wheat Infestation

Susceptible and resistant wheat plants were infested with aphids at the three-leaf stage by scattering the aphids evenly onto the plants (ca. 5 aphids/plant) and control samples (0 h) were harvested thereafter. Thereafter, plant tissue was harvested at 3 h intervals for another 30 h. The plant material was quick frozen using liquid nitrogen and stored at -80°C .

3.2.2. RNA Extraction

All apparatus used were first washed with soap, then in 10% (w/v) sodium dodecyl sulfate (SDS) and finally rinsed in water that was previously treated with 0.1% (v/v) dimethyl pyrocarbonate (DMPC) to destroy RNases. Solutions treated with 0.1% (v/v) DMPC were left in the fume hood overnight and autoclaved for 25 min at 125°C the next morning. The frozen tissue was ground to a fine powder in liquid nitrogen using a mortar and pestle that was baked at 260°C . The TriPure isolation reagent (Roche) was used to extract total RNA from the harvested plant material according to the manufacturer's specifications. The concentration of the RNA was determined (Sambrook *et al.*, 1989) and expressed as $\mu\text{g. ml}^{-1}$. To evaluate the quantity and quality of the extracted RNA, 500 ng total RNA of each treatment was separated on a 1% (w/v) agarose gel prepared in 0.5 x TAE

(20 mM Tris(hydroxymethyl)-amino-methane-hydrochloric acid (Tris-HCl), 0.5 mM ethylenediamine tetraacetic acid (EDTA), 0.28% (v/v) acetic acid) (Sambrook *et al.*, 1989). Ethidium bromide (EtBr) was added to a final concentration of $0.5 \mu\text{g}\cdot\text{ml}^{-1}$ to the gel to allow the visualization of the separated RNA. The running buffer used was a 0.5 x TAE solution. The RNA was diluted in loading buffer to a final concentration of 0.04% (w/v) bromophenol blue, 2.5% (w/v) ficoll, loaded on the gel and separated at $10 \text{ V}\cdot\text{cm}^{-1}$ for 1 h. The RNA was visualized under ultra violet light illumination (302 nm) and the gel photographed using a gel documentation system.

3.2.3. RT-PCR

Reverse transcription polymerase chain reaction (RT-PCR) was performed using a Titan One-Tube RT-PCR system (Roche) according to manufacturer's specifications with certain modifications.

In order to amplify putative protein kinase genes from the infested resistant wheat, two degenerate primers (Bovis 22 and 23) were designed (Table 3.1). Bovis 22 coded for the conserved amino acid sequence of sub domain VIb of the kinase domain from monocotyledonous protein kinases, while Bovis 23 coded for the same sequence from dicotyledonous protein kinases (Hanks *et al.*, 1988). During the RT-reaction, an anchored Oligo-dT primer (Bovis 32) containing an additional 5' tail sequence (Table 3.1) was used for the synthesis of the first strand cDNA. During the PCR step, Bovis 39, whose sequence was identical to this 5' tail, was used in combination with Bovis 22 and 23 respectively to amplify putative protein kinase genes.

Each RT-PCR reaction consisted of 10 ng total RNA, 2.5 pmol each of the respective degenerate, oligo-dT and tail specific primers, 0.25 mM deoxynucleotide triphosphates (dNTP's), 5 mM 1,4-dithiothreitol (DTT), 4 mM Tris-HCl pH 7.5, 20 mM KCl, 0.02 mM EDTA, 0.1% (v/v) Polyoxyethylene sorbitan monolaurate (Tween 20), 0.1% (v/v) 4-nonylphenolpolyethylenglycol (Nonidet P40), 10% (v/v) glycerol, 1.5 mM MgCl_2 , 0.5 μl of the Titan enzyme mix (Roche) and 10 μCi [α - ^{32}P]

Table 3.1. Nucleotide sequences of primers used in this study (Y = C or T, H = A or C or T, R = A or G, N = A or T or G or C and V = A or G or C). All oligonucleotides were designed using the Webprimer software (<http://seq.yeastgenome.org/cgi-bin/web-primer>).

| Primer | Primer Sequence | TM | Function |
|----------|--|--------|---|
| Bovis 22 | 5'- GAY ATH AAR CCN CAY AAY - 3' | 46.4°C | Degenerate primer for conserved subdomain VIb from monocotyledonous protein kinases |
| Bovis 23 | 5'- GAY GTN AAR CCN GAR AAY - 3' | 49.7°C | Degenerate primer for conserved subdomain VIb from dicotyledonous protein kinases |
| Bovis 26 | 5'- CAA CTT TCG ATG GTA GGA TAG - 3' | 51.3°C | Amplification of 18S rRNA gene as an internal control for RT-PCR |
| Bovis 27 | 5'- CTC GTT AAG GGA TTT AGA TTG - 3' | 49.4°C | Amplification of 18S rRNA gene as an internal control for RT-PCR |
| Bovis 32 | 5'- GAA GAA TTC TCG AGC GGC CGC TTT TTT TTT TTT TTT TVN - 3' | 65.5°C | Anchored oligo-dT with 5' tail primer for RT-PCR |
| Bovis 39 | 5'- GAA GAA TTC TCG AGC GGC - 3' | 53.5°C | 5' tail primer for PCR |
| Bovis 75 | P - 5' - AGG GTG GAT AAA CAG - 3' | 43.7°C | 5'-Phosphorylated primer for D20 RACE |
| Bovis 76 | 5' - AAT AAA GAA CCT GCT GTG AG - 3' | 50.1°C | Sense primer 1 for D20 RACE |
| Bovis 77 | 5' - CTT CGG AGA ACA CTT CCG AG - 3' | 55.1°C | Sense primer 2 for D20 RACE |
| Bovis 78 | 5' - CGG CAA CGA AAT GAA TAG AG - 3' | 51.5°C | Antisense primer 1 for D20 RACE |
| Bovis 79 | 5' - TGA ATA GAG GTT CTC GAC CG - 3' | 53.6°C | Antisense primer 2 for D20 RACE |
| Bovis 80 | 5' - TGA TGA TTC TGT TTA TCC ACC C - 3' | 52.4°C | Forward primer for D20 amplification |
| Bovis 81 | 5'- CAA CAG TGG ATC TTA AGT TGT C - 3' | 51.4°C | Reverse primer for D20 amplification |

deoxycytidine triphosphate (dCTP) in each 25 μ l reaction. Each reaction was covered with 30 μ l mineral oil. The reactions were performed in a Hybaid OmniGene thermal cycler using the following conditions: one cycle at 37°C for 30 min and 94°C for two min, 25 cycles at 94°C for 10 sec, 37°C for one min and 68°C for four min, 10 cycles at 94°C for 10 sec, 44°C for one min and 68°C for four min with an extension of 5 sec with each subsequent cycle followed by a final cycle at 68°C for seven min.

The amplified products were dissolved in loading buffer (50% (v/v) formamide, 0.0125% (w/v) bromophenol blue, 0.0125% (w/v) orange G), boiled for 5 min and separated on a 4% (v/v) Long Ranger™ denaturation gel (FMC Bioproducts) in 0.6 x TBE (6.48 mM Tris-HCl pH 8.0, 6.48 mM boric acid, 0.144 mM EDTA, 8 M urea, 0.55 μ l.ml⁻¹ N-N-N'-N'-tetramethylethylenediamin (TEMED), 4.5 μ l.ml⁻¹ ammonium peroxodisulfate (APS)). The running buffer used was 0.6 x TBE. The fragments were separated at 60 W for 2¼ h at constant current after the gel was pre-run for 30 min. The gel was dried on a gel drier for 1 h at 80 °C and exposed to an x-ray film (Agfa) for 4 days.

3.2.4. cDNA recovery

The recovery of the differentially expressed cDNA fragments was done by aligning the x-ray film and the dried gel. The bands of interest were excised using a sharp scalpel blade and placed into separate microcentrifuge tubes containing 100 μ l TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA). The tubes were incubated at room temperature for 15 min and then boiled for 10 min. The tubes were centrifuged at 12 000 g for three min and the supernatant transferred to a new tube.

The cDNA was re-amplified using Bovis 39 in combination with either Bovis 22 or 23. Each PCR reaction consisted of 1-3 μ l cDNA, 25 pmol of each primer, 0.25 mM dNTP's, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM MgCl₂, 0.001% (w/v) gelatin, 10% (v/v) dimethylsulfoxide (DMSO) and 2.5 U Taq

polymerase (Sigma-Aldrich). Each reaction was overlaid with 25 μl mineral oil.

The amplification regime was as follows: one cycle at 94°C for five min, 30 cycles at 94°C for one min, 44°C for 30 sec and 72°C for one min followed by a final cycle at 72°C for 10 min.

Following the PCR, the entire reaction was separated on a 1% (w/v) agarose gel (previously described in Section 3.2.2). The fragments of interest were excised from the gel and purified using the GFX PCR and gel purification kit (Amersham Biosciences) according to the manufacturer's specifications. The purified cDNA fragments were finally dissolved in 50 μl water.

3.2.5. Sub-cloning of amplified cDNA fragments

The re-amplified cDNA fragments were cloned into the pGemT-Easy vector (Promega). The amplified fragments (Section 3.2.4) were freeze dried and ligated into 12.5 ng pGemT-Easy plasmid in the presence of 3 Weiss units T4 DNA ligase, 30 mM Tris-acetate pH 7.9, 10 mM MgCl_2 , 10 mM DTT, 2 mM adenosine triphosphate (ATP) and 1% (w/v) polyethylene glycol 4000 (PEG 4000). The reaction was incubated at 4°C overnight.

The ligation mixtures were added to 25 μl competent *Escherichia coli* (JM109) cells (Promega) and incubated on ice for 20 min, after which they were placed at 42°C for 50 sec. The reactions were incubated at 37°C for 1 h after the addition of 965 μl LB medium (1% (w/v) tryptone, 0.5% (w/v) yeast-extract, 1% (w/v) NaCl) and shaken vigorously. The cells were finally plated onto LB plates (1% (w/v) tryptone, 0.5% (w/v) yeast-extract, 1% (w/v) NaCl, 3% (w/v) agar) containing 50 $\mu\text{g}\cdot\text{ml}^{-1}$ ampicillin, 250 $\mu\text{g}\cdot\text{ml}^{-1}$ 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) and 250 $\mu\text{g}\cdot\text{ml}^{-1}$ isopropyl- β -D-thiogalactopyranoside (IPTG).

White colonies containing putative recombinant plasmids were transferred to a new LB plate containing $50 \mu\text{g}\cdot\text{ml}^{-1}$ ampicillin and incubated overnight at 37°C .

3.2.6. Selection of recombinants

Plasmid DNA was extracted from the *E. coli* cells according to Sambrook *et al.* (1989). White colonies were inoculated into 5 ml LB shake cultures containing $50 \mu\text{g}\cdot\text{ml}^{-1}$ ampicillin and grown overnight at 37°C . The cells were harvested by centrifugation at $12\ 000\ \text{g}$ for 5 min and resuspended in 50 mM Tris-HCl pH 7.5, 10 mM EDTA, $10 \mu\text{g}\cdot\text{ml}^{-1}$ RNase A and incubated on ice for 5 min. The cells were lysed with 0.1 M NaOH, 0.5% (w/v) SDS and incubated on ice for 5 min. Ice-cold potassium acetate (pH 4.8) was added to a final concentration of 0.85 M to neutralize the lysate. The tubes were incubated on ice for 5 min and then centrifuged at $12000\ \text{g}$ for 5 min. The upper aqueous phase was transferred to a fresh tube and the plasmid DNA precipitated with 2.5 volumes ice-cold 100% (v/v) ethanol. The tubes were re-centrifuged, the pellet washed with ice-cold 70% (v/v) ethanol and was then left to air-dry. The plasmid DNA was finally dissolved in 50 μl distilled water. To verify the quality of the plasmid DNA, 2 μl of each sample was separated on a 1% (w/v) agarose gel (Section 3.2.2).

Two microlitres of the purified plasmid DNA were digested with 10 U *EcoRI* in the presence of 5 mM Tris-HCl pH 7.5, 1 mM MgCl_2 , 10 mM NaCl, 0.1 mM dithioerythritol (DTE) at 37°C for 1 h. The digestions were separated on a 1% (w/v) agarose gel (Section 3.2.2) and recombinant plasmids identified.

3.2.7. Reverse Northern Blot

Hybond N⁺ (Amersham) nylon membrane was used for the reverse Northern blot. A total of 200 ng DNA of each recombinant plasmid was digested with 2 U *SacI* in the presence of 3.3 mM Tris-acetate pH 7.9, 1 mM magnesium-acetate, 6.6 mM potassium-acetate, 0.05 mM DTT for 1 h at 37°C . A recombinant plasmid carrying the cloned actin gene was included as

a control and treated similarly. After digestion, the plasmid DNA was boiled for 5 min whereafter 1 volume 6 x SSC (90 mM NaCl, 9 mM sodium citrate pH 7.0) was added. The samples were then transferred to the membrane using a Slot blot apparatus. The individual wells were washed 3 times with 500 μ l 3 x SSC (45 mM NaCl, 4.5 mM sodium citrate pH 7.0) and the membrane dried for 15 min at 75°C.

The first strand cDNA probes used for the reverse Northern blot were prepared using 1 μ g total RNA isolated from infested plants harvested at the different time intervals. A mixture of 1 μ g total RNA and 25 pmol Bovis 32 primer (Table 3.1) were placed at 70°C for 5 min to denature the RNA and transferred to ice for 5 min. To this reaction mix, 200 units Improm-II Reverse Transcriptase (Promega), 10 μ Ci [α -³²P]-dCTP, 1 x ImProm-II reaction buffer, 3 mM MgCl₂, 0.5 mM dATP, 0.5 mM dTTP and 0.5 mM dGTP. The reactions were incubated at 25°C for 5 min and 42°C for 60 min for the extension of the first strand cDNA. The Improm-II Reverse Transcriptase was finally inactivated by incubation at 70°C for 15 min.

