The identification of genes involved in the interaction between *Triticum aestivum* and *Puccinia triticina*

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The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka! I found it!' but rather 'hmm....that's funny...' - *Isaac Asimov.*

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List of Abbreviations

A

APS	Ammonium peroxodisulphate
Avr	Avirulence genes

B

BR	Brassinosteroid
BYDV	Barley yellow dwarf virus

D

DTT	Dithiothreitol
DMSO	Dimethylsulfoxide
DD RT-PCR	Differential display reverse transcription PCR
dNTP	Deoxynucleotide triphosphate

E

ECM	Extra cellular matrix
EGF	Epidermal growth factor
EDTA	Ethylenedinitrilotetraacetic acid

H

H_2O_2	Hydrogen peroxide
h.p.i.	Hours post infection
HR	Hypersensitive response

I

IPTG	Isopropyl β-D-thiogalactopyranoside

K

kDa	Kilo dalton

L

Lr	Leaf rust resistance gene
LRR	Leucine-rich-repeat
LRR-RLK	Leucine rich repeat-receptor-like protein kinase
LZ	Leucine zipper

M

MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MBP	Myelin basic protein

N

NBS	Nucleotide binding site
NB-LRR	Nucleotide-binding/leucine-rich repeat

P

PAHBAH	p-Hydroxybenzoic acid hydrazide
PCR	Polymerase chain reaction
PIPES	Piperazine-N,N'-bis(2-ethane sulfonic acid)
PMSF	Phenylmethyl-sulfonylfluoride
PR	Pathogenesis related
PTB	Protein tyrosine-binding

R

RLK	Receptor-like protein kinase
RT	Reverse transcription
RTK	Receptor tyrosine protein kinase
RT-PCR	Reverse transcription PCR
ROS	Reactive oxygen species
R genes	Resistance genes

S

SA	Salicylic acid
SAR	Systemic acquired resistance
SDS	Sodium dodecyl sulphate
SI	Self-incompatibility
SH2	Src homology 2

T

TEMED	N, N, N', N'-tetramethylethylenediamine

Th/Lr34	Thatcher/Lr34
Tris	Tris (hydroxymethyl)-aminomethane
Tween™ 20	Polyoxyethylene sorbitan monolaurate
TNFR	Tumor necrosis factor receptor

U

UV	Ultraviolet

X

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

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Chapter 1:

Introduction

The origin of cultivated plants and the development of crops have always been considered important. In the ancient world mythology considered cultivated crops a generous gift given to man by the gods. Thus, the ancient Egyptians paid homage to Isis and Osiris for introducing wheat and barley into Egypt and for teaching the people the secrets of their cultivation. Similarly, the ancient Greeks ascribed the gift of these important cereals to Demeter and the Romans to the goddess Ceres (Feldman, 2001)

Over the centuries the evolution of wheat (*Triticum aestivum*) has progressed in parallel with that of humans. We are only now beginning to explore the use of techniques in molecular biology to gain a better understanding of many of the issues surrounding crop development such as the interaction between crops and pathogens. With the current demand on agriculture to increase yield but decrease the area of crops being planted, it is also particularly important to develop new and innovative solutions to improve both yield and quality for a growing world population (Pagesse, 2001).

Wheat is grown more extensively worldwide than any other crop and is a close third to rice and maize in yield. It is believed to have originated in South Western Asia. Archeological evidence has shown the presence of wheat in Syria, Jordan and Turkey. Primitive relatives of present day wheat have been discovered in some of the oldest excavations of the world in Eastern Iraq which date back 9000 years. Other archeological findings show that bread wheat was grown in the Nile Valley about 5000 B.C. as well as in India, China and even England at the same time (Feldman, 2001).

The first wheat in South Africa was planted in the winter of 1652 by Jan van Riebeeck. By 1684 wheat production was well established and there was even some wheat exported to India. Natural selection determined which varieties were able to adapt and survive in a new climate with the greatest problems experienced, being pathogen infection, periodic droughts and wind damage (Van Niekerk, 2001).

Leaf rust, caused by *Puccinia triticina,* contributes substantially to wheat yield losses in South Africa (Boshoff *et al.,* 2002) as well as in other wheat producing countries worldwide. Development of new cultivars with diverse and effective resistance against leaf rust is the most economical and environmentally safe method of reducing losses. About 45 leaf rust resistance genes have been identified to date (Kolmer, 1996; Saini *et al.,* 2002).

The plant's surveillance system to counter pathogen attack is based on the early recognition of the invading organism and the activation of defence mechanisms that result in the arrest of further pathogen invasion and resistance of the plant to the pathogen. Pathogen recognition is accomplished by the detection of elicitors (peptide-, oligosaccharide- or lipid-based signaling molecules) that originate from the pathogen or represent degradation products of the plant cell wall (Romeis, 2001).

In gene-for-gene plant pathogen interactions, race-specific elicitors are encoded by the pathogen avirulence genes (*Avr)* and specific pathogen recognition is conferred to the plant through corresponding plant disease resistance genes (Higgins *et al.,* 1998). Upon recognition of the pathogen by the host, signaling events are initiated triggering early cellular responses in ion flux, synthesis of reactive oxygen species (ROS), changes in gene transcription and a localized hypersensitive cell death characterized by necrotic lesions (Romeis, 2001). This is also known as the hypersensitive response (HR).

Long term responses include the production of antimicrobial compounds, cell wall fortification and the activation of systemic acquired resistance (SAR) that reflects a continued resistance established in non-infected areas of the plant (Hammond-Kosack and Parker, 2003). The accumulation of pathogenesis related proteins (PR) associated with the HR is well documented (Crute and Pink, 1996). PR proteins include β-1,3-glucanases, chitinases, peroxidases and proteases (Van Loon, 1997). β-1,3-glucanases function through their ability to hydrolyze β-1,3-glucans commonly present in fungal cell walls (Keen and Yoshikawa, 1983).

Over the past few years, protein kinases have been identified for both nonrace- and race specific elicitation of defence responses in plants. They often participate in the direct perception of elicitors or Avr products (Song *et al.*, 1995; Cervone *et al.*, 1997; Feuillet *et al.*, 1997; Thomas *et al.*, 1997), mediate signaling required for the induction of defence mechanisms and function as regulators of defence responses (Romeis, 2001).

In the future, the emphasis will be to identify the interactions between single signaling components functioning, for example, in the direct perception of pathogens with the involved downstream signal transduction mechanisms. This requires the exploration of other proteins functioning upstream or downstream of a given protein kinase. This will allow the characterization of regulators, phosphorylation targets and other interacting proteins that may incorporate the protein into a specific phosphorylation cascade or signaling complex.

The aim of this study was to investigate the early events of the infection between wheat and leaf rust, both on biochemical and molecular levels. This will be done by determining the activation of enzymes usually involved in defence responses as well as the polypeptide levels of the said enzymes. In addition, protein kinase genes involved in these early events will be cloned in order to elucidate the early signaling events in this particular interaction. It is thought that infection of wheat by the leaf rust fungus should lead to the rapid activation of plant defenses, most probably via several protein kinases. Therefore, by determining the earliest reaction of the plant to the infection, it would allow us to clone protein kinase genes that are induced by early signaling events.

Chapter 2:

Literature

2.1 Introduction

In animal, fungal and algal systems, the physical connection and communication between the extracellular matrix (ECM) and the cell play a fundamental role in cell growth and division (Wagner and Kohorn, 2001). Similarly, the plant cell wall forms an ECM of carbohydrate and protein that provides structure for individual cells and whole organs. Communication between the cytoplasm and the cell wall is also necessary since cell expansion (growth) and pathogen infection, amongst others, lead to altered biosynthesis and modification of cell wall components and downstream cytoplasmic events such as SAR.

In animals, receptors with an intrinsic protein tyrosine kinase activity, known as receptor tyrosine protein kinases (RTK's), play a key role in cellular processes to co-ordinate the development of multicellular organs (Walker, 1994; Schenk and Snaar-Jagalska, 1999). Because plant cells are encapsulated by cell walls, the idea of plant cell-to-cell communication via the recognition of polypeptide ligands by transmembrane receptors at the cell surface was once viewed with scepticism. The cloning of several receptor-like protein kinases (RLKs) in plants provided a breakthrough, showing that higher plants possess gene products that are structurally analogous to receptor protein kinases (Stein *et al.,* 1991; Chang *et al.,* 1992) and is evidence of the cross talk between the cell and its external environment.

2.2 Disease Resistance

Plants are constantly subjected to infection by a variety of pathogens and have developed through evolution a battery of defence mechanisms to fight disease. Upon infection by a pathogen, a resistant plant is able to recognize the invading pathogen through either endogenous signal molecules derived from the degradation of the cell wall components, or by exogenous molecules synthesized by the invading pathogen. Endogenous and exogenous signal compounds are termed elicitors and include, amongst others, proteins, glycoproteins, oligosaccharides and lipids (Morris and Walker, 2003). In most cases, elicitors are sufficient to induce a complete set of plant defence responses and they

presumably interact with specific plant receptors (Du and Chen, 2000; Takemoto *et al.*, 2000, Sessa and Martin, 2000).

Elicitors can be subdivided into race-specific and nonrace-specific elicitors. Race-specific elicitors are molecules that are encoded by *Avr* genes in the pathogen (De Wit, 1998). Resistance involves the specific recognition of the invading pathogen by a dominant or semi-dominant plant resistance gene (*R* gene) product. This interaction is called gene-for-gene interaction (Flor, 1971). For each gene that confers resistance in the host there is a corresponding gene in the pathogen that confers its virulence. This model suggests that the direct or indirect interaction of the AVR and R polypeptides triggers resistance (Blumwald *et al.*, 1998; Van der Biezen and Jones, 1998).

Nonrace-specific elicitors are able to activate the defence responses by mechanisms independent of plant *R* genes and their recognition is probably mediated by high-affinity receptors present in the cell membrane (Yang *et al.*, 1997; Sessa and Martin, 2000).

The ability to sense and rapidly respond to external stimuli allows plants to resist pathogen attack and colonization. Despite the large variety of phytopathogens present in nature, disease is rare and resistance is prevalent as an outcome of plant-pathogen interactions (Sessa and Martin, 2000). Plant disease resistance is mostly dependent on the genetic background of both the host and invading agent and often relies on complex mechanisms of molecular recognition and cellular signal transduction (Sessa and Martin, 2000).

The timely recognition of pathogens can trigger subsequent signal transduction events that are assumed to coordinate the activation of an array of defence responses, including the induction of a large number of defence related genes (Lease *et al.*, 1998). Successful pathogen recognition triggers the activation of several diverse defence responses. In many plant-pathogen interactions, resistance is manifested at the macroscopic level by the appearance of necrotic

lesions at the site of infection. This is the result of a rapid localized cell death, called the HR, which is thought to limit pathogen growth and it's spreading throughout the infected plant (Johal *et al.*, 1995).

Molecular responses associated with the HR include the production of ROS, the opening of ion channels, cell wall fortification, production of antimicrobial phytoalexins and synthesis of pathogenesis-related (PR) proteins such as glucanases and chitinases (Johal *et al.*, 1995; Ladyzhenskaya and Protsenko, 2002). In addition to responses localized at the site of pathogen infection, plants often induce defence mechanisms in uninfected areas of the plant. Defence responses at such secondary sites are collectively referred to as SAR (Ward *et al.*, 1991; Luan, 1998; Tang *et al.*, 1999; Sessa and Martin, 2000).

SAR results in non-specific plant immunity to a broad range of avirulent and virulent pathogens (Epple *et al.*, 1997; Oldroyd and Staskawicz, 1998). Available evidence suggests that SAR signaling is mediated, directly or indirectly, by salicylic acid (SA) produced or released from inactive conjugates during the HR (Johal *et al.*, 1995). Exactly how salicylic acid activates SAR is not known, but it is hypothesized that salicylic acid binds and inactivates a catalase that results in the accumulation of hydrogen peroxide (H_2O_2), which in turn induces the expression of the genes involved in SAR (Chen *et al.*, 1993).

The most important class of genes that has been used by breeders for disease control is the plant *R* genes. *R* genes are single determinants of effective and specific disease resistance that can often be characterized by localized necrosis at attempted infection sites (Rommens and Kishore, 2000). Although originally believed to provide durable resistance, only a few exceptional *R* genes proved able to control pathogens for an extended period of time. The limited durability of single *R* genes for many of the agronomical important diseases, including stem and leaf rust in wheat, made it necessary to continue the discovery and introgression of new *R* genes (Rommens and Kishore, 2000).

The most prevalent class of functionally defined *R* genes encodes intracellular nucleotide-binding/leucine-rich repeat (NB-LRR) proteins with variable *N*-terminal domains. Less common are the serine/threonine protein kinase class of proteins and extracellular LRR proteins that possess a single transmembrane domain and either a short intracellular C terminus or a kinase domain (Hammond-Kosack and Parker, 2003).

2.2.1 Pathogenesis Related Proteins

Amongst the most frequently observed biochemical events that follow plant infection by pathogens is the production and accumulation of a family of proteins collectively known as PR proteins (Stintzi *et al.*, 1993). PR proteins are grouped into classes based on homology in primary structure, serological relationships and enzymatic and biological activities (Van Loon *et al.*, 1994). PR proteins of groups two and three were found to display β-1,3-glucanase and chitinase activity respectively and their involvement in plant resistance against pathogens has been extensively demonstrated. Several members of these classes have been shown to mediate pathogen resistance by over expression of their genes in transgenic plants (Caruso *et al.*, 1999).

The induction of PR proteins has been studied in great detail in several plant species during the last decade and in particular on the proteins of classes two (glucanases) and three (chitinases), whereas less attention was paid to proteins belonging to class four (Caruso *et al.*, 1999).

2.3 Interplant Communication

SAR is the process whereby distal parts of the plant receive a signal from an infected part of the plant. This signal allows these distal parts to activate its defence mechanisms. In a similar way two neighboring plants could communicate so that the uninfected plant could activate its defenses based on an airborne signal coming from an infected neighbor. Intra-plant communication and signal transduction have been proven many times but what about the possibility of communication and signal transduction between plants? If there are receptor

proteins to facilitate intra plant communication, shouldn't there be ones to accept signals from other plants in the same area?

Plant communication is a loaded term that has come to encompass a broad definition. Most would accept a definition with the requirement that information can be exchanged, regardless of intent or fitness consequence for either party (Baldwin *et al.*, 2002).

Plants have developed a multitude of inducible defence mechanisms against aggressive biotic agents. Defensive actions by plants induced via specific signal transduction events may negatively affect an herbivore's physiology. An example is the induction of protease inhibitors in potato and soybean plants (Koiwa *et al.*, 1997). Plants may also defend themselves against herbivores indirectly by emitting specific blends of volatiles that attract carnivorous natural enemies of herbivores (Dicke *et al.*, 1990; Turlings *et al.*, 1990; Takabayashi and Dicke, 1996; Arimura *et al.*, 2001). In some cases these compounds are released when feeding ruptures pre-existing internal or external secretory structures in which volatiles are synthesized and stored and in other cases these volatiles are formed at the moment of damage (Gang *et al.*, 2001).

After herbivore attack, plants release complex bouquets of volatiles into the air from their vegetative tissues. Predators and parasitoids of insect herbivores are attracted to herbivore-induced volatile releases, showing a powerful indirect defence for plants (Baldwin *et al.*, 2002).

Among the compounds that are thought to be involved in interplant communication are two jasmonates [cis-jasmone (Preston *et al.*, 2001) and methyl jasmonate (Farmer and Ryan, 1990)], the methyl ester of salicylic acid which is often linked to pathogen attack (Shulaev *et al.*, 1997), several terpenes (Arimura *et al.*, 2001) and some C6-C10 alkenals and alkanals (Preston *et al.*, 2001). Of all these proposed signals, only methyl jasmonate is detectable in volatile collections

from sagebrush, as determined by comparison with chemical standards (Karban *et al.*, 2000).

However, methyl jasmonate is released by sagebrush irrespective of damage, so receiver plants need to distinguish the signal from 'background noise'. Karban *et al.*, (2000) and Preston *et al.*, (2001) found that sagebrush can increase methyl jasmonate production by up to 16 times the normal levels and can change the isomeric conformation of methyl jasmonate to the biologically more active cis isomer. It was also found that the trans:cis methyl jasmonate ratio changes from approximately 80:20 in undamaged plants to approximately 40:60 in damaged plants. It is thus hypothesized that the receiving plants use the more active cis isomer as an indicator of damage (Preston *et al.*, 2001).

Methyl jasmonate is a biosynthetic product of the lipoxygenase or octadecanoid pathway, which can be induced under stress caused by herbivory. Jasmonic acid and methyl jasmonate are known to induce various aspects of biochemically based defenses within the plant or in tissue cultures, but the volatility of the methyl ester potentiates aerial activity. When the plant is damaged by herbivory or simulated herbivory, methyl epi-jasmonate is predominantly released and this has greater activity on recipient wild tobacco plants (Pickett and Poppy, 2001; Karban, 2001). Methyl jasmonate released into the air from sagebrush also increases the production of proteinase inhibitors in tomato plants (Farmer and Ryan, 1990).

Ethylene emissions from lima bean leaves infested with spider mites have been observed and was reported by Xu *et al.* (1994) to activate some defence genes. Ethylene is thus also thought to be one of the candidate airborne signals involved in plant-plant communication (Arimura *et al.*, 2001).

Some studies found no evidence for the transfer of information between damaged and undamaged plants (Preston *et al.*, 2001) while many others presented evidence supporting the hypothesis of information exchange between damaged and undamaged plants (Dicke *et al.*, 1990; Arimura *et al.*, 2001; Karban *et al.*,

2000). An important question is whether information exchange between damaged and undamaged plants can be expected in all plant species. If plants of a certain species show the ability, the question is whether individuals of that species should always respond to information from damaged neighbors (Dicke and Bruin, 2001).

Studies on plant-to-plant communication are often received with scepticism. The major issues raised are as follows: (1) data suffers from statistical flaws such as pseudo replication, (2) the dose of chemicals applied in experiments is unrealistically high, (3) the mechanism is unknown or alternative mechanisms may explain the data and (4) experiments under realistic field conditions are lacking (Karban *et al*, 2000; Dicke and Bruin, 2001).

2.4 Signal Transduction

The response of a plant to environmental stimuli results in some inter and intracellular changes leading to a particular end response. A series of events ranging from the recognition of an environmental stimulus to a defined response constitute a signaling cascade pathway and the entire phenomenon is called signal transduction (Sopory and Munshi, 1998).

According to current concepts, signal transduction from the plasma membrane to the genetic apparatus is realized via systems of adenylate cyclase, mitogen-activated protein kinases (MAPK), phosphatidic acid, phosphatidylinositol, lipoxygenase, superoxide synthase and nitric oxide synthase, as well through receptors possessing histidine, tyrosine and serine or threonine kinase activity. Phosphorylation of other amino acids also takes place, but mostly only under special circumstances (Sopory and Munshi, 1998; Ladyzhenskaya and Protsenko, 2002). Signal transduction is realized through a number of reactions that are specific to each signaling system, resulting in the alteration of the activity of various protein kinases and phosphatases. These altered enzymes modify the activity levels of transcription factors that in turn lead to the inhibition or induction of RNA and protein synthesis necessary to respond to the incoming signal. Protein kinases play

a crucial role in signal transduction by phosphorylating specific amino acids of downstream substrates (Josso and Di Clemente, 1997).

2.4.1 Protein kinase phosphorylation

Recent studies on pathogen and environmental stress responses reveal the importance of protein phosphorylation and dephosphorylation in the signaling pathways triggered by elicitors and abiotic stress signals. In particular, reversible phosphorylation plays a key role in activation and inactivation of MAPKs (Luan 1998; Xing *et al.,* 2002).

Immediately downstream of the initial elicitor-receptor recognition, the initiation of ion fluxes and the production of H_2O_2 are the initial responses detected in plant cells (Higgins *et al.,* 1998). These processes which occur prior to the transcriptional activation of defence-related genes appear to be mediated through the regulation of plasma membrane-bound enzymes. These include changes in Ca^{2+}-ATPase and H^+-ATPase activities, the activation of plasma membrane-bound ion channels and the induction of a plasma membrane-bound NADPH oxidase (Xing *et al.,* 1997). A number of signal transduction pathways have been proposed to mediate these early responses in host cells, ensuring an elicitor-induced response that is quantitatively appropriate, correctly timed and coordinated with other activities of the host plant cells (Blumwald *et al.,* 1998; Xing *et al.,* 2002).

The phosphorylation of proteins, probably initiated by the receptor, is thought to relay the defence signal to different downstream effectors. In some cases, the receptor contains a kinase domain that may trigger the phosphorylation cascade, whereas in others a secondary messenger such as Ca^{2+} may trigger the protein kinases (Blumwald *et al.,* 1998).

2.5 Protein Kinases

Plant development is a dynamic phenomenon and includes a variety of complex processes. Various signaling cascades are operating and these are largely dependent on the specificity of the stimulus, the biochemical nature of the

receptor and the specific response. Some of the biochemical components of signaling are G-proteins, calcium, polyphosphoinositides, cAMP, hormones, growth regulators, jasmonates, salicylic acid and fungal elicitors to name a few (Sopory and Munshi, 1998). These molecules carry information into the cell and are generally known as messengers.

There are numerous protein kinases that play a role in physiological, biochemical and development pathways that leads to a particular response. Various protein kinases have been isolated, characterized and their roles well established in animal systems. In plants a large number of protein kinases were shown to exist, each having a specific function to perform (Stone and Walker, 1995).

2.5.1 Receptor-like protein kinases

Plants perceive and respond to both endogenous and exogenous stimuli such as chemical (hormones and polysaccharides) and physical stimuli (light, pathogens and wounding). The mechanisms by which plants detect and transduce these signals into the cell are however poorly understood. Recent evidence suggests that plants have many different types of transmembrane protein kinases that may function to transfer/translate extracellular information into the cell. These plant proteins are called receptor-like protein kinases (Stein *et al.*, 1991; Walker, 1994) and the cloning of the maize ZmPK1 gene provided a breakthrough, showing that higher plants possess a gene product structurally analogous to mammalian receptor protein kinases (Walker and Zhang, 1990).

Receptor protein kinases are a diverse group of proteins that span the plasma membrane that allow cells to recognize and respond to their extracellular environment. Many signals are initially perceived by transmembrane receptors, a large number of which functions by activation of an intrinsic protein kinase domain through phosphorylation (Lease *et al.*, 1998).

Over the past few years, our knowledge of the biological functions of RLKs has increased significantly. For instance, genetic evidence has identified the *S* receptor

kinase (SRK) as the female determinant of the self-incompatibility (SI) recognition response in *Brassica* (Nasrallah *et al.*, 1994). In addition, the RLK subfamily with an extracytoplasmic leucine-rich repeat (LRR) domain has been found to regulate various developmental processes, phytohormone perception and defence responses. This subfamily of developmental regulators includes the *Arabidopsis ERECTA* gene which specifies organ shape (Torii *et al.*, 1996), CLAVATA 1 (CLV1) which controls meristem cell fate (Clark *et al.*, 1993) and HAESA which plays a role in floral abscission (Jinn *et al.*, 2000). A putative brassinosteroid receptor, BRASSINOSTEROID INSENSITIVE 1 (BRI1) (Li and Chory, 1997) and a race-specific disease-resistance gene of rice, *Xa21* (Song *et al.*, 1995), also belongs to the LRR-RLK family (Lease *et al.*, 1998; Friedrichsen *et al.*, 2000; Torii, 2000).

2.5.1.1 Classification of RLKs

Common features of plant RLKs are a cytoplasmic catalytic protein kinase domain, a single membrane-spanning region, an N-terminal signal sequence and an extracellular domain that varies both in structure and in sequence. Plant RLKs are classified into several groups based on the structural features of the predicted extracellular domain (Walker 1994; Braun *et al.*, 1997; Feuillet *et al.*, 1997; Becraft 1998; Coello *et al.*, 1999; Czernic *et al.*, 1999; Torii and Clark, 2000;). The classes and the proposed functions of the different RLKs are summarized in Table 2.1.

The S-class of RLKs possesses an extracellular S-domain homologous to the self-incompatibility-locus glycoproteins (SLG) of *Brassica* (Nasrallah and Nasrallah, 1993). The distinguishing feature of the S-domain is an array of ten cysteine residues in combination with other conserved motifs (Walker, 1993; Torii and Clark, 2000).

In *Brassica*, the SRK gene is physically linked to the S locus, so that SLG and SRK are proposed to function together in the self-incompatibility recognition between pollen and the stigma (Nasrallah *et al.*, 1994). The cloning of several S-domain RLK genes from self-compatible plant species and their expression in vegetative tissues indicate that S-domain RLKs may play a developmental role in addition to self-incompatibility recognition (Nasrallah *et al.*, 1994; Walker, 1994; Bower *et al.*, 1996).