Hybridization solution (50% (v/v) formamide, 5 x SSPE (75 mM NaCl, 5 mM NaH₂PO₄ pH 7.4, 0.625 mM EDTA), 0.5% (w/v) SDS, 5 x Denhardtts (0.05% (w/v) polyvinylpyrrolidone (PVP), 0.05% (w/v) ficoll, 0.05% (w/v) bovine serum albumin (BSA)), 0.1 μ g.ml⁻¹ salmon sperm DNA) was added to the Hybond N⁺ membranes and the membranes were pre-hybridized for 2 h at 42°C. The probe was denatured at 94°C for 5 min, added to the hybridization solution and left to hybridize overnight at 42°C. The membranes were washed twice with 20 ml wash buffer 1 (30 mM NaCl, 3 mM sodium citrate pH 7.0, 0.1% (w/v) SDS) for 5 min at room temperature and twice with 20 ml wash buffer 2 (7.5 mM NaCl, 0.75 mM sodium citrate, 0.1% (w/v) SDS) for 15 min at 50°C. The membranes were finally exposed to x-ray film.

To evaluate the expression of each cDNA, the hybridization signal of each clone at each time interval was determined using a densitometer. This value was normalized against the value obtained for the control actin gene,

whereafter the values of each time interval was expressed relative to the 0 h control sample of that particular treatment. Clones were selected using the following criteria, namely 1) a large induction of expression, 2) differences of expression in the susceptible and resistant plants and 3) on an earlier induction time in the resistant plants compared to the susceptible plants.

3.2.8. Sequencing of clones

For each selected cDNA clone, 500 ng plasmid DNA was freeze-dried and sent to the Central Analytical Facility at Stellenbosch University for sequencing using the Big-DYE termination sequencing kit (Applied Biosciences). The plasmid DNA sequence was first deleted from the obtained sequences. Sequences were then translated to amino acid sequences using the ExPASy translate tool (<http://au.expasy.org/tools/dna.html>). The longest open reading frame (ORF) was used for a BLAST search on the GenBank website (<http://www.ncbi.nlm.nih.gov/Genbank>) to search for similarities on DNA and protein level with all known genes.

3.2.9. Confirmation of expression with RT-PCR

To confirm the expression of the identified D20 clone, RT-PCR was performed using a Titan One-Tube RT-PCR system (Roche) according to manufacturer's specifications with certain modifications. Each RT-PCR reaction consisted of 10 ng total RNA, 25 pmol of each primer (Bovis 80 and Bovis 81 (Table 3.1)), 0.25 mM dNTP's, 5 mM DTT, 4 mM Tris-HCl pH 7.5, 20 mM KCl, 0.02 mM EDTA, 0.1% (v/v) Tween 20, 0.1% (v/v) Nonidet P40, 10% (v/v) glycerol, 2.5 mM MgCl₂ and 0.2 µl of the Titan enzyme mix per 10 µl reaction. Each reaction was covered with 10 µl mineral oil. The reactions were performed in a Hybaid thermal cycler using the following conditions; one cycle at 42°C for 30 min, 94°C for two min, 10 cycles at 94°C for 10 sec, 50°C for 30 sec and 68°C for two min, 25 cycles at 94°C for 10 sec, 50°C for 30 sec, 68°C for two min with an extension of 5 sec with each subsequent cycle followed by a final cycle of 68°C for 10 min.

A control reaction was performed by similarly amplifying the 18S rRNA using Bovis 26 and 27 as primers (Table 3.1). The reactions were finally separated on a 1% (w/v) agarose gel (Section 3.2.2).

3.2.10. Genomic DNA extraction

Tugela, Tugela DN, Tugela DN2, Tugela DN3 and Tugela DN5 wheat were germinated and grown as described (Section 3.2.1). The tissue was harvested and ground to a fine powder in liquid nitrogen using a mortar and pestle. Extraction buffer (25 mM NaCl, 5 mM Tris-HCl pH 7.8, 2.5 mM EDTA, 1 mM sodium meta-bisulfate, 1.25% (w/v) SDS, 10 $\mu\text{g}\cdot\text{ml}^{-1}$ RNase A) was added to the ground tissue and incubated at 65°C for 30 min. One volume chloroform:isoamyl alcohol (24:1) was added to the tube, mixed and the tubes centrifuged at 12000 g for 15 min at 4°C. The upper phase was transferred to new tubes and 2 volumes 100% (v/v) ethanol were added to precipitate the DNA. The DNA was scooped out into clean tubes and washed with 5 ml 70% (v/v) ethanol. The DNA was air dried and dissolved in 2 ml water. The concentration of the DNA was determined and expressed as $\mu\text{g}\cdot\text{ml}^{-1}$ (Sambrook *et al.*, 1989). To evaluate the quantity and quality of the extracted DNA, 500 ng genomic DNA was separated on a 1% (w/v) agarose gel (Section 3.2.2).

3.2.11. Southern blot analysis

A total of 25 μg DNA of each cultivar was digested overnight with 50 U *EcoRI* in the presence of 5 mM Tris-HCl pH 7.5, 1 mM MgCl_2 , 10 mM NaCl and 0.1 mM DTE at 37°C. The digestions were separated on a 0.8% (w/v) agarose gel (Section 3.2.2). The digested DNA was transferred to a nylon membrane according to Chomczynski (1992). The DNA was first denatured in 3 M NaCl, 0.4 M NaOH for 30 min and then transferred to the membrane using a 1.5 M NaCl, 0.4 M NaOH transfer solution. After transfer, the membrane was neutralized in 0.2 M Na_2HPO_4 , 0.2 M NaH_2PO_4 pH 6.8 for 10 min and dried for 15 min at 70°C.

The membrane was pre-hybridised in 10 ml Ultrahyb solution (Ambion) for 1 h at 42°C. The probe was prepared by digesting 2 µg of the specific D20 clone with 10 U *EcoRI* in the presence of 5 mM Tris-HCl pH 7.5, 1 mM MgCl₂, 10 mM NaCl and 0.1 mM DTE for 1 h at 37°C. The reaction was separated on a 1% (w/v) agarose gel and the insert purified from the gel using the GFX PCR and gel purification kit (Amersham Biosciences) according to the manufacturer's specifications. The purified insert was finally dissolved in 50 µl water. Fifty nanogram of the insert was labelled with 40 µCi [α -³²P]-dCTP using the Rediprime kit (Amersham Biosciences) according to the manufacturer's specifications.

The labelled probe was purified on a Sephadex G75 column (Sambrook *et al.*, 1989), denatured at 94°C for 5 min and added to the Ultrahyb solution. Hybridization was allowed to proceed overnight at 42°C. The membranes were finally washed twice with 25 ml wash buffer 1 (30 mM NaCl, 3 mM sodium citrate pH 7.0, 0.1% (w/v) SDS) for 5 min at 42°C and twice with 25 ml wash buffer 2 (1.5 mM NaCl, 0.15 mM sodium citrate pH 7.0, 0.1% (w/v) SDS) for 15 min at 42°C. The membranes were finally exposed to x-ray film.

3.2.12. 5'-Rapid amplification of cDNA ends (RACE)

In order to obtain the full sequence for D20, a 5'-RACE reaction was performed (Fig 3.1). Gene specific primers were designed and amplification was done using the 5'-Full RACE Core Set (TaKaRa Bio Inc).

A 5' phosphorylated primer (Bovis 75) was designed together with two sense (Bovis 76 and 77) and two antisense primers (Bovis 78 and 79) (Fig 3.1a, Table 3.1). The phosphate group of Bovis 75 would enable the first strand cDNA fragment to be self ligated at its ends to form either a circular molecule or a concatemer. First strand cDNA synthesis was performed (Fig 3.1b) and the product from the RT-PCR reaction was ligated according to the manufacturer's specifications (Fig 3.1c).

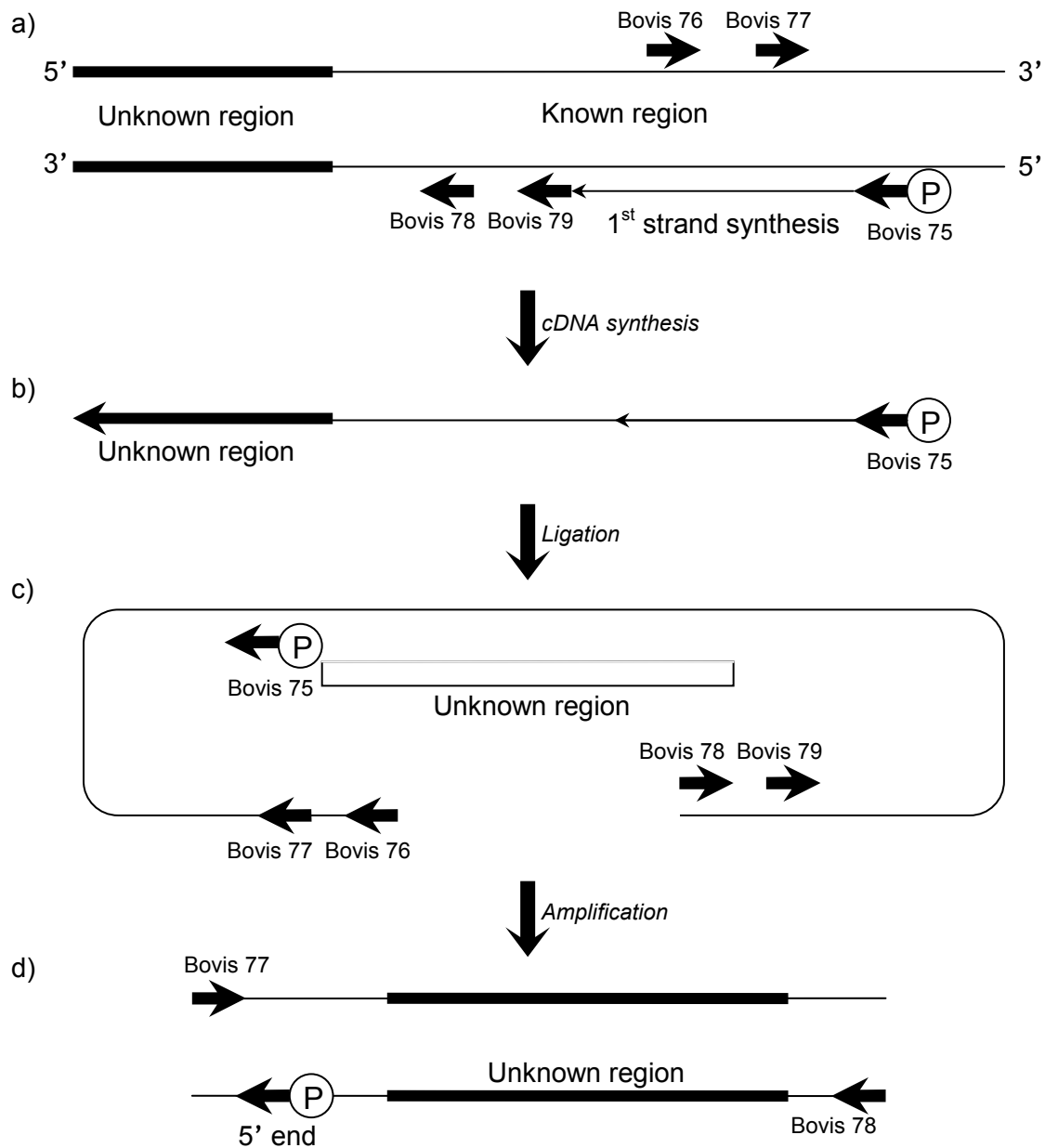


Figure 3.1. Schematic diagram of 5'RACE using TaKaRa 5'-Full RACE Core Set. (a) the designed primers are indicated. (b) RT-PCR was performed, (c) the single strand cDNA products were ligated and (d) the unknown regions are amplified. Arrows indicate the directions into which the primers will be extended, while the (P) indicates the phosphorylated primer.

Bovis 76 and 78 were used for the first PCR amplification step while Bovis 77 and 79 were used for the second amplification (Fig 3.1d). After the ligation of the first strand cDNA, the first amplification step was performed using 1 μ l of the ligation reaction (Fig 3.1c), 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01% (w/v) gelatine, 0.4 mM dNTP's and 5 U Taq polymerase. The reaction was covered with 30 μ l oil. The conditions for amplification were one cycle at 92°C for 3 min, 30 cycles at 94°C for 30 sec, 48°C for 30 sec and 72°C for two min. The second amplification step was performed using 2 μ l of the first PCR product, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01% (w/v) gelatine, 0.4 mM dNTP's and 5 U Taq polymerase. The conditions for amplification were one cycle at 92°C for three min, 30 cycles at 94°C for 30 sec, 53°C for 30 sec and 72°C for two min.

Following the second PCR, the products of the entire reaction were separated on a 1% (w/v) agarose gel (Section 3.2.2). The fragments of interest were excised from the gel and purified (Section 3.2.11). Sequencing and analysis of the obtained sequences were done as previously described (Section 3.2.8).

Chapter

4

Results

4.1. RWA infestation

Resistant (Tugela *DN*) and susceptible (Tugela) wheat plants were planted and infested with RWA. One month after infestation, the resistant Tugela *DN* cultivar appeared to be able to withstand the infestation, while the susceptible cultivar began to show early signs of wilting (Fig 4.1a). Necrotic spots caused by the degradation of chloroplasts (Ryan *et al.*, 1990) were visible in the Tugela *DN* one week after infestation. The RWA induced long, narrow, yellow streaks on the leaves of the Tugela plants (Fig 4.1b). The leaves of Tugela also began to roll inwards one week after infestation creating a shelter for the RWA. These results indicated that successful infestation took place and that the harvested tissue could be used as a source of RNA to be used for DD RT-PCR.