Table 2.1: Plant receptor-like protein kinases and their proposed biological functions

RLK Class	Plant species	Biological function (if not known, expression pattern)	**Reference**
S-domain class			
SRK	*Brassica oleracea*	Self-incompatibility recognition	Stein *et al.*, 1991
SFR2	*Brassica oleracea*	Defence response signaling	Pastuglia *et al.*, 1997
ARK1	*Arabidopsis thaliana*	(Leaf cell expansion)	Tobias *et al.*, 1992
ARK2	*Arabidopsis thaliana*	(Cotyledon, leaf, sepal)	Dwyer *et al.*, 1994
ARK3	*Arabidopsis thaliana*	(Flower pedicles)	Dwyer *et al.*, 1994
RLK1	*Arabidopsis thaliana*	(Rosettes)	Walker, 1993
RLK4	*Arabidopsis thaliana*	(Root-hypocotyl boundary, base of lateral root, base of the petiole)	Coello *et al.*, 1999
ZmPK1	*Zea mays*	(Seedling roots, shoots and silks)	Walker and Zhang, 1990
KIK1	*Zea mays*	(Husks, etiolated shoots)	Braun *et al.*, 1997
OsPK10	*Oryza sativa*	(Upregulated by light)	Zhao *et al.*, 1994
LRR class			
BRI1	*Arabidopsis thaliana*	BR perception	Li and Chory, 1997
CLAVATA1	*Arabidopsis thaliana*	Meristem and flower development	Clark *et al.*,1993
ERECTA	*Arabidopsis thaliana*	Organ elongation	Torii *et al.*, 1996
PRK1	*Petunia inflate*	Pollen development	Lee *et al.*, 1996
SERK	*Daucus carota*	Correlation with embryogenic potential	Schmidt *et al.*, 1997
Xa21	*Oryza sativa*	Resistance to *Xanthomonas oryzae*	Song *et al.*, 1995
LePRK1, 2	*Lycopersicon esculentum*	(Pollen-pistil interaction)	Muschietti *et al.*, 1998
RKF1	*Arabidopsis thaliana*	(Anther specific)	Takahashi *et al.*, 1998
RPK1	*Arabidopsis thaliana*	(Osmotic-stress induced)	Hong *et al.*, 1997
LRRPK	*Arabidopsis thaliana*	(Light-repressed)	Deeken and Kaldenhoff, 1997
TMK1	*Arabidopsis thaliana*	(Abscisic acid-, dehydration-, high salt- and cold-induced)	Chang *et al.*, 1992; Hong *et al.*, 1997
RLK5/HAESA	*Arabidopsis thaliana*	Floral abscission	Jinn *et al.*, 2000
LTK1, 2, 3	*Zea mays*	(Endosperm specific)	Li and Wurtzel, 1998

RLK Class	Plant species	Biological function (if not known, expression pattern)	**Reference**
OsTMK1	*Oryza sativa*	Gibberellin-induced cell division and elongation	Van der Knaap *et al.*, 1999
EILP	*Nicotiana tabacum*	Non-host disease resistance	Takemoto *et al.*, 2000
OsLRK1	*Oryza sativa*	Floral meristem activity	Kim *et al.*, 2000
SARK	*Phaseolus vulgaris*	Senescence induced	Hajouj *et al.*, 2000
SbRLK1	*Sorghum bicolor*	(Mesophyll cells)	Annen and Stockhaus, 1999
LRPKm1	*Malus x domestica*	Disease resistance	Komjanc *et al.*, 1999
TNFR class			
CRINKLY 4 (CR4)	*Zea mays*	Epidermal cell specification	Becraft *et al.*, 1996
EGF class			
WAK1, 2, 3, 4	*Arabidopsis thaliana*	Cell expansion and disease response	He *et al.*, 1996; Wagner and Kohorn, 2001
PR5 class			
PR5K	*Arabidopsis thaliana*	Disease/stress response	Wang *et al.*, 1996
Lectin class			
LecRK1	*Arabidopsis thaliana*	Development and adaptation	Riou *et al.*, 2002
Other class			
CrRLK1	*Catharanthus roseus*	(Cultured cells)	Schulze-Muth *et al.*, 1996
RKF2, 3	*Arabidopsis thaliana*	(Constitutively expressed)	Takahashi *et al.*, 1998
Lrk10	*Triticum aestivum*	Leaf rust resistance	Feuillet *et al.*, 1997
At-RLK3	*Arabidopsis thaliana*	(Induced by oxidative stress, pathogen attack)	Czernic *et al.*, 1999
PvRK20-1	*Phaseolus vulgaris*	(Plant-microbe interaction)	Lange *et al.*, 1999

Other S-domain RLKs include ZmPK 1 (Walker and Zhang, 1990) and KIK1 (Braun *et al.*, 1997) from maize, ARK1 (Tobias *et al.*, 1992), ARK2, ARK3 (Dwyer *et al.*, 1994), RLK1 (Walker, 1993) and RLK4 (Coello *et al.*, 1999) from *Arabidopsis* and SRK from *Brassica oleracea* (Stein *et al.*, 1991).

Leucine rich repeat-receptor-like kinases (LRR-RLK) comprise the largest class of plant RLKs. LRRs have been found in a variety of proteins with diverse functions in yeast, *Drosophila*, humans and plants and are implicated in protein-protein interactions. The LRRs are tandemly repeated and can occur in very divergent forms with gaps and insertions within or between repeats. The most conserved element of the LRR repeat is a β-sheet that is thought to be an exposed site involved in protein-protein interactions (Kobe and Deisenhofer, 1993; Walker, 1994).

The LRR class was first represented by the isolation of genes coding for TMK 1 (Chang *et al.*, 1992) and RLK5/HAESA (Jinn *et al.*, 2000). Several other LRR-RLKs that play a critical role in cell differentiation have since been discovered, such as, pollen receptor-like kinase 1 (PRK1) (Hong *et al.*, 1997), *Lycopersicon esculentum* pollen receptor-like kinase 1 (LePRK1) and LePRK2 that plays a role in pollen development (Muschietti *et al.*, 1998; Riou *et al.*, 2002).

LRR-RLKs also play a role in disease resistance. The tomato Cf9, Cf4 and Cf2 disease-resistance proteins contain extracellular domains but only very short cytoplasmic domains (Thomas *et al.*, 1997). Xa21 confers resistance to bacterial leaf blight in rice (Song *et al.*, 1995). The Xa21 mediated resistance conforms to a gene-for-gene interaction in which *Xa21* expressing plants are resistant to *Xanthomonas oryzae*, that has a corresponding, but yet uncharacterized, Avr product.

The maize *CR4* (*CRINKLY 4*) gene product is the only member of the TNFR-like (tumor necrosis factor receptor) class (Becraft *et al.*, 1996). The CR4 protein mediates cellular differentiation responses in tissues of the shoot and endosperm. This suggests that CR4 may function in the perception of positional cues that specify aleurone cell fate throughout endosperm development.

Cell wall-associated receptor kinases (WAKs) represent the epidermal growth factor (EGF) class (He *et al.,* 1996). EGF-like repeats are found in a variety of animal extracytoplasmic receptor domains and are known to play a role in protein-protein interactions (Kohorn *et al.,* 1992). The four WAKs (WAK1 to WAK4) identified in *Arabidopsis* all have extracellular EGF-like domains (He *et al.,* 1996). Although the structure of WAKs suggests a role in cell wall-membrane binding and signaling, their true function is unknown. It is suggested that WAKs may be involved in pathogenic responses as the induction of these receptors are necessary to survive high levels of SA (Wagner and Kohorn, 2001).

The *Arabidopsis* PR5K is the only known example of the PR class of RLKs (Wang *et al.,* 1996). The extracellular domain of PR5K exhibits sequence similarity to PR5 (pathogenesis related protein 5) whose expression is induced upon pathogen attack. The structural similarity between the PR5K receptor domain and PR5 suggests a role for PR5K in the pathogenesis response (Wang *et al.,* 1996; Epple *et al.,* 1997).

Lectin-type RLKs (LecRKs) are conserved among plants and in *Arabidopsis thaliana*, lecRK genes belong to a large super family (Shiu and Bleecker, 2001). Like many other RLKs, no physiological role has been assigned to lecRKs. In contrast to legume lectins which interact with foreign and endogenous oligosaccharides, the deduced amino-acid sequences of lecRKs suggest that their lectin-like domains may interact with complex glycans rather than with monosaccharides (Riou *et al.,* 2002). LecRK-a1 was shown to play an important role in the developmental and adaptive processes in *A. thaliana*.

A new type of receptor-like kinase was isolated from wheat. Lrk10 is the first member of this new structural class (wlrk class) of plant receptor-like kinases (Feuillet *et al.,* 1997). Lrk10 was mapped to the Lr10 disease resistance locus in wheat and it is possible that it is the same gene. It is also suggested that Lrk10 is a non-functional member of the Lr10 gene family.

Several other known RLKs possess an extracellular domain that shares no homology to known motifs. These include CnRLK1 of *Catharanthus roseus* (Schulze-Muth *et al.*, 1996), RKF3 (Torii and Clark, 2000) and LRRPK (Deeken and Kaldenhoff, 1997) from *Arabidopsis*.

The structural diversity in the extracellular receptor domains of RLKs perhaps reflects their functional diversity. The extracellular domain of RLKs is thought to function in the recognition and binding of a ligand, while the transmembrane domain anchors the protein in the membrane and the protein kinase domain transduces the resulting signal inside the cell (Sopory and Munshi, 1998).
Despite structural diversity and varied substrate specificity, the catalytic kinase domain of most protein kinases contains eleven blocks or subdomains of conserved amino acid sequence (Hanks *et al.*, 1988). Eukaryotic protein kinases are commonly classified as either serine/threonine-specific or tyrosine specific although dual-specificity protein kinases have also been reported (Mu *et al.*, 1994). Two subdomains, VIb and VIII, are used to differentiate serine/threonine protein kinases from tyrosine kinases (Sopory and Munshi, 1998). In plant RLK sequences, subdomain VIb matches the consensus DLKPEN found in serine/threonine kinases while DLAARN is conserved in tyrosine kinases (Walker, 1994; Torii and Clark, 2000).

Protein phosphorylation catalyzed by protein kinases plays an important role in plant metabolism and signaling cascades (Sopory and Munshi, 1998). Phosphorylation events are thus brought about by protein kinases.

All plant RLK genes encode polypeptides with a hydrophobic amino terminus. This region is thought to act as a signal peptide and targets the proteins to the endoplasmic reticulum during synthesis (Singer, 1990). A second hydrophobic transmembrane domain of a RLK occurs between the extracellular domain and the catalytic kinase domain. It is believed that the transmembrane domain serves to anchor the RLK to the plasma membrane.

2.5.1.2 Function of RLKs

Recent studies have established that some RLKs function in disease resistance and plant development (Braun *et al.,* 1997). Advances in molecular genetics, especially in the model plant *Arabidopsis*, have provided evidence that some RLKs regulate development in higher plants (Lee *et al.,* 1996; Torii *et al.,* 1996; Li and Chory, 1997; Becraft, 1998). The functions of several RLKs are known even though the proteins responsible for transducing the signal downstream of an activated receptor kinase have not been characterized.

2.5.1.2.1 RLKs in developmental roles

The epidermis is the outermost cell layer that covers plant organs and is essential for the separation of mature plant organs. In the leaf, the epidermal cells form thick cell walls with a cuticle layer and epicuticular wax which play a role in water retention and the prevention of wetting as well as pathogen invasion (Mauseth, 1995).

The maize CR4 (CRINKLY4) gene is required for proper development of the epidermis (Becraft *et al.,* 1996). Mutants for *cr4* have crinkly leaves without a rough surface. Morphological analysis has revealed that the mutant phenotype is due to a loss of proper epidermal cell patterning. Thus, the important function of the epidermis to restrict cell division patterns to anticlinal planes and prevent surface differentiation is severely compromised by the cr4 mutation. CR4 also has a high similarity to TNFR's, which play a crucial role in mammalian inflammatory response and cell death (Smith *et al.,* 1994).

Brassinosteroids (BR) are growth-promoting steroids found in plants. BRs were first purified from the pollen of *Brassica napus* (Grove *et al.,* 1979). Genetic evidence for the role of BRs in development was only recently uncovered. Several *Arabidopsis* mutants defective in BR-biosynthesis pathways have been isolated (Clouse, 1996; Li and Chory, 1997). All of these mutants displayed a characteristic dwarf phenotype with curled, round, dark-green leaves, very short stems, reduced fertility and delayed senescence. By providing the mutants with brassinolides or appropriate

intermediates, one could rescue all of the BR-deficient mutants (Friedrichsen *et al.*, 2000; Torii and Clark, 2000).

Genetic screens for brassinolide-insensitive mutants in *Arabidopsis* revealed a single locus gene, BRI1. BRI1 was isolated by map-based cloning and found to encode an LRR-RLK. The presence of LRRs suggests that the extracellular domain may interact with a peptide. Alternatively, a brassinolide binding protein might interact with the LRRs to activate the signal transduction pathway. It is also conceivable that BRI1 acts indirectly in cells where it is activated to confer developmental competence for the hormone response (Friedrichsen *et al.*, 2000).

Mutations at the three CLAVATA loci, namely CLV1, CLV2 and CLV3, result in plants with enlarged shoot meristems and consequently an increase in the number of floral organs and whorls (Clark *et al.*, 1993). Genetic evidence has identified CLV3 as a possible ligand for CLV1, an LRR-RLK (Clark *et al.*, 1995). Biochemical analysis has shown that CLV3 is required for the formation of the active CLV1 receptor complex. These results indicate that CLV3 acts as a ligand for the CLV1 receptor and that ligand binding activates the CLV1 receptor complex (Clark *et al.*, 1995). The identification of CLV3 raises the question of whether ligands for other LRR-RLKs such as ERECTA and HAESA/RLK5 are structurally analogous to CLV3. Although the *Arabidopsis* genome contains CLV3-like sequences, matching possible ligands to RLKs primarily based on their sequences is not a realistic proposition, if one takes the large number of LRR-RLKs present in *Arabidopsis* into account (Clark *et al.*, 1995; Jeong *et al.*, 1999).

Light not only supplies plants with energy but also plays a crucial role as a signal in many processes unrelated to photosynthetic activity (Deeken and Kaldenhoff 1997). Among these are cellular and morphogenic development as well as growth responses. Deeken and Kaldenhoff (1997) isolated a light-repressible receptor protein kinase (LRRPK) from *A. thaliana*. This gene exhibits a phytochrome A-like photophobic mode of expression and is restricted to the cotyledons of etiolated *A. thaliana* seedlings and the roots of plants cultivated under greenhouse conditions.

Thus, lrrpk is expressed solely in organs that are not subjected to any light. The encoded protein LRRPK might thus be involved in the early steps of light signal transduction.