4.2. Identification and cloning of differentially expressed cDNA fragments after RWA infection

Tugela *DN* leaves infested with RWA were harvested and total RNA was extracted. Before the DD RT-PCR was performed, the quality and the quantity of the extracted RNA were determined. Of each extraction, 500 ng total RNA was separated on an agarose gel (Fig 4.2). On the gel, the characteristic banding pattern of the ribosomal RNA was visible. The bands were sharp and not broken down. The smear in the background represents the mRNA. Furthermore, the RNA concentrations of all the samples were approximately the same. Since the quality and quantity of RNA was good, it was therefore used for all subsequent amplification reactions.

After the quality of the RNA was assessed, a DD RT-PCR was performed to identify differentially expressed cDNA fragments. A total of 25 cDNA fragments were found to be inducibly expressed using the Bovis 22 and 39 primer combination (Fig 4.3a), while 24 were found for the Bovis 23 and 39 primer combination (Fig 4.3b). The expression levels of these cDNA fragments differed substantially, with some being expressed at high levels

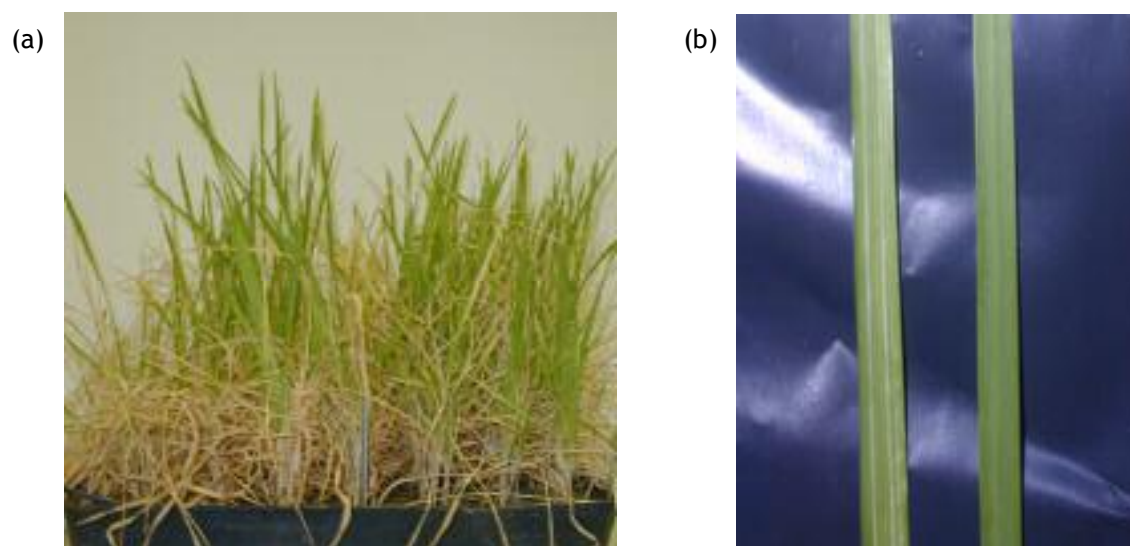


Figure 4.1. Infestation of Tugela and Tugela *DN* wheat with the RWA. In (a), the susceptible and the resistant plants were infested, while in (b), the effect of infestation on the individual leaves are indicated. In both (a) and (b), Tugela is presented on the left and Tugela *DN* on the right.

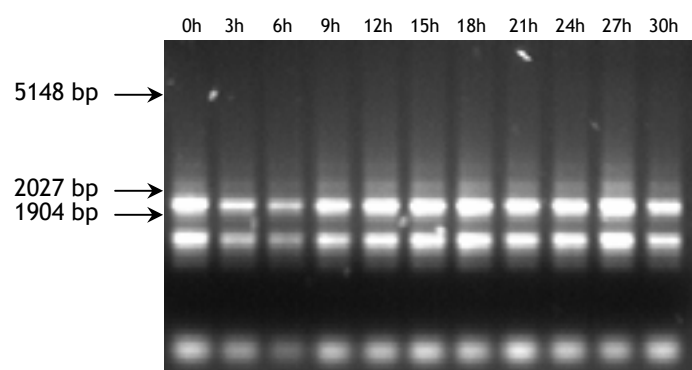


Figure 4.2. Total RNA extracted from infested resistant plants. The time intervals are as indicated. Sizes are as indicated.

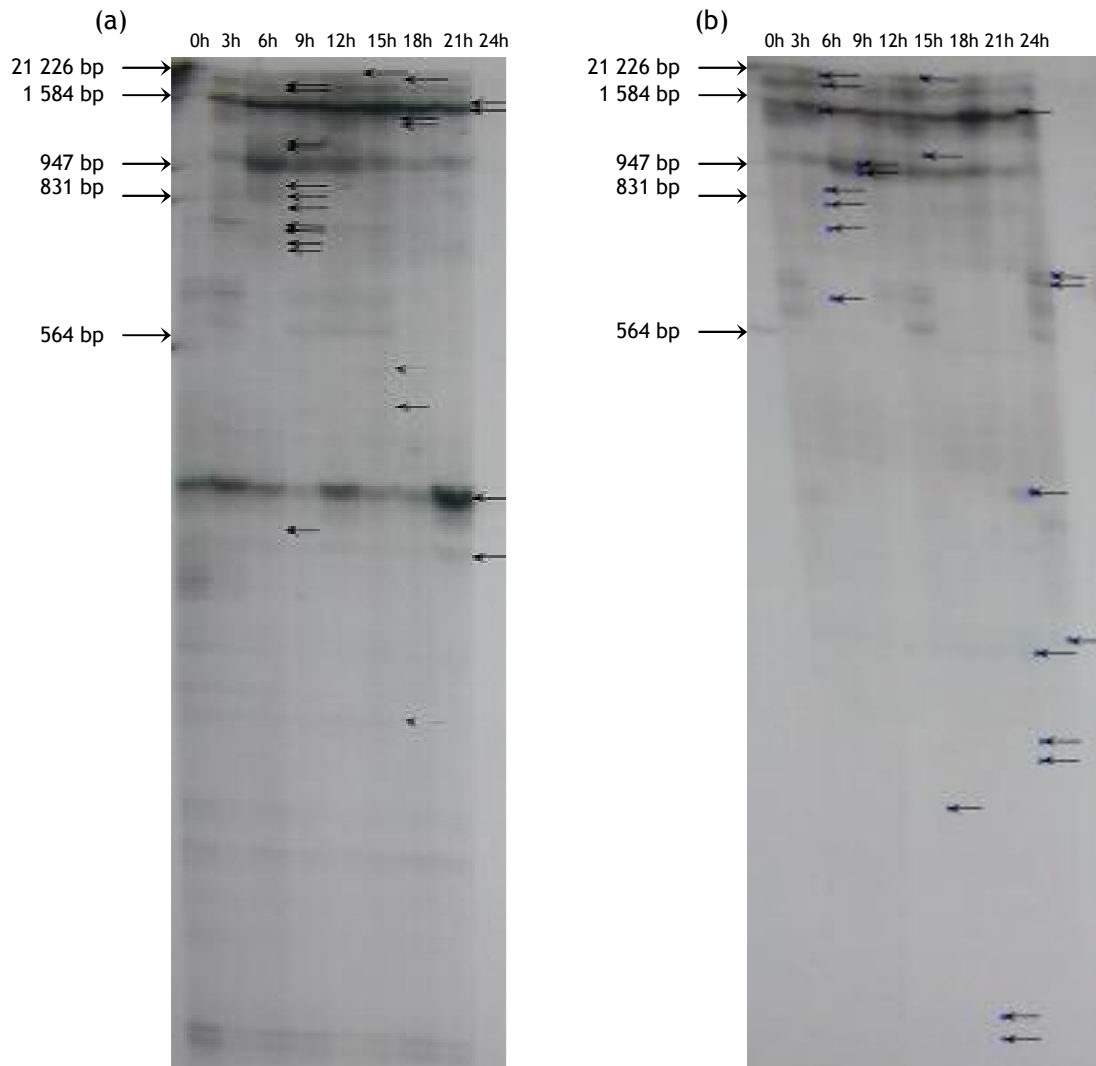


Figure 4.3. DD RT-PCR amplification of differentially expressed putative protein kinase genes from Tugela *DN* infested with RWA. In (a) the Bovis 22 and Bovis 39 combination and in (b) Bovis 23 and Bovis 39 combinations were used. The time intervals are as indicated. Arrows indicate differentially expressed cDNA fragments.

and others at extremely low levels. A number of these cDNA fragments were inducibly expressed during the early stages of infestation, while others were induced only at later stages (Table 4.1). These identified cDNA fragments were cut from the gel, purified and re-amplified. The differentially expressed genes were called M1-25 for those amplified using the monocot specific primer and D1-21 for those amplified using the dicot specific primer.

After re-amplification, the different fragments were cloned into the pGEM-T Easy plasmid vector and recombinants were selected using α -complementation (Fig 4.4). Blue colonies retained their ability to utilize X-gal as substrate because of an intact LacZ' gene present on the non-recombinant plasmid. This ability was lost in the white colonies due to the presence of an insert within the polycloning site of the plasmid, leading to a defective LacZ' gene whose encoded product was unable to utilize X-gal.

The white colonies were selected and screened for the presence of an insert in the plasmid by digesting the insert from the plasmid using *EcoRI* (Fig 4.5a and b). Lane 1 in both figure 4.5a and b represent *EcoRI* digested non-recombinant plasmids. The single 2 kb fragment therefore represented the digested plasmid DNA. The other smaller fragments present in the digested clones thus represented the cloned inserts. Most inserts were present as single intact fragments. The sizes of the inserts were compared to and verified with that of the original fragments present on the acrylamide gel (Fig 4.3). With the exception of a few, all corresponded in size to the original RT-fragment. Two inserts were visible for clone M10 (Fig 4.5a), D3 and D20 (Fig 4.5b). This was most probably caused by an internal *EcoRI* restriction site present within the insert. The unique band pattern for clones M5, M6 and M9 (Fig 4.5a) and clones D4, D6, D9 and D13 (Fig 4.5b) may have been caused by a mutation in one of the *EcoRI* restriction sites thus showing a larger single band that lies slightly above the normal plasmid band. Alternatively it is possible that these clones represent undigested plasmid leading to an altered band pattern on the gel. Even though some of

Table 4.1. Number of differentially expressed fragments induced at various time intervals.

| Primer combination | Time (h) | Number of fragments induced |
|----------------------------|----------|-----------------------------|
| <i>Bovis 22 and 39</i> | 0 | - |
| | 3 | 8 |
| | 6 | 6 |
| | 9 | - |
| | 12 | 1 |
| | 15 | 6 |
| | 18 | - |
| | 21 | 4 |
| | 24 | - |
| <i>Bovis 23 and 39</i> | 0 | - |
| | 3 | 7 |
| | 6 | 2 |
| | 9 | - |
| | 12 | 4 |
| | 15 | 2 |
| | 18 | - |
| | 21 | 5 |
| | 24 | 4 |

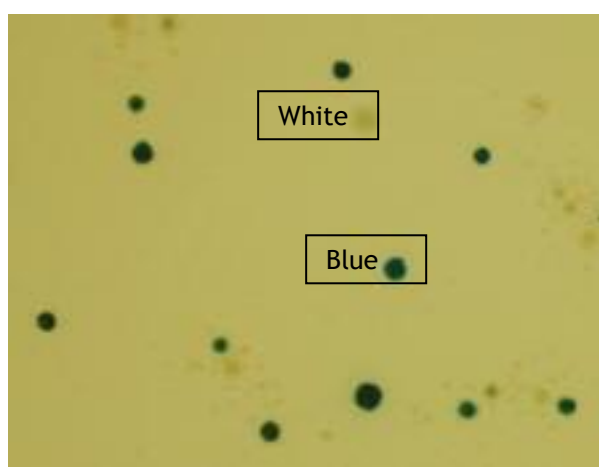


Figure 4.4. Selection of recombinant plasmids using α -complementation. Blue colonies contained non-recombinant plasmids, while the white colonies contained a recombinant plasmid.

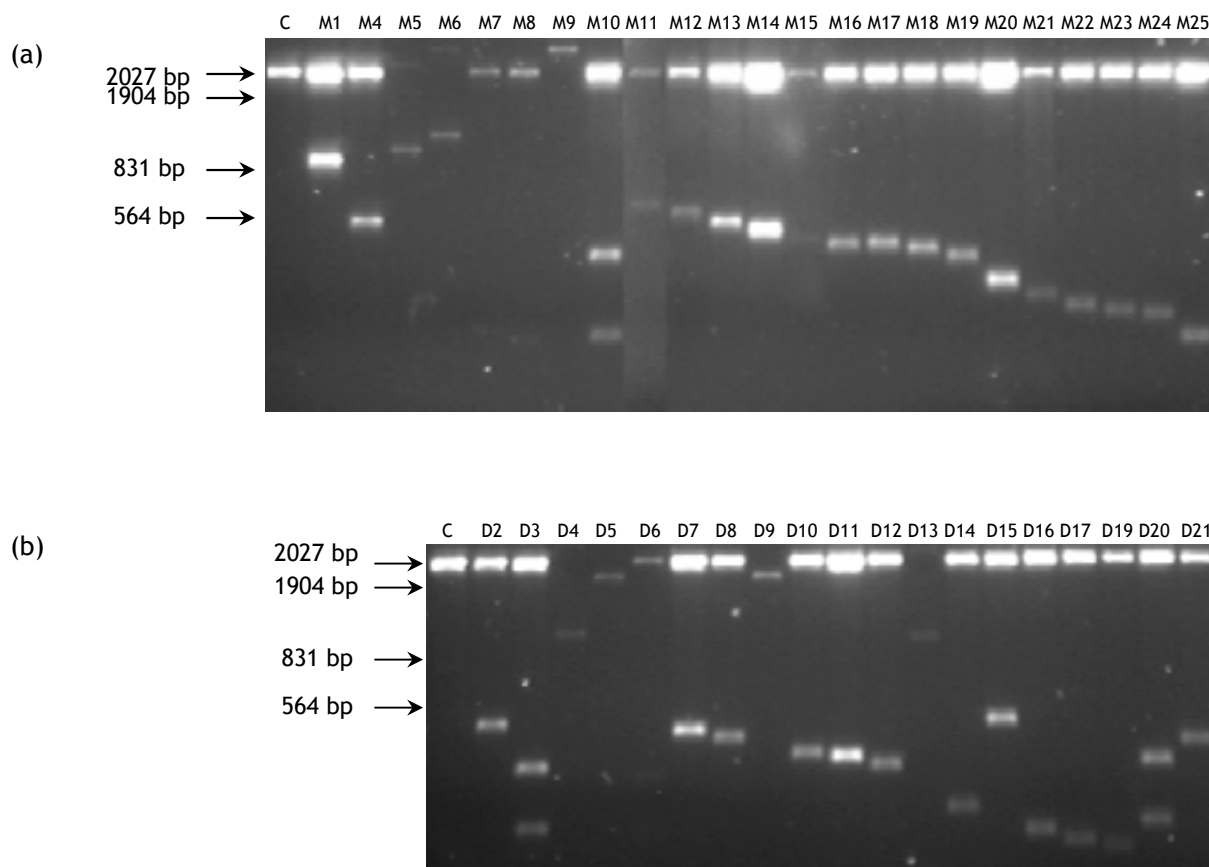


Figure 4.5. Re-amplification of the differential display fragments with the different primer combinations (a) Bovis 22 and 39, (b) Bovis 23 and 39.

the plasmids appear to be empty (e.g. M7 and M8) it is possible that the concentration of the digested plasmid was too low to see the insert. Three recombinant plasmids (D15, D20 and D21) contained inserts that were larger than the original fragments that were cut from the acrylamide gel. No explanation for these results can be given at this stage. Difficulties were experienced with the cloning of some of the larger fragments which were subsequently eliminated from the study.