2.5.1.2.2 RLKs involved in self incompatibility

In the *Brassica* family, a self-incompatibility system is present that prevents self-fertilization and is controlled by the multiallelic S locus. When the pollen parent shares the same S allele as the pistil on which the pollen has landed, germination is terminated. Thus, only pollen from a plant that contains a different S allele can germinate and so fertilize the ovule (Nasrallah and Nasrallah, 1993). To date, two different genes that are tightly linked to the S locus appear to be required for the self-incompatibility reaction namely *SLG* and *SRK* (Nasrallah *et al.*, 1994). The simplest explanation for the self-incompatibility reaction is that there is a pollen ligand present on the incompatible pollen that is responsible for activating SRK, which in turn is thought to activate the signaling pathway in the stigma leading to the rejection of the incompatible pollen (Nasrallah *et al.*, 1994; Bower *et al.*, 1996).

2.5.1.3 RLKs involved in disease resistance

In the gene-for-gene concept, a pathogen-derived avirulence gene product (elicitor) and a plant-derived resistance gene product (receptor) specifically interact with each other resulting in an incompatible plant pathogen interaction (Higgins *et al.*, 1998). Several recently cloned disease resistance genes, including *Xa21* from rice (Song *et al.*, 1995), *Pto* from tomato (Tang *et al.*, 1999) and *Lrk10* from wheat (Feuillet *et al.*, 1997) was shown to be RLKs and belong to the class of serine/threonine protein kinases (Song *et al.*, 1998).

Xa21 confers resistance to bacterial leaf blight caused by *Xanthomonas oryzae* (Song *et al.*, 1995). Pto confers resistance to bacterial speck disease caused by *P. syringae* (Tang *et al.*, 1999) while Lrk10 confer resistance to *Puccinia triticina* (Feuillet *et al.*, 1997). While *Pto* encodes for only a protein kinase domain, *Xa21* and *Lrk10* code for a receptor-like protein kinase with an extracellular leucine-rich

repeat domain, a membrane-spanning region and an intracellular kinase domain (Song *et al.*, 1995; Feuillet *et al.*, 1997).

It has been hypothesized that *R* gene products encode receptors capable of binding *Avr* products as ligands. Expression of these gene products in susceptible plants resulted in specific resistance, demonstrating that even susceptible plants possess the ability for resistance (Bent, 1996). The difference between resistance and susceptibility appears to lie in the recognition of the *Avr* product. Sometimes multiple products, some of which may be closely linked to the original *R* gene, are required for the manifestation of resistance. An example of this is the functional interaction between the tomato *Pto* and *Prf* gene products for expression of resistance to *P. syringae* (Oldroyed and Staskawicz, 1998). Figure 2.1 shows a proposed model for the Pto and Prf interaction. The *Pto* gene product is a serine/threonine kinase, whereas the *Prf* gene product contains the LRR domain, nucleotide binding sites (NBS) and leucine zipper (LZ) domains (Blumwald *et al.*, 1998).

It has been demonstrated that the Pto kinase interacts with the avrPto protein and mutations in Pto or avrPto that disrupts this interaction, abolish disease resistance (Gu *et al.*, 2000). Replacement of the weak endogenous promoter of Pto with the strong promoter of the cauliflower mosaic virus resulted in not only a further increase in resistance to *P. syringae,* but also a partial control of unrelated pathogens, such as *Xanthomonas campestris* and *Cladosporium fulvum* (Tang *et al.*, 1999; Rommens and Kishore, 2000).

Feuillet *et al.*, (1997) used a homology-based approach to test whether leaf rust resistance genes in wheat might encode serine/threonine kinases, as there are a high number of such kinases present in eukaryotic genomes. They used a set of near-isogenic wheat lines with different leaf rust resistance genes to relate a polymorphic DNA fragment to a specific resistance gene and isolated a new receptor-like kinase gene (Lrk10) encoded at the Lr10 disease resistance locus. The Lrk10 protein contains a new type of extracellular recognition domain, which was

not previously described. Genetically related wheat lines containing the Lr10 resistance gene were found to have identical alleles of Lrk10, whereas lines without Lr10 had different alleles at the same locus.

2.5.1.4 Proteins that interact with RLKs

Several proteins that interact with the Pto kinase have been characterized. The *Pti1* (Pto-interacting 1) gene encodes a serine/threonine kinase that is probably a downstream substrate of Pto (Zhou *et al.*, 1995). Three other proteins, encoded by the genes Pti4, Pti5 and Pti6, were also found to interact with the Pto kinase. Each of these proteins was shown to be a transcription factor that binds to the promoter region of other PR genes (Blumwald *et al.*, 1998; Van der Biezen and Jones, 1998).

Three proteins have been identified which may mediate a downstream step in an RLK initiated signaling pathway. They are a kinase associated protein phosphatase (KAPP) from *Arabidopsis* and two thioredoxins from *Brassica* (Bower *et al.*, 1996). KAPP was shown to interact *in vitro* with the phosphorylated form of RLK5, but not with the non-phosphorylated form (Stone *et al.*, 1994). KAPP can thus distinguish between the autophosphorylated, activated state of the kinase and the non-phosphorylated, inactive form and associates only with the former to presumably function in a signaling complex. The phosphorylation-dependent association between the kinase domain of RLK5 and the kinase interaction domain of KAPP is similar to the interaction between animal cell receptor tyrosine kinases and Src homology 2 (SH2) domains or protein tyrosine-binding (PTB) domains (Braun *et al.*, 1997).

Proteins that contain SH2 or PTB domains play important roles in animal cells in mediating cellular signaling based on phosphorylated tyrosine residues. Because the plant receptor-like protein kinases characterized to date are predicted to encode serine/threonine kinases and not tyrosine kinases, it is hypothesized that RLKs may use alternative means to transduce a signal to the next downstream protein in a signal transduction cascade.

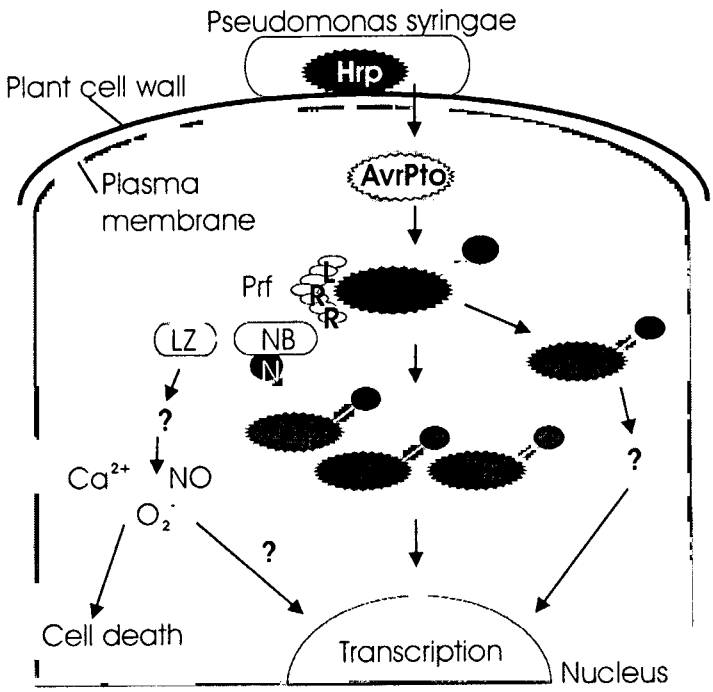


Figure 2.1: Model for the function of the tomato NB-LRR protein Prf in Pto-mediated recognition of the bacterial avrPto protein Hrp, which is a contact dependent bacterial (type III) secretion system. (Van der Biezen and Jones, 1998).

Therefore, it is hypothesized that the kinase interaction domain of KAPP would function in a manner analogous to SH2 or PTB domains, but would bind to phosphoserine and/or phosphothreonine residues of active RLKs (Stone *et al.*, 1994; Braun *et al.*, 1997).

Parsley cells recognize the fungal pathogen *Phytophthora sojae* through a plasma membrane receptor (Ligterink *et al.*, 1997). A pathogen-derived oligopeptide elicitor binds to this receptor and thereby stimulates a multicomponent defence response through sequential activation of ion channels and an oxidative burst. An elicitor-responsive MAPK was identified that acts downstream of the ion channels but independently or upstream from the oxidative burst (Ligterink *et al.*, 1997). Upon receptor-mediated activation, the MAPK is translocated to the nucleus where it might interact with transcription factors that induce expression of defence genes.

2.5.2 MAP Kinase Cascades

MAPK cascades are found in yeast, mammals and plants and function to translate extracellular stimuli into intracellular signals. MAP kinases are activated by MAP kinase kinase (MAPKK), which in turn is activated by a MAP kinase kinase kinase (MAPKKK). Approximately 20 different MAPKs have been identified in the *Arabidopsis* genome and other plants are likely to have similar amounts of MAPKs (Sessa and Martin, 2000; Zhang and Klessig, 2001).

Although the organization of plant MAPK cascades is likely to be similar to those in yeast and animals, not a single plant MAPKKK-MAPKK-MAPK model has yet been assembled based on *in vivo* evidence. A tobacco MAPKK, named salicylic acid-induced protein kinase kinase (SIPKK), was identified by a yeast two hybrid screening using salicylic acid-induced protein kinase (SIPK) as probe, but SIPKK could not phosphorylate SIPK (Zhang and Klessig, 2001).

MAPKKKs are the most divergent group of the three different MAP kinases. Several unrelated kinases can function as MAPKKKs to initiate the cascade for a single MAPK. Based on the similarity of the kinase domain, several plant kinases have been

identified as MAPKKKs, including CTR1, EDR1, NPK1/ANP and MEKK1-MEKK4. Although the downstream MAPKK is unknown, tobacco NPK1 and its *Arabidopsis* homologues (ANPs) have been shown to activate MPK3 and MPK6, two *Arabidopsis* MAPKs (Zhang and Klessig, 2001).

In spite of their highly conserved organization, plant MAPK cascades have evolved to play roles that are unique to plants, such as cytokinesis and phytohormone signaling. Recent studies also provided evidence that MAP kinases are also involved in the activation of stress-associated responses (Jonak *et al.*, 2002). The first evidence for the involvement of MAP kinases in defence responses was provided by the analysis of kinase activities from plant cell extracts that were able to phosphorylate myelin basic protein (MBP) (Sessa and Martin, 2000; Zhang and Klessig, 2001).

2.5.3 Transcriptional activators

Plant WRKY DNA-binding proteins recognize various WRKY-box elements with a TGAC core sequence that is present in promoters of a number of defence-related genes (Rushton and Somssich, 1998). A number of genes encoding WRKY proteins have been isolated from several plants, including some that are rapidly induced by pathogen infection or treatment with pathogenic elicitors or SA. DNA sequences similar to the W boxes have been found in promoters of the maize PR1 class gene *Prms* (Raventos *et al.*, 1995), the potato *Gst 1* gene (Hahn and Strittmatter, 1994), the *PR-10* gene from asparagus (Despres *et al.*, 1995), the *Vst1* gene from grapevine (Schubert *et al.*, 1997) and *RLK3, RLK4, RLK5* and *RLK6* from *Arabidopsis* (Du and Chen, 2000).

The pathogen-induced WRKY DNA-binding proteins may thus serve as common transcriptional activators that regulate the expression of a large set of pathogen-responsive genes. It may be possible to identify their possible target genes by analyzing potential WRKY DNA-binding sites within the *Arabidopsis* genome and predict pathways of transcriptional regulation. Subsequent molecular analysis

supported the hypothesis that these genes serve as targets of pathogen- and SA induced W-box binding proteins (Du and Chen, 2000).

2.6 Leaf Rust Resistance

Leaf rust on wheat (*Triticum aestivum*) is caused by *P. triticina* and found wherever wheat is grown and is the most regularly occurring of the three rusts, namely leaf rust, stripe rust and yellow rust found on wheat. Wheat cultivars that are susceptible to leaf rust suffer from yield reductions of 5 to 15% or more, depending on the stage of crop development when the initial rust infection occurs (Kolmer, 1996).

Genetic resistance is the most economical and preferable method of reducing yield losses due to leaf rust infection and can be fully utilized by knowing the identity of resistance genes in commonly used parental germplasm and released cultivars. Identification of the leaf rust resistance genes allows for efficient incorporation of different genes into germplasm pools. To date 46 leaf rust resistance genes (*Lr*) have been isolated and mapped to specific chromosomes. Near-isogenic Thatcher wheat lines for nearly all the leaf rust resistance genes were also developed (Kolmer, 1996).

Rust resistance in wheat has traditionally been based on the use of specific resistance genes but such resistance is often short lived. Durable resistance to wheat leaf rust has rarely been found and the basis for the most durable resistance to wheat leaf rust has been combinations of resistance genes *Lr*13 + *Lr*34 and *Lr*12 + *Lr*34 (German and Kolmer, 1992; Kolmer, 1996).

The resistance gene *Lr*34 is located at chromosome 7D. *Lr*34 is the only effective resistance gene in the Canadian cultivar Glenlea that has been resistant to leaf rust since its release in 1972, as well as in the American hard red winter wheat Sturdy, which has also shown durable resistance to leaf rust (German and Kolmer, 1992). In the field the presence of the *Lr*34 gene leads to variable pustule size and low percentages of infection. In the wheat variety Thatcher, *Lr*34 exhibited "slow rusting"

resistance and had a severity that was 50% lower than Thatcher without *Lr34*. *Lr*34 can be detected in the seedling stage, but it is best expressed in adult plants.