4.3. Selection of clones

Since the generation of false positives is a well known flaw of RT-PCR, a reverse Northern blot was performed to verify the expression of the cloned genes in both the infested resistant and susceptible plants. The recombinant plasmids containing the different cDNA fragments were transferred to a nylon membrane using a slot blot apparatus. Total mRNA isolated from different time intervals was reverse transcribed to cDNA, thereby generating cDNA probes. The results are as indicated (Fig 4.6 and 4.7).

In the resistant plants, clones M16, M17, M19, M20, D14, D15, D17, D19, D20 and D21 clones all showed an induction of expression as early as 3 hours post infection (h.p.i.) with the highest expression levels being reached at 9 h.p.i. for most of them. In contrast the expression levels in the infested susceptible plants remained more or less constant. In addition, the expression of both M16 and M17 are induced earlier in the resistant than in the susceptible plants (Fig 4.6). The most obvious of all the clones was M18 which showed a 4 fold higher expression level when compared to the other genes. In both infested susceptible and resistant plants, the expression of the gene is induced within 3 h.p.i. In the infested susceptible plants, the maximum increase was two fold 3 h.p.i. In the infested resistant plants, the maximal 4x induction was found only at 12 h.p.i. with a smaller 3 fold induced expression occurring at 3 h.p.i. Several clones produced no detectable levels of hybridization. It was thus impossible to quantify their expression.

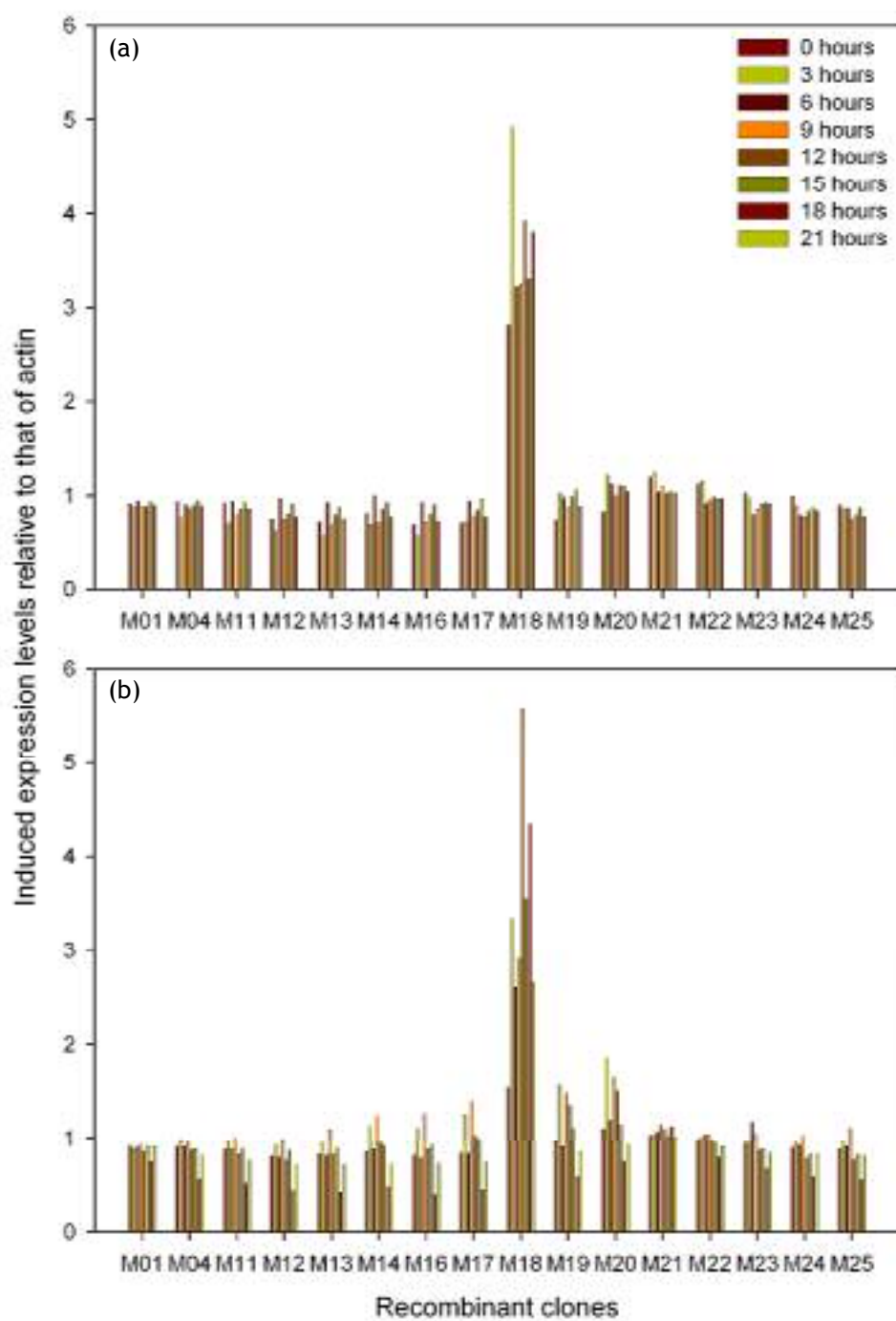


Figure 4.6. Reverse Northern blot of cDNA clones generated using the Bovis 22 and 39 combination. In (a) the cDNA probes were made using mRNA isolated from infested susceptible plants while in (b) total RNA from infested resistant plants were used. The time intervals are as indicated.

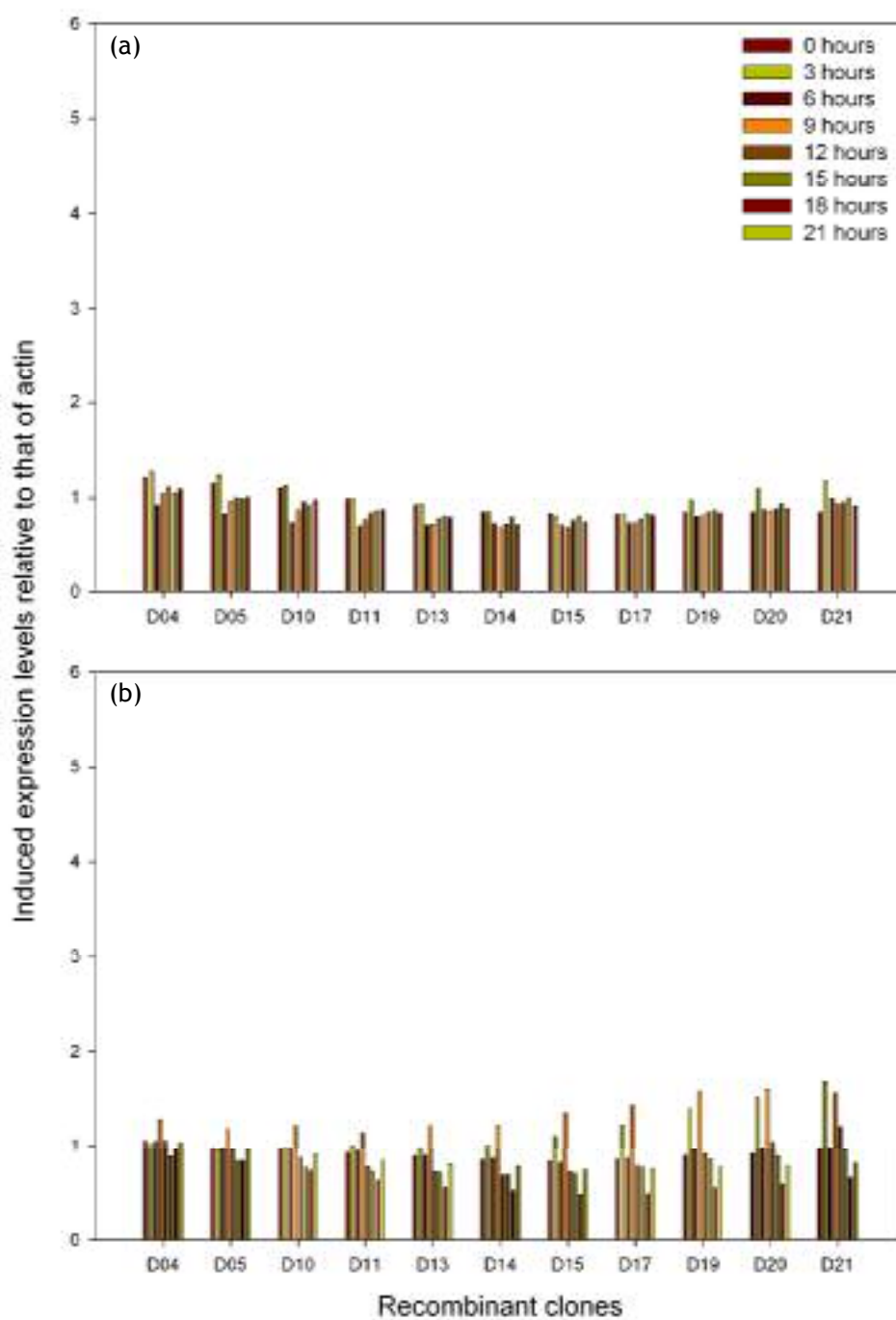


Figure 4.7. Reverse Northern blot of cDNA clones generated using the Bovis 23 and 39 combination. In (a) the cDNA probes were made using mRNA isolated from infested susceptible plants while in (b) total RNA from infested resistant plants were used. The time intervals are as indicated.

4.4. Sequencing of differentially expressed genes

From the pool of cloned cDNAs obtained after DD RT-PCR, 11 were selected to be sequenced and analysed. The other clones were discarded since they did not meet the criteria used for selection. The clones either did not show induction or was induced in both the susceptible and resistant plants.

Once the DNA sequences were obtained, the plasmid sequences were removed leaving only that of the inserts. These sequences were then translated into amino acid sequences. The longest open reading frames were then compared to known sequences in the GenBank database. Sequence homologies, identities and E-values are indicated in table 4.2.

Clone M17 consisted of 721 bases with the longest open reading frame (ORF) coding for a polypeptide 166 amino acids in length (Fig 4.8). On amino acid level, it showed homology to indole-3-glycerol phosphate lyase from *Oryza sativa*, *Triticum aestivum* and *Zea mays*. It also shows homology to tryptophan synthase from *Zea mays*. Indole-3-glycerol phosphate lyase catalyzes the formation of free indole and tryptophan synthase catalyzes the biosynthesis of L-tryptophan.

Clone M18 was 566 bases in length and the longest ORF coded for a polypeptide 206 amino acids in length (Fig 4.9). On amino acid level, it showed homology to ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) from *Hordeum comosum*, *Hordeum vulgare* subsp. *vulgare* and *Triticum aestivum*. RuBisCO is a catalyst of the Calvin-Benson-Bassham cycle that enables inorganic atmospheric carbon dioxide to be converted into organic cellular constituents.