For some corresponding gene pairs the interactions differ from the classical one-to-one relationship. For example, three different patterns for inheritance of virulence corresponding to resistance genes in allelic sets in wheat have been found. Although the *Lr*2 locus in wheat has three alleles, *Lr*2a, *Lr*2b and *Lr*2c, avirulence is conditioned by a single gene in *P. triticina* (Kolmer, 1996).

In addition to enhanced resistance to leaf rust, *Lr*34 has been found to contribute to improved resistance to stem rust *(P. graminis)*, since Th/*Lr*34 has considerably more stem rust resistance than Thatcher. It is also tightly linked with resistance genes to stripe rust (*P. striiformis*) and barley yellow dwarf virus (BYDV) and selection for *Lr*34 would also select resistance to both stripe rust and BYDV (German and Kolmer, 1992; Kolmer, 1996).

It is remarkable that *Lr*34 has contributed to provide an effective level of resistance despite being in cultivars that have been grown extensively for extended periods in many wheat-growing areas of the world. There is no clear explanation for the longevity of *Lr*34's effectiveness. For example, the wheat leaf rust fungus is present year-round in the wheat growing areas of South America and wheat lines with *Lr*34 have maintained effective levels of resistance in this region despite the large number of yearly uredinial generations that should give ample opportunity for isolates with virulence to develop (Kolmer, 1996).

2.7 Concluding remarks

Numerous RLKs have been cloned but only a few have undergone molecular characterization. Functions for RLKs have been shown in diverse biological processes such as development, disease resistance and self incompatibility and although their biological roles are diverse, they may share common signaling elements. Many important insights into how plants perceive and respond to their

environment will be gained by characterizing RLKs and their signaling pathways (Lease *et al.*, 1998).

Both the *Pto* tomato resistance gene and the *Xa21* resistance gene in rice have been found to encode serine/threonine protein kinases, suggesting that other resistance genes might also belong to this class of proteins. By exploiting the similarity between serine/threonine protein kinases, the *Lrk10* gene was isolated from the complex wheat genome (Feuillet *et al.*, 1997).

An approach taking advantage of the coding sequence of the new extracellular domain of *Lrk10* has already identified putative candidates for a number of additional leaf rust resistance genes (Feuillet *et al.*, 1998). In addition, race-specific resistance genes against other biotrophic fungal pathogens such as powdery mildew or stem rust could possibly be identified by this approach (Feuillet *et al.*, 1997).

The most durable leaf rust resistance is almost inevitably provided by adult-plant resistance genes, often *Lr*34. However, over reliance on *Lr*34 would be foolish. There is no reason to assume that isolates of *P. triticina* with virulence to this gene will not eventually appear and quickly be selected for in the pathogen population. The continuous genetic examination of wheat and its related species for the presence of new resistance genes and their signaling pathways will to help maintain a diversity of effective resistance genes in released cultivars (Kolmer, 1996).

Chapter 3:

Material and Methods

3.1 Materials

3.1.1 Plant Material

The plant material used in this study consisted of susceptible and resistant plants from the *Thatcher* wheat line and susceptible plants from the *Karee* wheat line. The resistant *Thatcher* plants contained the *Lr34* leaf rust resistance gene. The Karee seeds were planted in pots containing a sterilized 1:1 soil: veen mixture and grown in a greenhouse at 25°C with a 16 h day and 8 h night cycle. When they were 5 days old, each pot received 50 ml of a 10 $mg.l^{-1}$ maleic hydrazide solution which is a growth inhibitor. The *Thatcher* and *Thatcher/Lr34* plants were grown in a climate cabinet where cool-white fluorescent tubes emitting 120 $\mu E.m^{-2}.s^{-1}$ provided a 24 h day length. When the plants were 10 days old, each pot received 20 ml of a 10 $g.l^{-1}$ liquid fertilizer (1part nitrogen: 2 parts phosphorous: 3 parts of potassium).

3.1.2 Leaf Rust Material

The leaf rust material consisted of spores of the leaf rust fungus, *Puccinia triticina* pathotype UVPrt9.

3.1.3 Chemicals

ρ-Hydroxybenzoic acid hydrazide (PAHBAH) (Sigma-Aldrich) was used in the colorimetric determination of β-1,3-glucanase activity as it was stated by Lever (1972) to be the most sensitive reagent for β-1,3-glucanase activity determination. The ECL system (Amersham Pharmacia Biotech) was used for the chemiluminescent detection of β-1,3-glucanase polypeptides transferred to PVDF membranes (Amersham Pharmacia Biotech). An anti-rabbit horseradish peroxidase antibody from Amersham Pharmacia Biotech was used as secondary antibody during the Western blot analysis.

The Titan RT-PCR system (Roche) was used to reverse transcribe and amplify putative differentially expressed protein kinase genes. A GFX-Gel and PCR cleaning kit from Amersham Pharmacia Biotech was used to purify the differentially expressed genes

where after a pGemT-Easy vector System II (Promega) was used for the cloning of these expressed genes.

All other chemicals were of the highest quality and purity.

3.2 Methods

3.2.1 Leaf rust augmentation

The *Karee* plants were used to propagate the *P. triticina* UVPrt9 strain. The spores were mixed (approximately 65000 spores.ml^{-1}) with mineral oil and sprayed on 10-day-old *Karee* plants. The plants were incubated overnight in a dew-simulation cabinet at 18 to 20°C for 12 h and then transferred to the greenhouse. When the fungus on the infected plants began to sporulate, the spores were collected and used to inoculate adult *Thatcher* and *Thatcher/Lr34* plants.

3.2.2 Leaf rust inoculation

The adult *Thatcher* and *Thatcher/Lr34* plants were sprayed with freshly harvested spores of *P. triticina* pathotype UVPrt9 that were suspended in distilled water containing a drop of polyoxyethylene sorbitan monolaurate (Tween™ 20). The control plants were sprayed with distilled water containing Tween™ 20. The plants were left to dry and then placed in the dark in a dew-simulation cabinet at 18 to 20°C for 12 h to facilitate spore germination and was then transferred to the greenhouse. For the first time study, material was collected from infected and uninfected plants at six-hour intervals for the first 24 h and then every 12 h thereafter up to 78 h. For the second time study, leaf material was collected at three-hour intervals for 15 h where time zero was directly after inoculation. Every sample was quick-frozen in liquid nitrogen and stored at -70°C.

3.2.3 Protein Extraction

Total cellular protein was extracted as follows. The plant tissue was ground in liquid nitrogen to a fine powder. Extraction buffer (50 mM Tris-HCl pH 7.5, 2.0 mM ethylenedinitrilotetraacetic acid (EDTA), 2.0 mM phenylmethyl-sulfonylfluorid (PMSF) and 10 mM β-mercaptoethanol) was added to the powdered plant tissue in a 3:1

ratio and mixed thoroughly. The homogenate was centrifuged at 10 000 *g* for 15 min and the supernatant containing the total cellular proteins was kept at -70°C.

The concentration of the extracted proteins was determined according to Bradford (1976) using the protein assay reagent from Bio-Rad with 0.5 $mg.ml^{-1}$ gamma globulin as standard. A microplate reader (Bio-Rad Model 3550) was used according to the method described by Rybutt and Parish (1982). The following formula was used to calculate the protein concentration: protein concentration = absorbancy of sample x (concentration of standard. $absorbancy^{-1}$ of standard) x dilution of the sample.

3.2.4 Biochemical Analysis

3.2.4.1 β-1,3-Glucanase activity

The determination of the β-1,3-glucanase activity was done according to Lever (1972). The first step was to prepare a standard curve which was used to determine the linear region for β-1,3-glucanase activity. This was done by determining the A_{410nm} values of different concentrations of glucose and then drawing a graph of the A_{410} values against the glucose concentrations. For the first curve the following glucose concentrations were used: 1 nM, 10 nM, 100 nM, 0.001 mM, 0.01 mM, 0.1 mM, 1 mM, 10 mM and 100 mM.

A second curve was drawn to better define the linear region of the curve with the following concentrations: 0.02 mM, 0.039 mM, 0.078 mM, 0.156 mM, 0.313 mM, 0.625 mM, 1.25 mM, 2.5 mM, 5 mM and 10 mM. The different glucose dilutions were incubated at 37°C for 30 min. One and a half milliliter PAHBAH solution (5% (w/v) PAHBAH dissolved in 0.5 M HCl) was added to the tubes, boiled for 10 min at 94°C and the A_{410} determined. The plant samples were treated exactly the same, but a known volume of protein extract was added in place of the glucose. The activity was calculated as follows: $((A_{410} - C).\ M^{-1})$ x $(\text{amount of protein x incubation time})^{-1}$, where C is the x-cutoff and M is the gradient of the standard curve.

3.2.4.2 SDS-PAGE

Twenty microgram total cellular protein was separated on a 12% (w/v) SDS-Page gel according to Laemmli (1970). The SDS-Page gel consisted of a running gel and stacking gel. The running gel was made up of 12% (w/v) acrylamide: bis-acylamide (30:0.8), 0.34 M Tris (pH 8.8), 0.09% (w/v) sodium dodecyl sulphate (SDS), 0.04% (w/v) ammonium peroxodisulphate (APS) and 0.067% (v/v) N,N,N',N'-tetramethylethylenediamine (TEMED). The 6% (w/v) stacking gel solution consisted of 6% (w/v) acrylamide: bis-acrylamide (30:0.8), 0.12 M Tris (pH 6.8), 0.09% (w/v) SDS, 0.1% (w/v) APS and 0.16% (v/v) TEMED. Prior to the loading of the proteins on the gel, loading buffer was added to each sample to a final concentration of 1% (w/v) SDS, 4% (v/v) glycerol, 50 mM dithiothreitol (DTT), 13 mM Tris (pH 6.8) and 0.04% (w/v) bromophenol blue to the proteins and heated to 96°C for 10 min. The proteins were separated for 1 h at 200 volt and constant current in running buffer (0.1 M Tris, 0.15 M Glycine and 0.1% (w/v) SDS). The gel was stained with Coomassie (2 $mg.ml^{-1}$ Coomassie Blue R250, 1 $mg.ml^{-1}$ Coomassie Blue G250, 7% (v/v) acetic acid and 50% (v/v) methanol) for 30 min at 60°C and destained in 43% (v/v) methanol and 7% (v/v) acetic acid. The gels were then photographed.

3.2.4.3 Immunological detection of β-1.3-glucanase levels

Western blots were done to verify the induction of β-1.3-glucanase protein levels after infection. Immediately after separation of the polypeptides on a SDS-PAGE gel (3.2.4.2), they were transferred to PVDF membranes using a Hoefer wet transfer system for 1 h at 350 mA current in a transfer buffer consisting of 25 mM Tris (pH 8.3), 192 mM glycine and 20% (v/v) methanol according to the manufacturer's specifications.

After transfer, the membrane was quenched for 1 h in TBST (10 mM Tris-HCl, 1.5 M NaCl, 0.1% (v/v) Tween™ 20) and 8% (w/v) fat-free milk powder) and washed twice for two minutes each in TBST. A rabbit-anti-β-1.3-glucanase antibody (Anguelova *et al.*, 1999) was used as primary antibody diluted 1: 9000 in TBST containing 4% (w/v) fat-free milk powder. The membrane was probed for one hour at room temperature with the primary antibody where after it was washed once for fifteen

minutes and three times for five minutes each in TBST. The secondary antibody, an anti-rabbit horseradish peroxidase antibody (Amersham Pharmacia Biotech), was diluted 1: 2000 in TBST and the membrane was probed for 1 h at room temperature, washed once for 15 minutes followed by three times for five minutes each in TBST. Detection was then done using the ECL detection system according to the manufacturers' specifications. To identify induced expression, the band intensities were determined using the Molecular Analyst software (Biorad). Each time point was expressed relative to time 0.

3.2.4.4 Hydrogen peroxide concentration

H_2O_2 levels were determined according to Patterson *et al.* (1984). A standard curve was drawn for the A_{415} values of different concentrations of H_2O_2 using the following concentrations: 0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM, 1 mM, 1.2 mM, 1.4 mM and 1.6 mM.

For the determination of H_2O_2 levels in the plants, fresh tissue was homogenized in cold acetone and centrifuged at 1250 *g* for 20 min. Titanium reagent (20% (v/v) $TiCl_4$ in concentrated HCl), was added to a known volume of supernatant to a final concentration of 2% (v/v) titanium. The solution was agitated and one-fifth strength NH_4OH was added drop wise while mixing thoroughly. The samples were centrifuged at 1250 *g* and the precipitate washed repeatedly with acetone until the supernatant was colorless. The precipitate was solubilized in 2 *N* H_2SO_4 and filtered prior to determining the A_{415nm} value against a control lacking enzyme extract, treated the same as the other samples. The H_2O_2 levels were determined using the formula: $(((A_{415nm}\text{-}C).M^{-1}).vol^{-1}).mass^{-1}$, where C is the x-cutoff, M is the gradient of the standard curve, vol is the volume enzyme extract and mass is the starting fresh mass of the sample.