Clone D14 consisted of 639 bases with the longest ORF coding for a 120 amino acid polypeptide (Fig 4.10). On amino acid level, it showed homology to triosephosphate isomerase from *Secale cereale*, *Triticum aestivum*, *Hordeum vulgare* and *Zea mays*. Triosephosphate isomerase

Table 4.2. Identities and E-values of genes showing homology to the cloned cDNA fragments. The clones indicated in bold show no homology to any know gene in the database. The clones in bold and italics only shows homology on DNA level.

| | Clone | Accession nr. | Similar gene | Identity | E-value |
|----------------|--------------|----------------------|---|-----------------|----------------|
| Unknown | M19 | | Unknown | | |
| | M20 | A44803 | pG1 protein - human (fragment) | 26/41 (63%) | 5e-05 |
| | | ZP_00203331 | Hypothetical protein Avar026203 [<i>Anabaena variabilis</i> ATCC 29413] | 17/21 (80%) | 0.43 |
| | | ZP_00203430 | Hypothetical protein Avar020180 [<i>Anabaena variabilis</i> ATCC 29413] | 17/21 (80%) | 0.6 |
| | | ZP_00226931 | COG1173: ABC-type dipeptide/oligopeptide/nickel transport systems, permease components [<i>Kineococcus radiotolerans</i> SRS30216] | 15/36 (41%) | 0.69 |
| | D13 | CAE26945 | Hypothetical protein [<i>Rhodopseudomonas palustris</i> CGA009] | 19/51 (37%) | 3.4 |
| | D15 | CAE04608 | OSJNBb0004G23.6 [<i>Oryza sativa</i> (japonica cultivar-group)] | 57/73 (78%) | 3e-14 |
| | | AAN15495 | Unknown protein [<i>Arabidopsis thaliana</i>] | 39/71 (54%) | 0.003 |
| | | G96652 | Protein F23N19.15 [imported] - <i>Arabidopsis thaliana</i> | 39/71 (54%) | 0.005 |
| | D20 | | Unknown | | |
| D21 | | Unknown | | | |

| | Clone | Accession nr. | Similar gene | Identities | E-value |
|--------------|--------------|----------------------|--|-------------------|----------------|
| Known | M17 | AAP44667 | Putative indole-3-glycerol phosphate lyase [<i>Oryza sativa</i> (japonica cultivar-group)] | 119/162 (73%) | 1e-55 |
| | | BAC81205 | Indole synthase [<i>Triticum aestivum</i>] | 107/154 (69%) | 6e-50 |
| | | AAG42687 | Indole-3-glycerol phosphate lyase [<i>Zea mays</i>] | 108/164 (65%) | 9e-50 |
| | | CAA54131 | Tryptophan synthase, alpha subunit [<i>Zea mays</i>] | 103/154 (66%) | 5e-49 |
| | M18 | AAN27974 | Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [<i>Hordeum comosum</i>] | 202/214 (94%) | 0e-112 |
| | | P11383 | Ribulose bisphosphate carboxylase large chain precursor (RuBisCO large subunit) | 202/214 (94%) | 0e-111 |
| | | AAN27989 | Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [<i>Hordeum vulgare</i> subsp. vulgare] | 202/214 (94%) | 0e-111 |
| | | AAP92166 | Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [<i>Triticum aestivum</i>] | 202/214 (94%) | 0e-111 |
| | D14 | CAA81487 | Triosephosphate isomerase [<i>Secale cereale</i>] | 116/122 (95%) | 2e-53 |
| | | CAC14917 | Triosephosphate-isomerase [<i>Triticum aestivum</i>] | 115/122 (94%) | 2e-52 |
| | | AAB41052 | Cytosolic triosephosphate isomerase [<i>Hordeum vulgare</i>] | 114/122 (93%) | 2e-51 |
| | | AAB81110 | Triosephosphate isomerase 1 [<i>Zea mays</i>] | 110/122 (90%) | 7e-51 |
| | D17 | M63251 | <i>Buchnera aphidicola</i> (primary endosymbiont of <i>Diuraphis noxia</i>) 16S ribosomal RNA | 382/388 (98%) | 0 |
| | | AB033776 | <i>Buchnera</i> sp. gene for 16S rRNA, partial sequence | 384/392 (97%) | 0 |
| | | M63247 | <i>Buchnera aphidicola</i> (primary endosymbiont of <i>Rhopalosiphum maidis</i>) 16S ribosomal RNA | 381/392 (97%) | 0 |
| | | AP001118 | <i>Buchnera aphidicola</i> str. APS (<i>Acyrtosiphon pisum</i>) | 383/392 (97%) | 0 |
| | | AP000398 | genomic DNA, complete sequence, segment 1/2 | | |
| | D19 | NP_343764 | 2-isopropylmalate synthase, putative (leuA-3) [<i>Sulfolobus solfataricus</i> P2] | 14/28 (50%) | 3 |

a)

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GAAGAATTCTCGAGCGGGCGAGGGACTGGGAGCTTCACCGCCGCGCCAGAGAGGCCGGTGTACGAGGTC
TTATAGTACCCGATCTTCCTTACACGGAGGCAAGTGTCTGAGCATTGAAGCCAAGAAGAACGAGATCG
AGCTGGTGCTGCTTACAACACCAACTACAAAGGCAGAGAGGATGAATGAGATCACATCAGCTTCCGAAG
GGTTTGTGTACCTTGTAAGTATCAACGGATTAACAGGGGGCCCGCCCAAGTGTGAATCCGCACGTTAAGG
ATCTTCTCAGGGAGATTAAGCAGGTCACTGACAAAAGCAGTTGCTGTTGGATTTGGCATAATCCACCCCTG
ACCATGTTAGGCAGGTTGCTCAGTGGGGTGCAGACGGTGTGATAATCGGCAGTGCATGGTGAAGCAGC
TAGGTGAAGCAAATTCTCCGAGAGAAGGATTGAAGAGGCTGGAAGTATATGCCAGGAGTCTCAAGGATG
CACTGCATGCCGTGATATGTACAATATAGGACAATGTACTAAATATCGACGAACCGGAATTGCGATGG
CAAGTTAGCTTGGTGTTCCTAGTAATACAAGTGGTGGTAGAAGCAATAAGCTAGAAGTATTTGTTAGTT
TTTTCTTGTATGGCATGCAAATGATGGTGGTGGAGTACCCGGTCCTTTCCTTCTAGCCAATTAATTA
GTTCAATTAAGTTTAAAAAAAAAAAAAAAAAAAAGCGGNCGCTCGAAAATCTTCAATCNCTAGN

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b)

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M17 -----ERRGTGSFTAAREAGV 17
AAP44667 VIQASAARALAAGATMDGVMSMLAEVTPELSCPVVLFSYFGPIVRRGPANFTAAREAGV 180
                                     ***...*****:***

M17 RGLIVPDLPHYTEASVLSIEAKKNEIELVLLTTPPTKAERMETNEITSASEGFVYLVLSING 77
AAP44667 QGLIVPDLPHYVETSTFRSEAIKNNLELVLLTTPATPADRMKA--ITAASGGFVYLVSVNG 238
:*****.*.*.: ** *.:*****.* *.*.:..*:* ** **.*:*

M17 LTGARPSVNPVHKDLLREIKQVTDKAVAVGFGISTPDHVRQVAQWADGVIIGSAMETVK 137
AAP44667 VTGSIQNVNPRVEHLLQEIKQVTDKAVCVGFGISTPDHVRQIADWGADGVIIGSAM--VR 296
:*: .***.*:.*:*****.*****.*:*****.*:*

M17 QLGEANSPREGLKRLEVYARSLKDALHAVICTI 170
AAP44667 QLGEAASPKQGLKRLEKYARSLKDALP----- 323
***** *.:***** ***** : .:

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Figure 4.8. Sequence analysis of M17. (a) DNA sequence of M17. (b) Amino acid alignment of the largest ORF to indole-3-glycerol phosphate lyase (AAP44667 from *Oryza sativa*). The conserved amino acids are indicated with an asterisk. The letters in bold in a) indicate the poly A tail and the letters in bold italics is the stop codon.

a)

TATTAGCTAAAGCATTGCGTATGTCTGGGGGAGATCATATCCACTCCGGTACAGTAGTAGGTAAGTTAG
 AAGGGGAACGCGAAATGACTTTAGGTTTTGTTGATTTATTGCGCGATGATTTTATTGAAAAGGATCGTG
 CTCGCGGTATCTTTTTCACTCAGGACTGGGTATCCATGCCAGGTGTTATACCGGTAGCTTCAGGTGGTA
 TTCATGTTTGGCATATGCCAGCTCTGACCGAAAATCTTTGGGGACGATTCTGTATTACAATTTGGTGGAG
 GAACTTTAGGACATCCTTGGGGAAAATGCACCTGGTGCAGCAGCTAATCGAGTGGCTTTAGAAGCCTGTG
 TACAAGCTCGTAACGAAGGGCGCGATCTTGCTCGCGAAGGTAATGAAATATCCGAGCAGCTTGCAAAT
 GGAGTCTGAACTAGCCGAGCTTGTGAAGTATGGAAGGCGATCAAATTCGAGTTCGAGCCGGTAGATA
 CTATTGATAAG ***TAG***AATAAACTAAAGATAAAGAAGAGAAGGTATAAATAAAAAAGAAACGAAAG***AAAAA***
AAAAAAAAAAAAAAAAAGCGCCGCTCGAGAATTCTTC

b)

| | | |
|----------|--|-----|
| M18 | -----AANTTLAHYCRDNGLLLHIHRAMETH | 29 |
| AAN27974 | NATAGTCEEMIKRAVFARELGVPIVMHDYLTGGFTANTTLAHYCRDNGLLLHIHRAM--H | 298 |
| | :***** * * | |
| M18 | AVIDRQKNHGMETHFRVLAKALRMETSGGDHIHSGTVVGKLEGEREMETTLGFVDLLRDD | 89 |
| AAN27974 | AVIDRQKNHGM--HFRVLAKALRMS--GGDHIHSGTVVGKLEGER--EMTLGFVDLLRDD | 352 |
| | ***** . ***** * ***** | |
| M18 | FIEKDRARGIFFTQDWVSMETPGVIPVASGGIHVHMETPALTEIFGDDSVLQFGGGTLG | 149 |
| AAN27974 | FIEKDRARGIFFTQDWVSMP--GVIPVASGGIHVHMP--ALTEIFGDDSVLQFGGGTLG | 408 |
| | ***** ***** ***** | |
| M18 | HPWGNAPGAAANRVALEACVQARNEGRDLAREGNEIIRAACKWSPELAAACEVWKAIKFE | 209 |
| AAN27974 | HPWGNAPGAAANRVALEACVQARNEGRDLAREGNEIIRAACKWSPELAAACEVWKAIKFE | 468 |
| | ***** | |
| M18 | FEPVDTIDK-- | 218 |
| AAN27974 | FEPVDTIDKKV | 479 |
| | ***** | |

Figure 4.9. Sequence analysis of M18. (a) DNA sequence of M18. (b) Amino acid alignment of the largest ORF to Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (AAN27974 from *Hordeum comosum*). The conserved amino acids are indicated with an asterisk. The letters in bold in a) indicate the poly A tail and the letters in bold italics is the stop codon.

a)

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GAAGAATTCTCGAGCGGCGAGAAGCTGGATCAACCATGGCAGTTGTCGCTGAACAGACAAAAGCAATTG
CTGGCAAGATCAAGGACTGGACCAATGTGGTTGTCGCTTACGAACCAGTGTGGGCCATTGGCACCGGTA
AAGTTGCGACACCGGCTCAGGCGCAGGAAGTCCATGCCAATCTGAGGGACTGGCTCAAGACCAATGTGA
GCCCCGAGGTTGCTGAATCCACGAGGATCATCTATGGAGGATCTGTAAGCTGGCGCAAGCTGCAAGGAGT
TGGCAGGGCAGCCCGACGTTGATGGTTTCCTCGTTGGTGGAGCTTCTTTGAAGCCTGAGTTCATTGACA
TCATCAACGCCGCCACCGTGAAGTCCGCCTAAGAAAAATCTTGATGTGGCTCCTCTCGGTGCGGAGGAA
GACAGCGAAGATGAAGAGACTCCTTTTTTCTCCTGTGTTTATTATGTGTGTACTATGCTAGTAATTTTG
TGGTGGAGCAGCTGCTACTGTTTTCTCCCCCCTGACTTGAGCTTGCAAGCCAATAATGATAATGTT
TGCTACTGAACATATATCAGCAATAATCTTTAGCTCCTATTAATGATAACAATGTGTATCGGCTGAAAA
AAAAAAAAAAAAAAAAAAAAGCGGCCGCTCGA

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b)

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D14 -----RREAGSTMETAVVAEQTKAIAGKIKDWTNVVAYEPVWAIIGTKVAT 47
CAA81487 GLKVIACVGETLEQREAGSTME--VVAEQTKAIADKIKDWTNVVAYEPVWAIIGTKVAS 178
          :*****:*****.*****:
D14 PAQAQEVHANLRDWLKTNVSPEVAESTRIYGGSVTGASCKELAGQPDVDGFLVGGASLK 107
CAA81487 PAQAQEVHANLRDWLKTNVSPEVAESTRIYGGSVTGASCKELAAQPDVDGFLVGGASLK 238
          *****.*****
D14 PEFIDIINAATVKSA 122
CAA81487 PEFIDIINAATVKSA 253
          *****

```

Figure 4.10. Sequence analysis of D14. (a) DNA sequence of D14. (b) Amino acid alignment of the largest ORF to triosephosphate isomerase (CAA81487 from *Secale cereale*). The conserved amino acids are indicated with an asterisk. The letters in bold in a) indicate the poly A tail and the letters in bold italics is the stop codon.

a)

```

GAAGAATTCTCGAGCGGCATATAGTCAAAGGAGATATGATTTTGCTTGCGGTATATACAGAGGCTAAGG
CCAGTAGTGGTACCTTCCACCCAAGTCTGTTCTTGCAGATGCATATAATCTTTTTTGTTCGGGGAAAGAA
TAAAAGTAACCCTGCACTTCCCTAGTGCTCCACGGAGAAAAATCCTTTGTACCTTTTACTCTCGAGTT
ATACTTCTGCGGCACGTTCAAACCTCGCAGATTAATTTTGTAGTCCTTCCACCACAAAGATAAGCAAAGG
GCCAGTTTGGACCTGTTTTTGTTCAAAAAAAAAAAAAAAAAAAAAGCGGCCGCTCGAGAATTCTTC

```

b)

```

D19 -----LERHIVKG 8
NP_343764 MIARKLADLGVDVIEAGFPASSEGEFIATRKIFEEIGDQVEVIGLSRSNKNDIDKTISTG 60
                                         ::: * .*

D19 -----DMETILLAVYTEAKASS----- 25
NP_343764 ISSIHLFIATSELHMKYKLMKTKEEVLNKIYESVKYAKDHGMIVEFSPEDATRTEEDFLF 120
                                         * :* :* .* :.