3.2.4.5 Peroxidase activity

Peroxidase activity assays were done according to Zieslin and Ben-Zaken (1991). This was done by determining the rate of conversion of guiacol to tetraguiacol by means of peroxidase at A_{470nm}. Each reaction consisted of the following components: 80 mM potassium phosphate buffer containing 200 μM EDTA pH 5.5,

50 mM guiacol, 8.2 mM H_2O_2 and 10 μl of enzyme extract. The absorbancy was determined every 10 seconds over 180 seconds at 30°C. The difference in absorbancy was used to determine the activity using the following formula: Specific enzyme activity = $[\Delta A \times \text{dilution factor} \times 10^9].[\Delta t \times \varepsilon \times \text{protein concentration}]^{-1}$ where ΔA is the difference in absorbancy, Δt is the difference in time (10 sec or 0.6 min), 10^9 is the conversion of mol to nmol, ε is 2.66×10^7 which is the molar extinction coefficient of guiacol and the dilution factor ([total volume in cuvette.volume enzyme^{-1}] x dilution of enzyme).

3.2.4.6 Possible Interplant Communication

A time study was performed to determine a possible interplant communication between infected and uninfected plants. *Th/Lr34* plants were infected with leaf rust and grown for 24 h to allow the fungus to colonize the plant. Uninfected control plants were kept in an environment free of any rust contamination.

After inoculation, samples of each group of plants were taken 0 hours post inoculation (h.p.i), 12 h.p.i and 24 h.p.i. Both groups of plants were then placed under bell jars. The bell jar of one set of infected plants was connected with a pipe with a closed glass tap to the bell jar of the set of uninfected plants. Another set of uninfected plants was kept isolated as a control. The plants were allowed to grow for a further 24 h and another sample were taken after this period (48 h.p.i).

After this acclimation stage (48 h.p.i), the glass tap connecting the infected and uninfected plants was opened and gas exchange was allowed between the two sets of plants for 24 h after which samples of the plants were taken (72 h.p.i). β-1.3-glucanase activity was determined (3.2.4.1) on total cellular proteins (3.2.3).

3.2.5 Molecular analysis

3.2.5.1 Total RNA extraction from wheat

Total RNA was extracted from wheat leaf tissue according to the method of Chomczynski and Sacchi (1987). The RNA concentration was determined with the following formula: $((A_{260} \times \text{dilution}).\text{total volume in cuvette}^{-1}) \times$ constant which is 40

ng.μl^{-1} for single stranded RNA. Extracted RNA was visualized on a 1% (w/v) agarose gel in 0.5 x TAE (20 mM Tris, 0.5 mM EDTA and 0.28% (v/v) acetic acid) with the addition of loading buffer (0.005% (w/v) bromophenol blue, 0.06% (w/v) orange G and 2.5% (w/v) Ficoll) to determine the quality of the extracted RNA (Sambrook *et al.*, 1989).

3.2.5.2 RT-PCR amplification of differentially expressed genes

Differential display reverse transcription polymerase chain reaction (DD RT-PCR) was performed to identify inducibly expressed putative protein kinase genes.

Two separate forward primers (Bovis 22 and Bovis 23) were used in combination with an anchored oligo dT primer (Bovis 21) as the reverse primer to amplify protein kinase genes. To obtain the sequences for the two forward primers, the kinase domains of several protein kinases were aligned. From this alignment it was determined that there is a difference of two amino acids in the conserved sub domain VIb of monocot and dicot plants. The sequences of the two forward primers were as follows: Bovis 22 5'- GAY ATH AAR CCN CAY AAY – 3' and Bovis 23 5'-GAY GTN AAR CCN GAR AAY – 3', where Y = C or T, H = A ,C or T, R = A or G and N = A, G, C or T. The reverse primer, Bovis 21, had the following sequence: 5'-dT_{12}VN – 3', where V = A, G or C and N = A, G, C or T (Appendix A).

Each RT-PCR reaction consisted of 10 ng total RNA, 2.5 pmol of one forward and the oligo dT primer, 0.25 mM deoxynucleotide triphosphates (dNTP's), 5 mM DTT, 1x RT-PCR buffer, 1.5 mM $MgCl_2$, 1 μl of the Titan enzyme mix and 10 μCi [α-^{32}P] dCTP. Each reaction was overlaid with 30 μl mineral oil and the reactions were performed in a Hybaid thermal cycler using the following conditions: 1 cycle at 37°C for 30 minutes, 1 cycle at 94°C for two minutes, 25 cycles at 94°C for 10 seconds, 35°C for one minute and 68°C for four minutes, 10 cycles at 94°C for 10 seconds, 35°C for one minute, 68°C for four minutes with an extension of 5 seconds with each subsequent cycle followed by a final cycle at 68°C for seven minutes. The RT temperature was reduced from the recommended 50°C to 37°C due to the low

Tm values of the primers used. During the optimization of the RT-PCR the *Arabidopsis At-RLK3* gene was used as a control since it contained a poly-A tail.

The DD RT-PCR products were resolved on a 6% (v/v) non-denaturing Long Ranger™ (FMC Bioproducts) gel in 0.6 x TBE (54 mM Tris pH 8.0, 54 mM boric acid and 1.2 mM EDTA) with the addition of loading buffer [0.005% (w/v) bromophenol blue, 0.06% (w/v) orange G and 2.5% (w/v) Ficoll] for 1.5 h at 120 volts and constant current after a pre-run of 30 minutes. The gels were dried between two sheets of dehydrating cellophane and exposed to x-ray film for between 1 to 4 days.

3.2.5.3 cDNA recovery

For the recovery of the differentially expressed genes, the developed x-ray film was aligned with the dried gel and the bands of interest were cut from the gel with a sharp, sterile scalpel blade. Each gel fragment was placed in a microcentrifuge tube and 100 μl distilled water was added. The tubes were incubated for 15 min at room temperature where after it was incubated at 94°C for 10 min. The tubes were centrifuged at 10 000 *g* for three minutes and the supernatant containing the cDNA was transferred to a new microcentrifuge tube.

The cDNA was re-amplified using the polymerase chain reaction (PCR) and the original primer combinations. Each reaction consisted of 5 μl cDNA, 2.5 pmol of each primer, 0.25 mM dNTP's, 1 x PCR buffer, 1.5 mM $MgCl_2$, 10% (v/v) dimethylsulfoxide (DMSO) and 3U of Taq polymerase (Sigma-Aldrich). Each reaction was overlaid with 20 μl mineral oil and performed in a Hybaid thermal cycler using the following conditions: 1 cycle at 94°C for two minutes, 35 cycles at 94°C for 10 seconds, 35°C for one minute and 72°C for four minutes followed by a final cycle at 72°C for seven minutes.

After completion of the PCR reaction, five microlitres of each reaction were separated on a 1% (w/v) agarose gel in 0.5 x TAE to confirm the amplification (3.2.5.1).

3.2.5.4 Cloning of differentially expressed fragments

For the cloning of the re-amplified fragments, competent *Escherichia coli* JM109 cells were prepared according to Inoue *et al.* (1990). An *E. coli* culture was streaked on a fresh LB plate (1% (w/v) tryptone, 0.5 % (w/v) Yeast-extract, 1% (w/v) NaCl and 1.5% (w/v) agar) and incubated overnight at 37°C. Five colonies were inoculated in SOB shake cultures [2% (w/v) tryptone, 0.5% (w/v) yeast-extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$ and 10 mM $MgSO_4$] and grown overnight at room temperature until an A_{600} = 0.6 was reached. The cells were incubated on ice for 10 min and centrifuged for 10 min at 2500 *g* at 4°C. The supernatant was discarded and the cells re-suspended in cold TB-buffer (10 mM Piperazine-N,N'-bis(2-ethane-sulfonic acid) (Pipes) pH 6.7, 15 mM $CaCl_2$, 250 mM KCl and 55 mM $MnCl_2$). The re-suspended cells were incubated on ice for 10 min and then re-centrifuged. The pellet was resuspended in ice-cold TB-buffer containing 1.4 % (v/v) DMSO and incubated on ice for 10 min. The cells were divided into microcentrifuge tubes, snap-frozen in liquid nitrogen and stored at -70°C.

The re-amplified fragments were ligated into the pGemT-Easy vector according to the manufacturer's specifications. Three microlitres of the PCR reaction was added to 50 ng pGemT-Easy plasmid, 1U T4 DNA Ligase in a ligation buffer (33 mM Tris-acetate pH 7.9, 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM DTT). The reaction was incubated at 4°C overnight.

The ligation mixture was added to 100 µl JM109 competent cells, incubated on ice for 30 min and heat shocked at 42°C for 30 seconds. The reaction was then incubated in a shaking water bath at 37°C after the addition of 1 ml LB medium. The cells were finally plated on LB plates containing 50 $\mu g.ml^{-1}$ ampicillin, 1 $mg.ml^{-1}$ 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) and 1$mg.ml^{-1}$ Isopropyl β-D-thiogalactopyranoside (IPTG) (Sambrook *et al.*, 1989).

3.2.5.5 Sequencing of fragments

White colonies containing recombinant plasmids were grown overnight in LB shake cultures at 37°C after which the cells were harvested by centrifugation at 12000 *g*

for one minute at 4°C. The cells were resuspended in a resuspension solution (25 mM Tris pH 7.5, 10 mM EDTA, 50 mM glucose) and incubated for five minutes on ice, where after a cell lysis solution [0.2 M NaOH and 0.1% (w/v) SDS] was added, mixed by inversion and incubated for five minutes on ice.

Ice-cold 0.3 M potassium acetate solution, pH 4.8 was added to neutralize the lysate and incubated on ice for five minutes and then centrifuged at 12000 *g* for five minutes. The upper, aqueous phase was transferred to a fresh tube and 2.5 volumes ice-cold absolute ethanol were added to the supernatant. The tubes were mixed and incubated on ice for five minutes, centrifuged and the pellet washed with ice-cold 70% (v/v) ethanol, centrifuged and dried under vacuum. The dried pellet was dissolved in 50 μl distilled water and a 5 μl aliquot was separated on a 1% (w/v) agarose gel (3.2.5.1).

To confirm that the extracted plasmids did indeed contain inserts, 500 ng of each plasmid was digested with 10U *Eco*R1 in a buffer consisting of 90 mM Tris-HCl, 10 mM Mg_2Cl and 50 mM NaCl, pH 7.5. The reactions were incubated at 37°C for 2 h, where after it was separated on an agarose gel (3.2.5.1) together with undigested plasmid.

After confirmation of the presence of the inserts in the plasmids, 100 ml LB shake cultures containing 50 $\mu g.ml^{-1}$ ampicillin were inoculated with the recombinant colonies. The cultures were allowed to grow overnight where after the plasmid DNA was extracted with a Qiagen midi-prep kit (Qiagen) according to the manufacturers' instructions. After extraction the plasmid concentration was determined (3.2.5.1). The standard value used was 50 $\mu g.ml^{-1}$ for double stranded DNA

Aliquots of the extracted plasmids were freeze dried and sent to the Central Analytical Facility at Stellenbosch University for sequencing in one direction using the SP6 primer. The resulting sequences were used to do a BLAST search on the

GenBank website (http://www.ncbi.nlm.nih.gov/Genbank) to search for similarities to known genes.

3.2.5.6 Re-amplification of differentially expressed protein kinase genes

Following the results of the BLAST search, the DD RT-PCR reaction was repeated with an oligo dT primer having a higher Tm-value to exclude random annealing of the primers. The new oligo dT primer contained an additional 5' tail to improve specificity of the PCR reaction. This region acted as the attachment site of Bovis 39 during the PCR step. The oligo dT primer, Bovis 21, was substituted for Bovis 32 during the RT part of the RT-PCR with the addition of Bovis 39 for the PCR part of the reaction. The sequences for the primers were as follows: Bovis 32 5' – GAA GAA TTC TCG AGC GGC CGC dT_{19}VN – 3' where V is an A, G or C and N is an A, G, C or T and Bovis 39 5' – GAA GAA TTC TCG AGC GGC – 3'. The RT-PCR reactions were performed as described previously (3.2.5.2) except for an increase in the PCR annealing temperature from 35°C to 43°C.

To each RT-PCR reaction an equal volume of loading buffer (95% (v/v) formamide and 0.09% (w/v) bromophenol blue) was added. The RT-PCR products were resolved on a 6% denaturing acrylamide gel (6% (v/v) Long Ranger™ (FMC Bioproducts), 8 M Urea and 0.6 x TBE (54 mM Tris pH 8.0, 54 mM boric acid, 1.2 mM EDTA), 0.1% (w/v) APS and 0.1% (v/v) TEMED) in 0.6 x TBE as running buffer. The gel was run for one and a half hours at 60 Watts after a pre-run of 30 minutes. The gels were dried and exposed to x-ray film for between 1 to 4 days. The resulting differentially expressed bands were recovered as described previously (3.2.5.3).

Chapter 4:

Results

4.1 Rust Inoculation

The aim of the study was to clone a wheat gene involved in the specific recognition of *P. triticina* infection. Since the wheat flag leaves were harvested before any visible signs of infection were present, some infected plants were grown until infection was visible. One week after inoculation, the susceptible, inoculated *Thatcher* plants showed clearly visible brown markings of fungal colonies on the leaves (fig. 4.1). The resistant, inoculated *Thatcher/Lr34* showed localized lesions where infection was terminated. Both uninoculated *Thatcher* and *Thatcher/Lr34* showed no signs of infection.

4.2 The effect of leaf rust infection on the wheat defence response

4.2.1 Long term activation of plant defenses

The infected *Thatcher* plants showed an initial increase in β-1.3-glucanase activity from time zero to 12 h.p.i., with a further increase in activity until 54 h.p.i were after there was a sudden decrease in glucanase activity. The uninfected control plants showed a similar profile, but at lower levels than that of the infected plants. The glucanase activity proceeded to increase to 54 h.p.i, where the uninfected plants' glucanase activity was as high as those of the infected plants, when a decrease in activity similar to that of the infected plants occurred (fig. 4.2a). In the later time intervals, the activity profile of the infected and uninfected plants is almost the same, but there are visible differences over the earlier times (12 h.p.i and 18 h.p.i).

The infected *Thatcher/Lr34* (*Th/Lr34*) (fig. 4.2b) plants showed an increase in β-1.3-glucanase activity from time zero to 12 hours after inoculation with a subsequent increase until 24 hours after infection where after the activity remained stable with a sudden decrease in activity 54 h.p.i. The initial activity of the infected *Th/Lr34* was lower than that of the uninfected plants, but quickly increased to levels higher than that of the uninfected control plants.

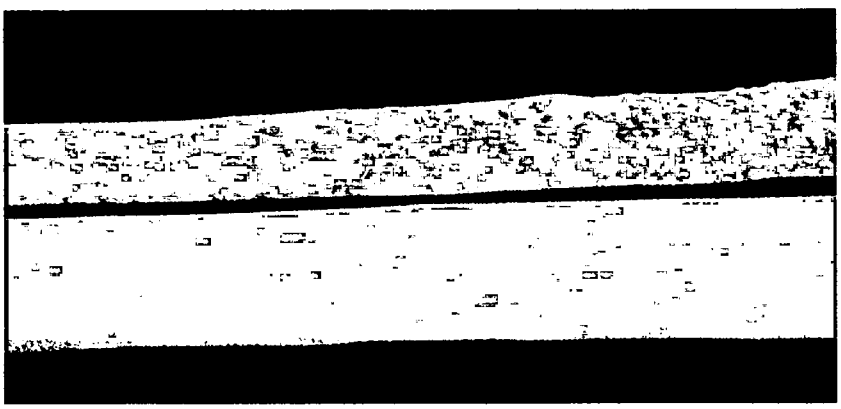


Figure 4.1: Flag leaves of adult *Thatcher* and *Thatcher/Lr34* plants one week after infection with *P. triticina*

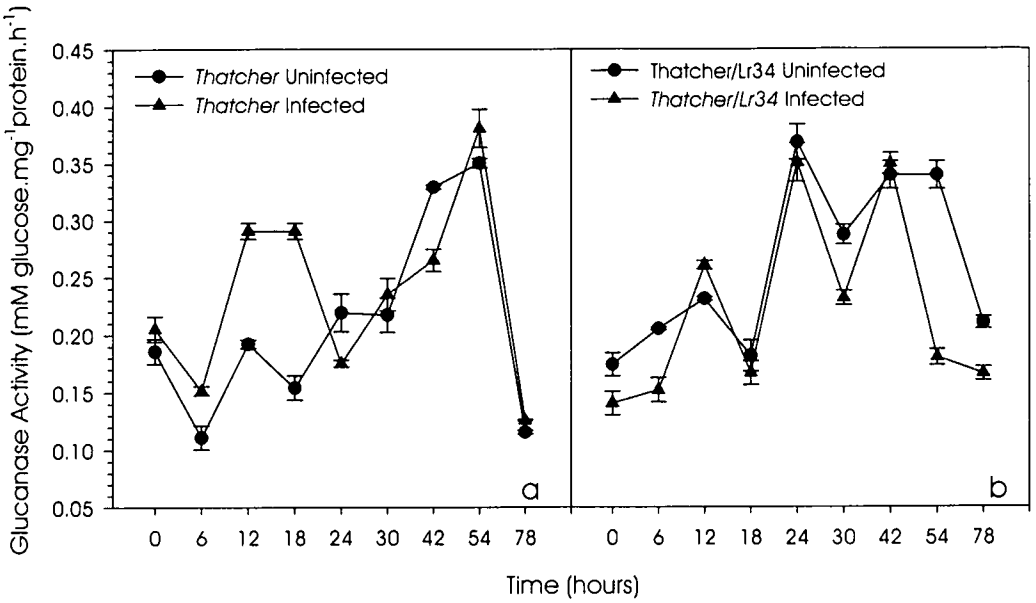


Figure 4.2: β-1.3-Glucanase activity in infected and uninfected (a) *Thatcher* plants and (b) *Thatcher/Lr34* plants. Error bars indicate standard deviation and n=3.

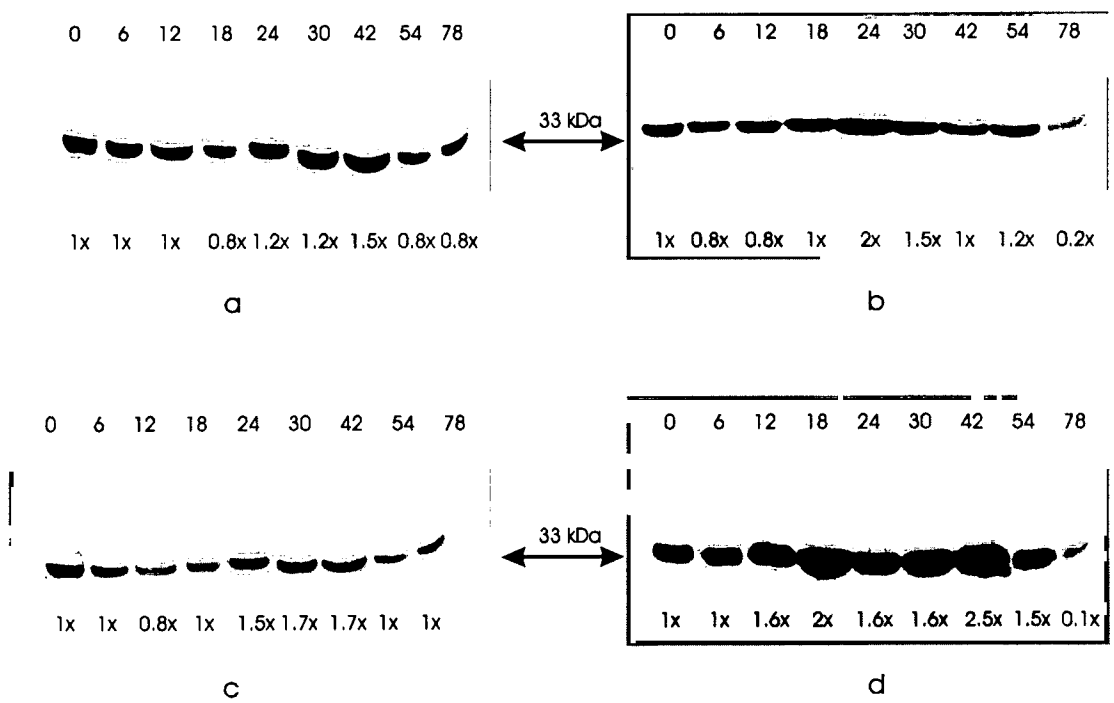


Figure 4.3: Immunological detection of β-1.3-Glucanase levels of (a) *Thatcher* uninfected, (b) *Thatcher* infected, (c) *Thatcher/Lr34* uninfected and (d) *Thatcher/Lr34* infected plants.

The uninfected *Th/Lr34* control plants showed a similar induction pattern to that of the infected plants, with an increase in activity from zero to 12 h.p.i and a subsequent increase to 24 h.p.i. The final decrease also occurred 24 h later than the resistant plants (fig 4.2b).

When the β-1.3-glucanase activity between resistant and susceptible infected plants was compared, it is evident that the initial activity levels of the resistant plants were marginally lower than that of the susceptible plants. Both showed an initial increase in activity 12 h.p.i., but that of the susceptible plants was higher than that of the resistant plants. The maximum activity for both plants was similar with that of the resistant plants occurring 24 h.p.i., while the maximum activity of the susceptible plants occurred at 54 h.p.i.

On the β-1.3-glucanase polypeptide profiles (Western blots), only one 33 kilo Dalton (kDa) band was visible (fig 4.3). The size of the polypeptide is within the size range of other β-1.3-glucanases (Anguelova *et al.,* 1999).

The infected *Thatcher* plants showed a notable increase in β-1.3-glucanase polypeptide levels 24 h.p.i (fig. 4.3b). The β-1.3-glucanase polypeptide levels reached a maximum at 24 h.p.i where after it remained constant with a decrease at 78 h.p.i. The uninfected *Thatcher* plants showed no visible increases in β-1.3-glucanase (fig 4.3a) levels although there were increases in activity (fig. 4.2a).

The infected *Thatcher/Lr34* plants showed increases in β-1.3-glucanase protein levels at 12 h.p.i with a further increase at 18 h.p.i reaching a maximum at 42 h.p.i where after the protein levels decreased again (fig. 4.3d). The uninfected *Thatcher/Lr34* plants showed an increase in β-1.3-glucanase levels 24 h.p.i, with a decrease in protein levels at 78 h.p.i, (fig. 4.3c) which is similar to that of the infected plants.

4.2.2 Determination of the earliest activation of plant defenses

The aim of the shorter time interval experiments was to determine the earliest reaction of the wheat towards the leaf rust fungus, since this implies that a signal transduction event has already taken place within the infected plants. Any protein kinase gene that was differentially expressed during this short time interval is therefore implicated to have played a role in the signaling. Biochemical confirmation of this early reaction was done by determining both β-1.3-glucanase and peroxidase activity.

The infected *Thatcher* plants showed an increase in β-1.3-glucanase activity within six hours and another increase 15 h.p.i (fig. 4.4a). This differed from the longer time intervals due to possible different inoculation conditions that occurred in the two separate studies. The uninfected control plants showed a similar pattern with similar activity levels. The infected *Th/Lr34* plants showed an initial decrease in enzyme activity, reaching the lowest point at 9 h.p.i. followed by an increase in enzyme activity up to 12 h.p.i., where after there was a decrease in activity 15 h.p.i (fig. 4.4b). The uninfected *Th/Lr34* plants again showed a similar profile, but its lowest enzyme activity was at 6 h.p.i compared to the infected plants' 9 h.p.i. Following the decrease, there was an increase in activity to 12 h.p.i followed again by a decrease in activity at 15 h.p.i.

Western blots were again performed on total extracted proteins and probed with the same antibody used in 4.2.1.1. The infected *Thatcher* plants showed an increase in β-1.3-glucanase levels after 12 hours (fig. 4.5b), which correlated with the increase in activity, but showed no increase in enzyme levels corresponding with the increased activity at 6 h.p.i. The uninfected *Thatcher* plants showed an increase in β-1.3-glucanase levels at 3 h.p.i and 15 h.p.i (fig. 4.5a) which correlates with the enzyme activity.

The infected *Th/Lr34* plants showed an increase in β-1.3-glucanase levels 9 h.p.i and 12 h.p.i. (fig. 4.5d) which confirms the increase in activity in the plants. It also showed a decrease in enzyme levels which corresponded with the decrease in

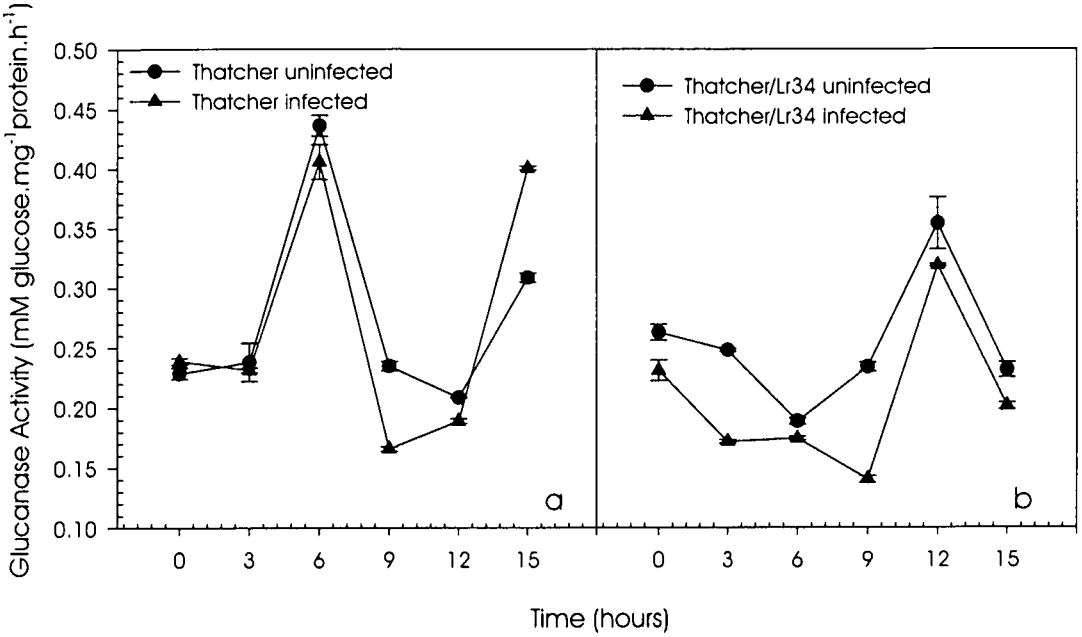


Figure 4.4: β-1.3-Glucanase activity in infected and uninfected (a) *Thatcher* plants and (b) *Thatcher/Lr34* plants. Error bars indicate standard deviation and n=3