D19 -----GTFHPSLFLQMETHIIFVGERIKVTLH----FPSAPRRK 61
NP_343764 TAIRTAIEAGADRINIPDVTGTMHFPKYDMIKKIVNFVGERIIVSVHCHNDFGLATANS 180
                                         **:** : :* .:* :***** *::* * *. ..

D19 -----LSSYTSAAARS-NSQINFVVLPPQR----- 90
NP_343764 LAGVYAGARQAHVTVNGIGERAGNASLEEVVMGIKLLNYETNVKTKWLYEVS RFVAEMT 240
: .: :. . *:* :*::: **: :.

```

Figure 4.11. Sequence analysis of D19. (a) DNA sequence of D19. (b) Amino acid alignment of the largest ORF to 2-isopropylmalate synthase, putative (leuA-3) (NP_343764 from *Sulfolobus solfataricus*). The conserved amino acids are indicated with an asterisk. The letters in bold in a) indicate the poly A tail and the letters in bold italics is the stop codon.

catalyzes the isomerization between dihydroxyacetone phosphate and glyceraldehyde-3-phosphate.

Clone D19 consisted of 321 bases and the longest ORF coded for a polypeptide 86 amino acids in length (Fig 4.11). On amino acid level, it showed homology to 2-isopropylmalate synthase from *Sulfolobus solfataricus*. The enzyme catalyzes the transfer of an acetyl group from acetyl-CoA to 2-ketoisovalerate to produce 3-carboxy-3-hydroxyisocarpoate (isopropylmalate) in the leucine biosynthesis pathway. The homology was however not very high and therefore not significant.

Clone M20 consisted of 566 bases and the longest ORF coded for a polypeptide of 153 amino acids (Fig 4.12). Two large ORFs were translated and blasted. On amino acid level, it showed homology to the pG1 protein from humans, ABC-type dipeptide/oligopeptide/nickel transport systems from *Kineococcus radiotolerans* and two hypothetical proteins from *Anabaena variabilis*. Both the pG1 and hypothetical proteins had no defined function.

Clone D13 consisted of 653 bases and the longest ORF coding for a polypeptide was 191 amino acids (Fig 4.13). On amino acid level, it showed low homology to a hypothetical protein from *Rhodospseudomonas palustris*.

Clone D15 consisted of 601 bases and the longest ORF coding for a polypeptide was 65 amino acids (Fig 4.14). On amino acid level, it showed homology to OSJNBb0004G23.6 from *Oryza sativa*, an unknown protein and protein F23N19.15 from *Arabidopsis thaliana*.

Clones M19, D20 and D21 did not show homology on DNA or protein level to any gene present in the current databases.

For the continuance of the project, it was decided to further analyze one of the unknown cDNAs. Of the three clones, it was decided to use clone

a)

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GAAGAATTCTCGAGCGGCATTGCCCTTCTTCACTCACGCGATATTGCTGGATCGGGCTTTCGCCCATG
TCCAAGATTCCCCACTGCTGCCCCCGTGGGAGTCCGGGCCGTGTCTCAGTCCCAGTGTGGCTGATCAT
CCGAAAAGACCAGCTAAGCATCATTGGCTTGGTCAGCCTTTACCTGACCAACTACCTAATACTACGCAG
GCTCATCAAACAGCGCTTTTGGAGCTTCTTTCAGGATTTGGCCCGAACTGTTCCGGCAGATTCCCACGCGT
TACGCACCCGTTTCGCCACTTCGTTCTCAACTATTCGATTCAGCAAACGGAGGCCGTTAGGTCAAAGC
CGTAGCGCGCGCCCTGTAGTTTTGAAGCAGGGCGAGACAACCTTTAGCTAGGAGCCTCTTTTCCTTCTGC
CCAGCTCCCCGAAAACAACGTTTCGACTTGCATGTGTTAAGCATATAGCTAGCGTTCCTTCTGAGCCAGG
ATCAAACTCAGATTTTGACTATGAGCAAAAAAAAAAAAAAAAAAAAGCGGCCGCTCGAGAATTCTTC

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b)

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M20          --LVIEEFSSGIALLHSRDIAGSGFRPLSKIPHCCPPWESGPCXSPSVADHPKRPKHHW 58
A44803       FSLITAVYIPKNFILHAVSLHQASL--IVENSLLLPPVESGPYLSPSVAVQPLGPAKHHR 58
              *:   : .   :**: .:  .: :  : .   ** ***** ***** :* *****

M20          LGQPLPDQLPNTTQAHQTALLSFLQDLARTVRQIPTRYAPVRHFVNLNYSRSSKRRPLGQS 118
A44803       LGRPLPYQLTN-----VPHPHF----- 75
              **:*** **.*                               .* **

```

Figure 4.12. Sequence analysis of M20. (a) DNA sequence of M20. (b) Amino acid alignment of the largest ORF to pG1 protein (A44803 from human). The conserved amino acids are indicated with an asterisk. The letters in bold in a) indicate the poly A tail and the letters in bold italics is the stop codon.

a)

```

GAAGAATTCTCGAGCGGCTCACCAACATCCTGCAATCCAAAGACAGGAAGAAAACCTCTGCTCGACATGG
TCGAACGGAACGAGCTCAACATGTCCATATTGACGCTTCTTGATGAGAACATAGCGAGTGCCAAGACGA
GTAACCAGGAGGAGGCTGTGGCTTTCATGGAGGATGTGCGCTCATCTATGCTGAAATACATAACAGTAT
AAATTTTGCCTACATATGGCGTGACCAGTTATCTGTGGCATTGGAGTCGACTCCCTTCAGCACATAGTT
GTTTCGAATAAATTCTGAAGGAGCTGTAATTCAGAAGGCTATCTTGTTGCTATAATTTTGTTCGGTTGT
ATTTAGTTGTTTTGTTTCATATATGTTCCAATTGTCAATAGCTTTTACTGGCAAAGAGAGATTCTACAAT
CATATATACATGTACGGCTGTACCACAAAACAAACACAACAAGACTATCATCTGTTCTCCTGGTCGGTTA
ACTGGTTTAGGAATGTATGATTTGCTAGTGATTTCTTACAGTAAATCTGTAACAATTTGTAACAATTAG
AGACAGTACCTATGTTTGAGAATGATCTGCAAAAAAAAAAAAAAAAAAAAGCGGCCGCTCGAGAATTCCTT
C

```

b)

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D15          -----ERLTNILQSKDRKKTLLDMETVERNELNMETSILTLLDENIASAKTSNQE EAVA 54
CAE04608     DLVSAKERLTKILQSSDKKSTLLEM--VERNELNMS--ILTLLDENIASAKTNNQE EAVA 236
              ****:*:**.*:*:**.* *****. *****.*****.*****

D15          FMETEDVRSSMETLKYITV 73
CAE04608     FMEN--VRSS--ILKYITV 251
              ***.  ***  *****

```

Figure 4.14. Sequence analysis of D15. (a) DNA sequence of D15. (b) Amino acid alignment of the largest ORF to OSJNBb0004G23.6 (CAE04608 from *Oryza sativa*). The conserved amino acids are indicated with an asterisk. The letters in bold in a) indicate the poly A tail and the letters in bold italics is the stop codon.

D20, since it was only induced in the infested resistant plants and not in the susceptible plants. The cDNA was further analyzed by reconfirming its expression pattern, by determining the copy number of the gene and attempting to clone the full-length gene.

4.5. Confirmation of clone D20 expression using RT-PCR

To confirm the expression levels of the D20 clone, an RT-PCR was performed. The expression of the 18S rRNA molecules was used as a control. Gene specific primers were synthesised for clone D20 and the gene was amplified using total RNA from the different time intervals. The expression pattern of the gene was expressed relative to that of the 18S rRNA and compared with the results obtained with the reverse Northern blot. D20 showed an induction in both the infested resistant and infested susceptible plants (Fig 4.15). Infested resistant plants showed the highest induced expression at 6 h.p.i. with a 2.5 fold increase compared to time zero. Infestation of the susceptible plants only led to a 1.4 fold increase at the similar stage. As a whole, the expression of D20 was several times higher in the infested resistant plant compared to the infested susceptible plants.

Even though both the levels of induction and the timing thereof differed from the results obtained in the reverse Northern blot, it is clear that clone D20 gene expression is confined to the infested resistant plants shortly after infestation. This result allowed us to continue with the analysis of clone D20.

4.6. Southern blot analysis of D20

To determine whether clone D20 is present in the genomes of the Tugela and the different Tugela *DN* cultivars, a Southern blot was done (Fig 4.16).

Even though the hybridization signal was very weak, D20 hybridized with complementary DNA in Tugela, Tugela *DN*, Tugela *DN2*, Tugela *DN3* and

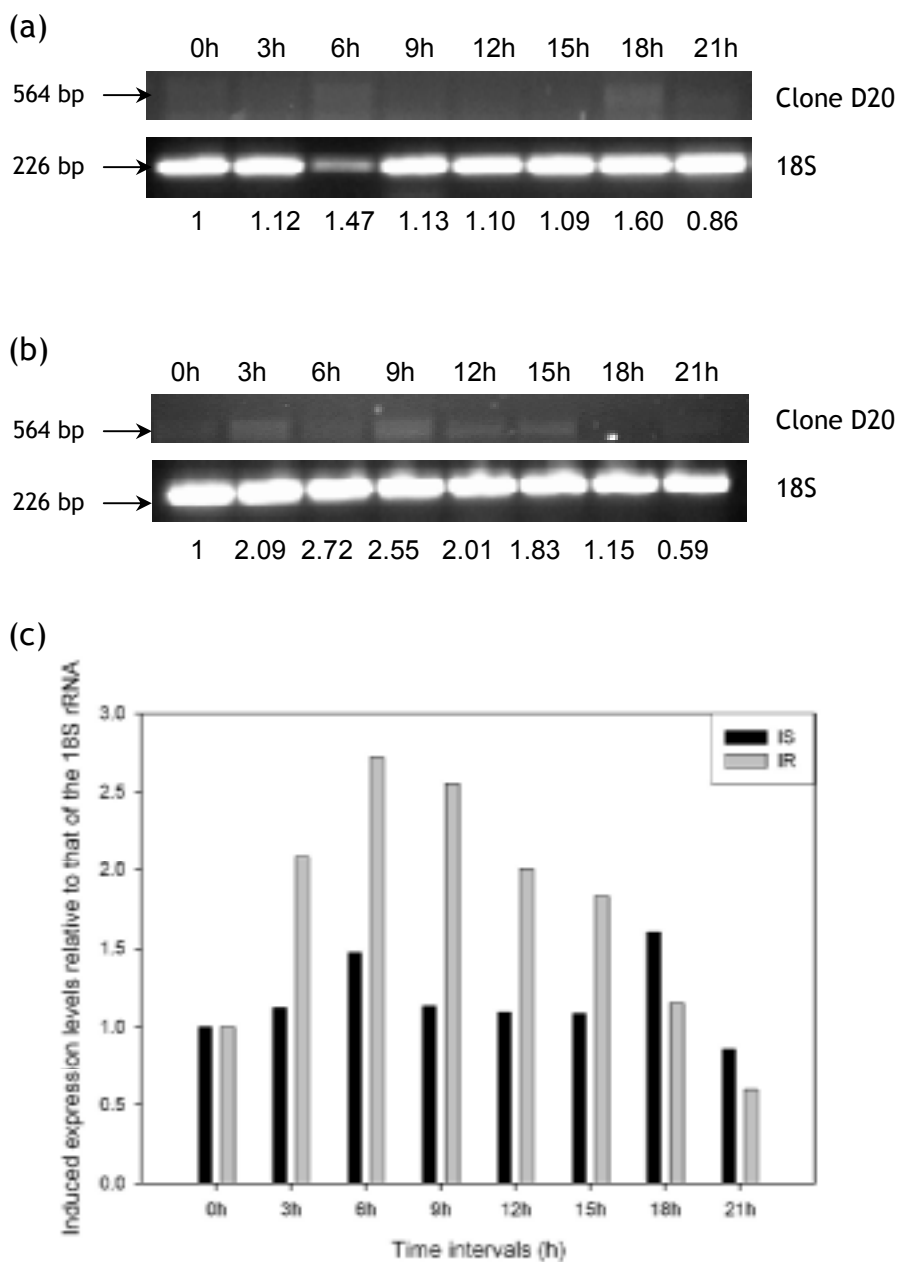


Figure 4.15. Expression profile of clone D20 using RT-PCR. In (a) both clone D20 and 18S rRNA were amplified using total RNA extracted from infested susceptible plants while in (b) total RNA from infested resistant plants were used. Sizes of the amplified fragments as well as the induction levels are indicated. (c) represents a bar graph of the expression patterns.

Tugela *DN5* with an approximate size of 5,300 bp. Since a single fragment hybridized at high stringency, the gene is most probably present as a single copy in the genome. The hybridized fragments were similar in size in all the Tugela lines, therefore no polymorphisms. Although faint, a fragment is present in Tugela while it is present in all the other lanes.

4.7. 5'-RACE on D20

To obtain the full DNA sequence of clone D20, 5'-RACE was performed. During RACE, gene specific primers were used to amplify the full length cDNA fragment (Fig 4.17). In total two PCR reactions were performed. For the first PCR reaction (Fig 4.17a), single stranded cDNA fragments were used as template while during the second PCR reaction amplified products from the first PCR reaction were used (Fig 4.17b). As can be seen in the second PCR reaction, a number of distinct bands are visible even though no amplification was evident during the first PCR reaction. Of the six amplified fragments both the second and the fifth largest fragments, as indicated with arrows, were sequenced. The multiple bands may have been caused by the primers annealing on different areas of the RACE product. No sequence information was however obtained for the larger fragment, only for the smaller fragment. The new DNA sequences were combined with the initial sequence. This larger sequence was then analyzed to putatively identify clone D20.

Sequence analysis of clone D20 on DNA level, showed homology to various mRNA clones (Table 4.3). The longest ORF coded for a 68 amino acid polypeptide. Protein BLAST was performed but showed extremely low homology on short sequences. D20 showed the best homology with an mRNA sequence from *Zea mays*, a portion of a cDNA clone from *Oryza sativa* and predicted mRNA from *Oryza sativa* (Fig 4.18). The genes clone D20 showed homology, were all unknown genes or hypothetical proteins, therefore the identity of clone D20 remains inconclusive.

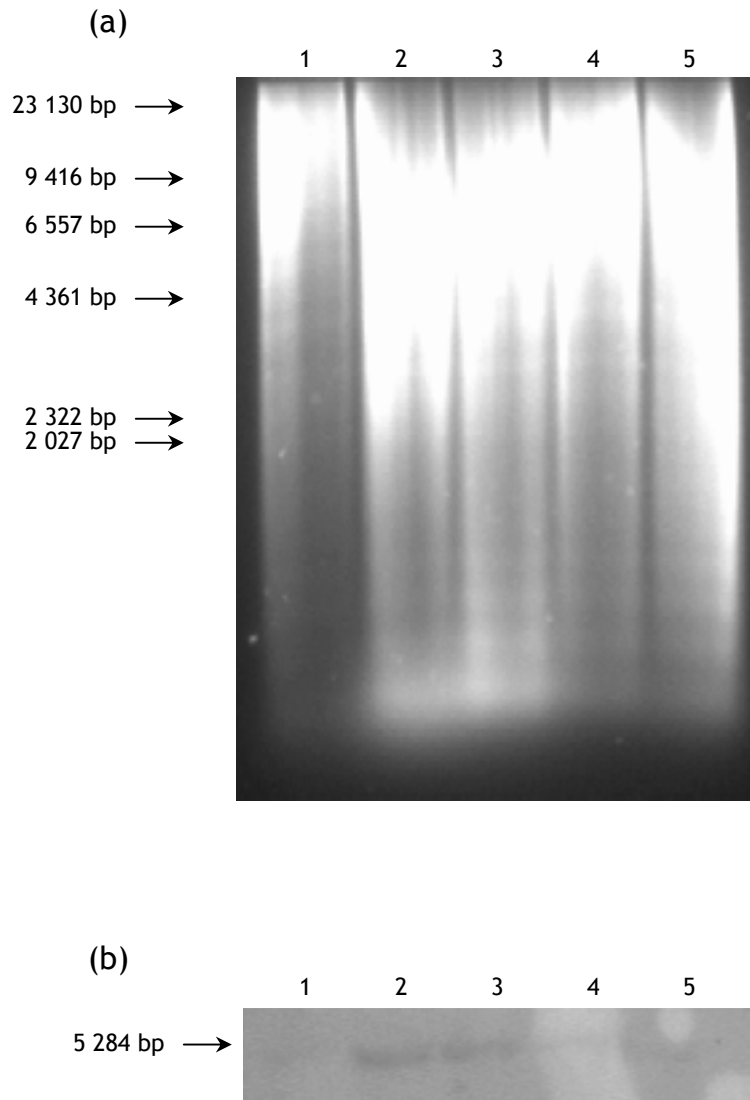


Figure 4.16. Southern blot analysis of clone D20. Lane 1 contains digested genomic DNA from Tugela, lane 2 from Tugela *DN*, lane 3 from Tugela *DN2*, lane 4 from Tugela *DN3* and lane 5 from Tugela *DN5*. In (a) the agarose gel containing the digested genomic DNA is indicated and in (b) the x-ray film hybridizing with clone D20. The sizes of the hybridizing fragments is indicated.

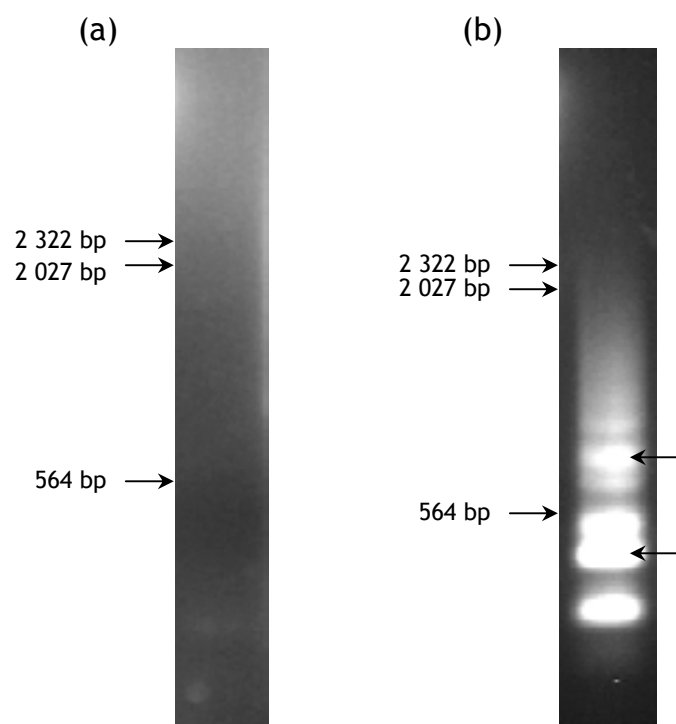


Figure 4.17. Amplification of D20 for RACE after ligation (a) first amplification, while (b) is the second amplification.

Table 4.3. Identities and E-values of genes showing homology to the cloned D20 cDNA fragment.

| <i>Clone</i> | <i>Accession nr.</i> | <i>Similar gene</i> | <i>Identity</i> | <i>E-value</i> |
|--------------|----------------------|--|-----------------|----------------|
| D20 | AY103628 | <i>Zea mays</i> PCO109231 mRNA sequence | 77/87 (88%) | 3e-16 |
| | AK120899 | <i>Oryza sativa</i> (japonica cultivar-group) cDNA clone:J023030J05, full insert sequence | 73/83 (87%) | 6e-14 |
| | XM_474211 | <i>Oryza sativa</i> (japonica cultivar-group), predicted mRNA | 71/81 (87%) | 1e-12 |
| | AL606608 | <i>Oryza sativa</i> genomic DNA, chromosome 4, BAC clone: OSJNBa0015K02, complete sequence | 45/48 (93%) | 1e-09 |

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AK120899      -GGAGAGGATCGGAGAGCTTTCCGC---CGGCCATGGGGTTCGGGTGACCACGC-TCAT
XM_474211      -----ATGGGGTTCGGGTGACCACGC-TCAT
AY103628      CGGAGGAGCTCGCCGAGGCCGCCGAAGACGACCATGGGGTTCGGGCGACGACGC-TCAT
D20            ACAACAATAATGATGATTCTGTT-----TATCCACCCTGTTGCGGTTGACCAACCGCCAT
                                *      **** *   *** * *   ***
AK120899      CTCCTCCTCGCCGGCTTCGTCGCCTCCCTCTTCTCGCTGCTCTGCTGCAACCGCGGGCC
XM_474211      CTCCTCCTCGCCGGCTTCGTCGCCTCCCTCTTCTCGCTGCTCTGCTGCAACCGCGGGCC
AY103628      CTCGTCCTCGCCGGCGTCGCCGCATCCCTCTTCACGCTCCTCTGCTGCAACCGCGGGCC
D20            CCTCTCCTCGCCGGCTTCATCGCCTGCCTCTTCTCGCTCCTCTGCTGCAACCCAGTGGT
                                * * * ***** **  *** * ***** **** ***** ***** * *
AK120899      GTCGACCAACCTATTCCATTT-GACCTTGGTTATTACAGCAACGGTTTGCTGTTGGATGA
XM_474211      GTCGACCAACCTATTCCATTT-GACCTTGGTTATTACAGCAACGGTTTGCTGTTGGATGA
AY103628      CTCCACAAACCTGTTCCATTT-GACCTTGGTGACTACAGCTGTAATTTGTTGCTGGATGA
D20            GATGTACACTTATTTGGATTTAGAATCTGGATATAACCGCTACAGTTTGTTGTTGGATGA
                                *      **  **** *   *** * *   ** *   **** * * *****
AK120899      TGTGGGCAATCGTCTATCTTGCGCAGCTGAAGCCACTGATCAACCCTATCCTAAGCGGCG
XM_474211      TGTGGGCAATCGTCTATCTTGCGCAGCTGAAGCCACTGATCAACCCTATCCTAAGCGGCG
AY103628      TGTATGCAATCGTCTACCTTGCTCAGATGAAGCCATTGATCAACCCATTCTGAGCGGAG
D20            TGTGGGCAATCGTGTACCTCGCCAGATGAAGCCTTTGATCAACCCATCCTGAGCGGCG
                                *** ***** ** * * *   *** ***** ***** ***** ** * * ***** *
AK120899      AGTGAGC-----TGTGTT---ACAATTTGAAGTCTGCTCATGCTGTATCACT
XM_474211      AGTGA-----
AY103628      AGTGAGCGCTGCAT----CTGTATTGA--ACAGAACGAAAGCGAGACCTGCTGTACCATT
D20            AGTGAGCCCTGCCCTGCCCTGCCCGGAGACAACCTAAGATCCACTGTTGATGTATCATT
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Figure 4.18. Homology of D20 sequence. AK120899 = cDNA clone isolated from *Oryza sativa*. XM_474211 = predicted mRNA sequence from *Oryza sativa*. AY103628 = mRNA sequence from *Zea mays*.

Chapter

5

Discussion

5. Discussion

All the more, molecular genetic tools are used to identify genes involved in various plant-pathogen interactions. The techniques range from simple differential display as used in this study to the more complicated suppression subtractive hybridization (Lu *et al.*, 2004) and micro-array analysis (Voelckel *et al.*, 2004). The reason for this increased activity is the need to understand the complex interaction that takes place between the plant and the pathogen. Once this interaction is better understood, ways could be found to improve the resistance of plants against the invasion of potential pathogens.

What is clear from various studies is that plant-pathogen interactions are extremely complex with several different aspects of plant cellular life being involved. These include primary and secondary metabolism, plant defence and senescence, communication and signal transduction, cellular transport and ultrastructure (Lacock *et al.*, 2003; Lu *et al.*, 2004; Voelckel *et al.*, 2004). In addition, every study yields a number of cloned fragments that either share homology with DNA sequences for which no known function has been described, or do not share homology with any known genes at all. It is these genes that usually attract the most attention, for the fact that they are novel and that the encoded proteins could therefore perform a unique function during the plant-pathogen interaction.

Russian wheat aphid infestation is a major problem for wheat growing farmers (Du Toit and Walters, 1984). The insect is responsible for huge losses in crop yield, since they attack the plants by withdrawing phloem sap from the veins that leads to the wilting of the plants (Miles, 1999). Control of the aphids is very difficult, since they are protected from pesticides (Hein *et al.*, 1998) as the leaves roll into a tubular structure (Dixon, 1973).

Aphids are also responsible for the injection of a possible toxin into the leaves upon feeding, thereby creating yellow and white streaks on the

leaves (Du Toit, 1986). The toxins are further responsible for the degradation of the chloroplast membranes leading to decreased photosynthesis (<http://insects.tamu.edu/extension/bulletins/b-1572.html>). In addition, the damage of Rubisco upon RWA infestation might contribute to the poor survival abilities of susceptible cultivars (van der Westhuizen and Botha, 1993).

Much research on the biochemical defences employed in resistant and susceptible wheat has been done in the past (Botha *et al.*, 1998; van der Westhuizen *et al.*, 1998a; van der Westhuizen *et al.*, 1998b; Mohase and van der Westhuizen, 2002a; Mohase and van der Westhuizen, 2002b). It was found that the activation of the defence reaction in the resistant cultivars was more efficient in the resistant cultivars than in the susceptible cultivars.

In this study, an attempt was made to identify genes whose encoded proteins are directly involved in the very early events following RWA infestation in the resistant Tugela *DN1* line. A total of 49 cDNA fragments that showed induced expression after RWA infestation of Tugela *DN1*, were amplified and cloned (Fig 4.3). This amplification was done using a primer combination that was specifically designed for the amplification of protein kinase genes. The upstream primer (Bovis 22 or 23) was specific for the conserved subdomain VIb of protein kinases, while the downstream primer (Bovis 39) annealed to the tail sequence of the anchored oligo-dT primer that was used for first strand cDNA synthesis (Table 3.1).

This technique was however flawed, since out of the 11 fragments sequenced, only two (D13 and M18) displayed the correct primer sequences on the respective 5' and 3' ends of the cDNA sequence. The others were all amplified with Bovis 39 annealing on both sides of the cDNA fragments. This explains the fact that out of the 11 cloned and sequenced cDNA clones, none was a putative protein kinase gene.

Instead, the isolated cDNA fragments coded for a variety of both known and unknown genes (Table 4.1). All the identified cDNA clones except one, were inducibly expressed exclusively within the infested resistant Tugela *DN1* line (Fig 4.6 and Fig 4.7). No induced expression of the identified cDNAs were visible within the infested susceptible Tugela line. The only exception was clone M18 that coded for the large subunit of Rubisco that was inducibly expressed in both the infested resistant and susceptible lines. The induced expression in the resistant line was however at least 4.5 fold compared to the 1.5 fold induction level within the susceptible line (Fig 4.6).

Even though 49 different cDNA clones were identified as being inducibly expressed upon infestation, only 11 were selected for further analysis based on their unique expression patterns. All eleven cDNAs that showed induced expression within the infested resistant Tugela *DN1* line, were sequenced (Fig 4.8 - 4.14). Of the eleven, five cDNA clones showed homology to known genes (Table 4.1). Each clone will now be discussed, as well as its possible relevance during the infection of wheat by the RWA.

Firstly, M17 showed 73% homology to indole-3-glycerol phosphate lyase (IGL) which catalyzes the formation of free indole (Fig 4.6b). The E-value is 1×10^{-55} which shows that the homology is significant. The closer the E-value is to zero, the more significant the homology. Indole is converted from indole-3-glycerol phosphate to a defence-related secondary metabolite 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (Frey *et al.*, 1997). IGL was shown to be induced in maize by volicitin that acts as an elicitor isolated from the regurgitant of beet armyworm (Alborn *et al.*, 1997). Treatment with the elicitor induces the production of volatiles. These volatiles are believed to attract parasitic wasps which are natural enemies of the beet armyworm, to the damaged maize. This clone showed an induction in the infested resistant line at 3 h.p.i. and the highest levels of expression were reached around 9 h.p.i. (Fig 4.6b). Much lower expression levels were observed in the infested susceptible line (Fig 4.6a).

Clone M18 was induced at high levels within the infested susceptible and resistant plants (Fig 4.9b). This clone showed 94% homology to Rubisco, which plays a major role in photosynthesis and photorespiration and was located in the chloroplasts (Staehein and Newcomb, 2000). The E-value was 0×10^{-112} which shows that the homology is significant. The RWA causes degradation of the chloroplasts leading to the development of necrotic spots (Ryan *et al.*, 1990). Photosynthesis is then severely compromised, since contents of the chloroplasts leaks out because of the damage. The highest induced expression of the clone was detected within 3 h.p.i. in the infested susceptible lines (Fig 4.6a) while for the infested resistant lines, the maximum induction was reached at 12 h.p.i. even though continuously induced expression was found from the beginning (Fig 4.6b).

This activated expression of a Rubisco large subunit gene was also found by Lacock *et al.* (2003). In addition Rubisco gene expression has also been shown to be induced under stress conditions (Hanson and Tabita, 2001). Regarding the wheat RWA interaction the induced expression of Rubisco indicates that the plants compensate for the breakdown of the chloroplasts by producing more Rubisco protein that could increase the photosynthetic rate. Since the induction levels were bigger in the resistant plants, one can assume that the resistant plants are more effective in this compensation allowing the plants to survive RWA infestation.

D14 showed 95% homology to triose-phosphate isomerase (Fig 4.10b). The E-value was 2×10^{-53} , signifying that the homology was significant. Triose-phosphate isomerase plays a role during glycolysis and catalyzes the interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate and is one of the most active enzymes known (Dennis and Blakeley, 2000). This clone showed an induction within 3 h.p.i. and reached the highest expression around 12 h.p.i. in the infested resistant plants (Fig 4.7b). The infested susceptible plants did however not show any substantial induction of the cDNA (Fig 4.7b).

Clone D17 showed 98% homology with a 16S rRNA from an endosymbiont of *D. noxia* and had a zero E-value which is highly significant. The cloning of a 16S rRNA gene from the *D. noxia* endosymbiont may be due to the presence of the endosymbiont in the saliva or stylet of the RWA that might have been transferred to the plants during infestation. This gene showed a 2 fold induction at 9 h.p.i. in the infested resistant plants (Fig 4.7a) while infested susceptible plants did not show any significant induction (Fig 4.7b). It is however very difficult to explain this result, since the role of the *D. noxia* endosymbiont in the activation of the defence response is not clear. It is furthermore not known whether this organism plays any role in the initiation of the defence responses of wheat. It was thus decided not to investigate this fragment any further.

When clone D19 was used for a BLAST analysis, only one previously cloned gene showed any homology. This homology was however very low (Fig 4.11) with a high E-value. The homology was also found only within a short portion of the isopropylmalate synthase gene. 2-isopropylmalate synthase plays a key role in leucine biosynthesis. Leucine is an essential dietary amino acid and also serves as a precursor for secondary metabolism (Coruzzi and Last, 2000). This clone is induced within the first three h.p.i. and reached its highest expression at 12 h.p.i. in the infested resistant plants (Fig 4.7a) while no significant induction was found in the infested susceptible plants (Fig 4.7b).

Three clones (M20, D13 and D15) showed homology to either hypothetical or unknown genes (Table 4.2). These hypothetical genes were sequenced as part of the genome projects and no functions were assigned to them as yet. In addition, the homology was very low with quite high E-values, indicating that the identities were not significant. These clones were induced within the first three hours after infestation and may encode for proteins or enzymes that play a role in the activation of the defence response. Despite this D15 showed homology to WRKY binding proteins (Eulgem *et al.*, 2000). WRKY binding proteins play a role in disease

resistance, senescence and germination and contains a zinc finger motif (<http://www.btny.purdue.edu/WRKY/>) that could bind to the promoters of genes containing the WRKY *cis*-acting element.

M20 showed homology to pG1 protein from human. It showed 63% homology with an E-value of 5×10^{-5} which is partially significant. No function has as yet been assigned to the pG1 protein. Even though the homology between the clones and their respective partners were very low, when one considers the complete cDNA fragments, the best homology was obtained in short stretches where the identity values were very high, 63% and 78% for M20 and D15 respectively (Table 4.1). These regions could therefore represent conserved active domains indicating a common function.

Three clones (M19, D20 and D21) showed little or no homology to any known genes in the current databases (Table 4.2). These genes could play important roles in the resistance interaction between the RWA and wheat since the genes were differentially expressed in the leaves of the infested resistant wheat. These genes could probably be involved in the signalling of the defence response due to the early induction of the genes.

Based on the fact that clone D20 showed no homology to any known gene and that it was induced preferentially in the infested resistant plants, it was decided to analyze it further. In addition, it showed very low homology to a socE signalling gene from *Myxococcus xanthus* (Crawford and Shimkets, 2000). This gene is involved in cell contact-dependent C-signalling system mediated by CsgA.

The gene encoding clone D20 was present in both Tugela and all the different Tugela lines containing the resistance genes included in this study (Fig 4.16b). This confirms the reverse Northern blot results (Fig 4.15) where it was found that the gene is expressed in both the infested susceptible and resistant plants. The gene is present as a single copy in the genomes of all of the different lines.

The expression profile of D20 was confirmed with RT-PCR by using gene specific primers (Fig 4.15a and b). The highest expression level occurred at 6 h.p.i. in the infested resistant plants while the infested susceptible plants showed the highest expression level at 18 h.p.i. (Fig 4.15c). The expression profile also matched that obtained during the reverse Northern blots (Fig 4.7) with only minor differences in expression levels. Since D20 is induced within the first three hours after infestation, it may be directly involved in the recognition of the RWA by wheat. It may therefore play a role in either the signalling or activation of the defence response events.

Since clone D20 showed potential to perform an essential function during the response of wheat towards the RWA, it was decided to attempt to clone the full length gene using 5'-RACE. Gene specific primers (Table 3.1) were designed for clone D20 to be used in the RACE reaction (Fig 3.1).

Although 6 fragments were amplified after the second PCR reaction it was decided to sequence the 2 most distinct fragments (Fig 4.17b). Sequencing of the smaller amplified fragment was successful, however no results were obtained for the larger amplified fragment. This new nucleotide sequence was combined with the previously obtained sequence and the longer DNA sequence was used for BLAST analysis (Table 4.3; Fig 4.18).

Whereas the original fragment showed no homology with any known plant genes, the longer DNA sequence showed homology to several plant DNA sequences from maize and rice (Table 4.3). Even though these genes did not have any function assigned to them, the E-values indicated that the homology was significant. In a multiple alignment of clone D20 and the two showing the best homology indicated significant similarities between the fragments.

At this stage it was still impossible to identify clone D20 since none of the homologous genes had a function. Analysis of the amino acid sequence also did not yield any putative conserved domains that could indicate a putative function. Therefore it is impossible at this stage to speculate on a particular function for clone D20 within wheat infested with RWA. Since it is induced very early after infestation it is tempting to assume that it plays a key role in the adaptation of resistant wheat upon infestation. This could be either in a signalling or defence activation role. Future research will however have to prove this theory right.

The results obtained in this study closely resemble that from two other related studies (Lacock *et al.*, 2003; Lu *et al.*, 2004). In both, the interaction between a plant and pathogen was investigated. Lu *et al.* (2004) studied the interaction between the rice blast fungus (*Magnaporthe grisea*) and rice to identify genes whose products are involved in this interaction. They identified 47 induced cDNA fragments from infected resistant plants whose expression was induced upon infection. After sequencing, all these genes fitted into 8 groups that define different cellular functions within rice. The different groups were cell rescue and defence, signal transduction, transcription, transport, metabolism, energy, protein destination and finally a group of unknown genes.

Similarly, Lacock *et al.* (2003) did a study where genes encoding proteins that contain NBS and LRR regions, were cloned. The authors used a similar DD RT-PCR approach as was done in the current study to identify possible defence-related genes whose expression was induced upon RWA infestation of Tugela DN1, DN2 and DN5. The degenerate primers that were designed for the DD RT-PCR approach were specific for the NBS domain located on defence related proteins. The authors managed to identify 37 genes that were inducibly expressed within the infested resistant plants. Again, the identified genes were placed into 6 groups, namely structural proteins, protein synthesis, regulatory proteins, primary and secondary metabolism and miscellaneous.

It is therefore clear from all three studies, and there are several more like these, that the defence reaction of the plant towards a pathogen or insect attack is a complex issue. This study has however broadened our knowledge of the genes whose encoded protein products play an important role in the response of the wheat when infested by the Russian wheat aphid. It is important to note that several of the cloned cDNAs are currently unknown and might therefore still have a unique function that needs to be discovered.

From all three these studies, especially the two performed on the RWA wheat interaction, it is clear that the metabolic adaptation of the resistant plants forms a crucial part of its defence. It is likely that this enhanced metabolic activity is responsible for the better survival and adaptation of the resistant plants after RWA infestation. The validity of this hypothesis will however need to be confirmed in the future.

To conclude, the project was partially successful in that the initial aims of the study were not completely met. Due to a technical flaw, no protein kinase genes were cloned and identified from the resistant plants. This could have been prevented by using two kinase specific primers instead of one. However several unknown cDNA fragments that show great promise were identified and will be analyzed in the future.

Chapter

6

References

6. *References*

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Summary

Summary

The interaction between wheat (*Triticum aestivum*) and the Russian wheat aphid (*Diuraphis noxia*) was investigated on a molecular level. The resistant cultivar, Tugela DN1, was infested with the Russian wheat aphid and tissue harvested at 3 hour time intervals for 24 hours. Differential display RT-PCR was used to clone genes that were inducibly expressed using primers specific to the conserved subdomain VIb of the kinase domain of protein kinases. Differentially expressed genes were found as early as three hours after infestation. These fragments were cloned and sequenced.

A total of 11 differentially expressed cDNA fragments were analyzed. Four showed homology to genes encoding proteins involved in normal cellular metabolism, such as the Rubisco large subunit, indole-3-glycerol phosphate lyase that produces indole that could act as a volatile messenger molecule, triose-phosphate isomerase that is involved in glycolysis and 2-isopropylmalate synthase that is involved in leucine biosynthesis.

Of the other cDNAs, three clones showed homology to several hypothetical proteins with no defined functions, while the other three showed no homology at all with any other known genes.

One of the unknown clones (D20) was selected for further analysis. The gene was inducibly expressed only in the infested resistant plants, but not in the infested susceptible plants. The expression of the gene was induced within 3 h after infestation reaching a maximum expression level 6 h.p.i. The gene is present as a single copy within the genomes of susceptible and 5 different resistance lines. An attempt to clone the full length gene was only partially successful since after the sequences were combined homology was found with several hypothetical genes from rice and maize. However, no identity and function could be given.

Keywords: *Triticum aestivum*, *Diuraphis noxia*, plant defence, DD RT-PCR

Opsomming

Tydens hierdie studie is die interaksie tussen koring (*Triticum aestivum*) en die Russiese koringluis (*Diuraphis noxia*) op molekulêre vlak ondersoek. Tugela DN1, wat weerstandbiedend is teen die Russiese koringluis, is met die luise geïnfesteer. Blaarweefsel is elke 3 uur versamel oor 'n tydperk van 24 uur. Gene wat gedurende die tydperk differensieel tot uiting gekom het, is met behulp van DD RT-PCR geamplifiseer. Die inleiers wat gebruik was, kodeer spesifiek vir die hoogs gekonserveerde subdomein VIb van plant proteïenkinases. Verskeie fragmente is gevind waarvan die transkripsie binne drie uur na infestering geïnduseer is. Differensieel uitgedrukte cDNA-fragmente is gekloneer en die nukleotiedvolgordes daarvan bepaal.

Elf gekloneerde cDNA-fragmente is verder ge-analiseer. Vier van die fragmente het homologie getoon met gene waarvan die gekodeerde proteïene 'n rol speel tydens metabolisme. Ingesluit hierby was gene wat kodeer vir die groot subeenheid van Rubisco wat betrokke is by fotosintese, indool-3-gliserolfosfaatliase wat indool sintetiseer wat kan dien as 'n vlugtige seinmolekule, triose-fosfaatisomerase wat betrokke is by glikoliese en 2-isopropielmalaatsintase wat vermoedelik betrokke is by leusienbiosintese.

Van die oorblywende ses klone het drie homologie getoon met verskeie hipotetiese proteïene waarvoor daar tans nog geen funksie bestaan nie. Die oorblywende drie het egter geen homologie met enige bekende gene getoon nie.

Een van die onbekende klone (D20) was gekies vir verdere studie. Die uiting van die kloon word slegs in die weerstandbiedende geïnfesteerde plante geïnduseer en glad nie in die geïnfesteerde vatbare plante nie. Induksie van uiting was binne drie ure na infestering. Die maksimum uitsingsvlak is ses ure na infestering bereik. Die geen kom voor as 'n enkelkopie in die genome van vatbare, sowel as vyf verskillende weerstandbiedende, koringlyne. 'n Poging om die DNA-volgorde van die volledige geen te bepaal, was egter slegs gedeeltelik suksesvol. Nadat alle beskikbare DNA volgordes saamgevoeg was, kon homologie

alleenlik met verskeie hipotetiese proteïene uit rys en mielies verkry word. Tans kan daar egter nog geen funksie of positiewe identifikasie aan die gekloneerde D20 cDNA-fragment gegee word nie.

Sleutelwoorde: *Triticum aestivum*, *Diuraphis noxia*, plantverdediging, DD RT-PCR