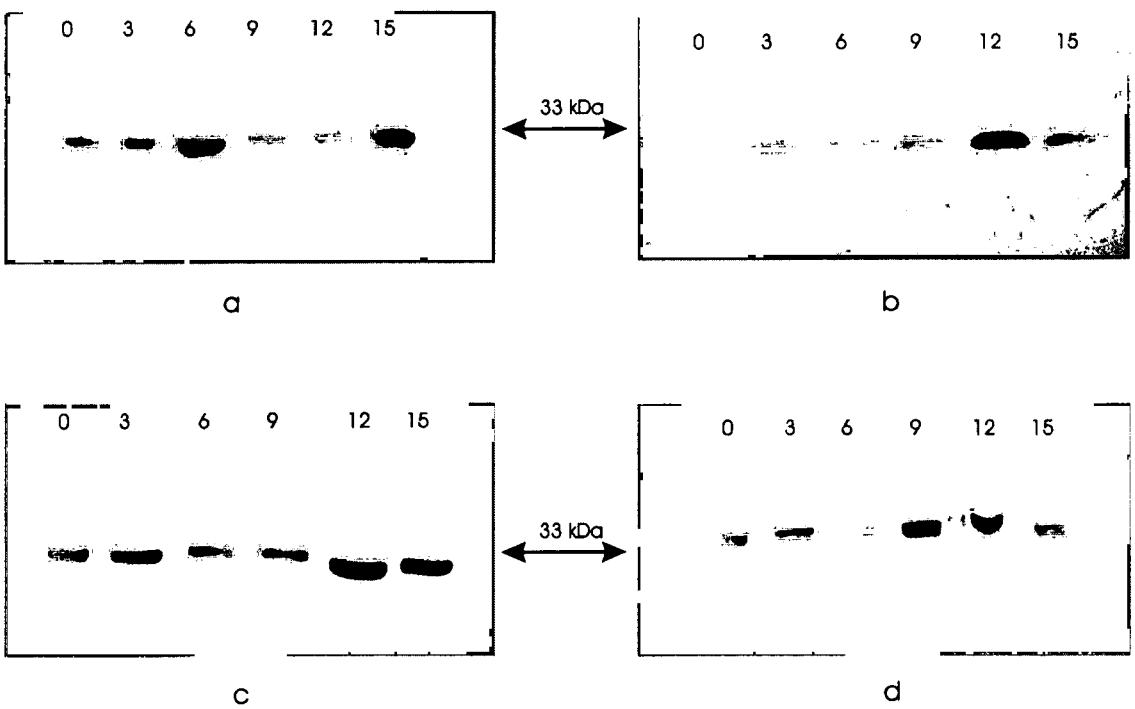


Figure 4.5: Immunological detection of β-1.3-glucanase proteins of (a) *Thatcher* uninfected, (b) *Thatcher* infected, (c) *Thatcher/Lr34* uninfected and (d) *Thatcher/Lr34* infected plants.

enzyme activity 15 h.p.i. The uninfected *Th/Lr34* plants showed an increase in β-1.3-glucanase levels 12 and 15 h.p.i. (fig. 4.5c) which is similar albeit later than that of the infected *Th/Lr34* plants. It also supports the activation of β-1.3-glucanase activity in the infected and uninfected susceptible plants.

4.2.3 Hydrogen peroxide levels

From the standard curve of H_2O_2 concentrations against absorbancy (fig. 4.6), the gradient of the regression plot was determined and was used in the calculation of the hydrogen peroxide concentrations. The gradient of the line was 0.1678075397 and the y cut-off was –0.00578.

The H_2O_2 levels of the infected *Thatcher* plants increased continuously from 6 h.p.i to 15 h.p.i. while those of the uninfected plants stayed basically the same until 15 h.p.i where there was an increase (fig. 4.7a). Although the initial H_2O_2 levels of the uninfected plants were higher than that of the infected plants, the hydrogen peroxide levels of the infected plants increased at an earlier stage of infection.

In the infected *Th/Lr34* plants the H_2O_2 levels decreased initially until 6 h.p.i where after it increased to a maximum at 12 h.p.i with a decrease again at 15 h.p.i (fig. 4.7b). In the uninfected control plants there was a sharp decrease of H_2O_2 levels 9 h.p.i that was followed by a sharp increase to 15 h.p.i but even then it was almost at the same starting level.

4.2.4 Peroxidase activity

In the infected *Thatcher* plants, there was an initial doubling in peroxidase activity within six hours after inoculation (fig. 4.8a), a sharp decrease 9 h.p.i followed by another increase to 15 h.p.i. The initial increase in enzyme activity correlated with the increased H_2O_2 levels 9 h.p.i, as did the later increase in enzyme activity 12 h.p.i. The uninfected control plants showed the same basic profile as the infected plants, but at much lower levels.

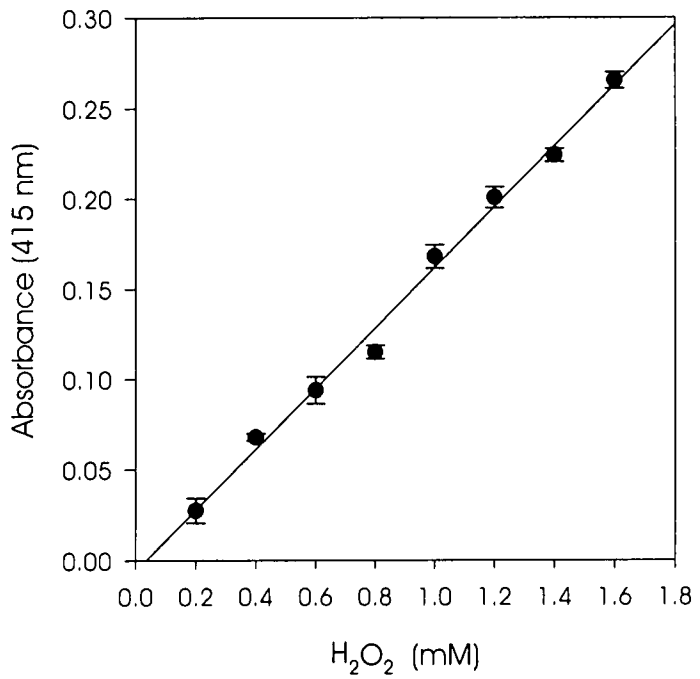


Figure 4.6: Hydrogen peroxide standard curve with A_{415} values plotted against hydrogen peroxide concentrations. Error bars indicate standard deviation and n=3.

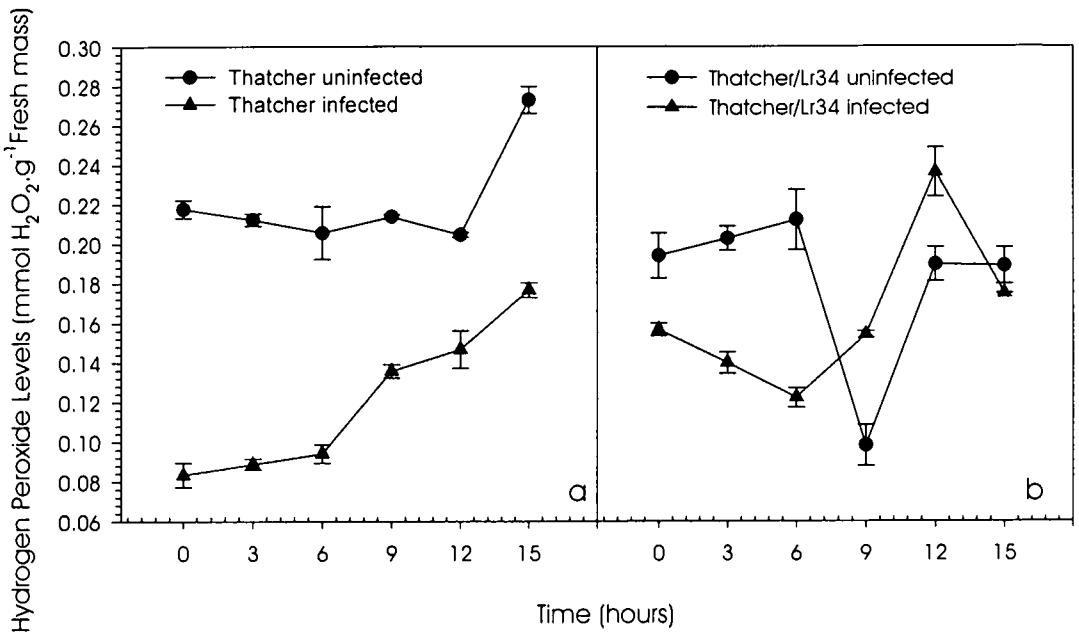


Figure 4.7: Hydrogen peroxide levels in infected and uninfected (a) *Thatcher* plants and (b) Thatcher/Lr34 plants. Error bars indicate standard deviation and n=3.

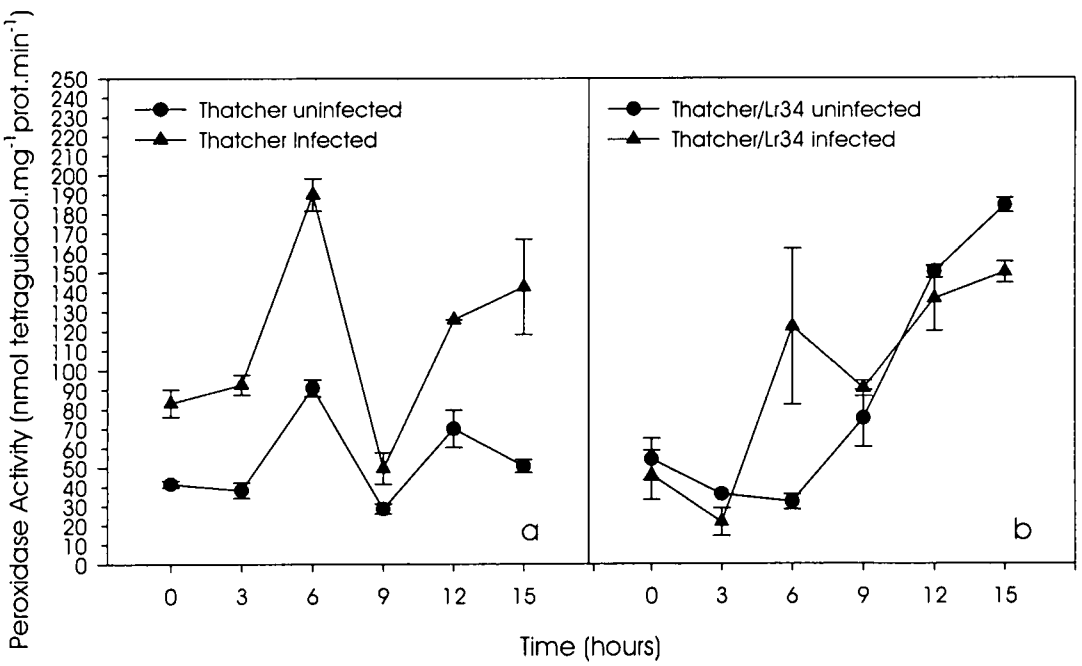


Figure 4.8: Peroxidase activity of infected and uninfected (a) *Thatcher* and (b) *Thatcher/Lr34* plants. Error bars indicate standard deviation and n=3.

In the infected *Th/Lr34* plants there was a sharp, prominent increase of peroxidase activity 6 h.p.i followed by a further smaller increase in activity (fig. 4.8b).

This increase occurred at the same time that the H_2O_2 levels started to increase at 6 h.p.i. The uninfected control plants showed lower peroxidase activity until 6 h.p.i where after it increased reaching a maximum at 15 h.p.i. This 6 h.p.i increase occurred at the same time that the H_2O_2 levels started to increase after an initial drop (fig 4.7b).

4.2.5 Possible interplant communication

Since the shorter time interval activities of both β-1.3-glucanase and peroxidase between infected and uninfected plants were similar in profile, a possible communication between plants was investigated. The fungus was allowed to sporulate and colonize *Th/Lr34* plants for 24 h before the plants were isolated in glass chambers. β-1.3-glucanase activity over this time period (0 h.p.i to 24 h.p.i) was thus low (fig. 4.9). After this infection stage, at 24 h.p.i, the plants were placed in isolated glass chambers and grown for 24 hours to acclimate. At 48 h.p.i a connection was established between one set of infected *Th/Lr34* plants and a set of uninfected *Th/Lr34* plants.

The increase in β-1.3-glucanase activity between, 24 h.p.i and 48 h.p.i (fig. 4.9) showed that the plants reacted to the restricted environment provided by the bell jars. The β-1.3-glucanase activity of the infected plants rose up to a maximum at 72 h.p.i, which was much later compared to the previous experiments (fig 4.2). the activity levels were however much lower. At 48 h.p.i, when the connection was established between the infected and uninfected plants, there was a drop in β-1.3-glucanase activity in the uninfected *Th/Lr34* plants, while that of the infected plants increased. The uninfected control plants showed a slight increase in activity.

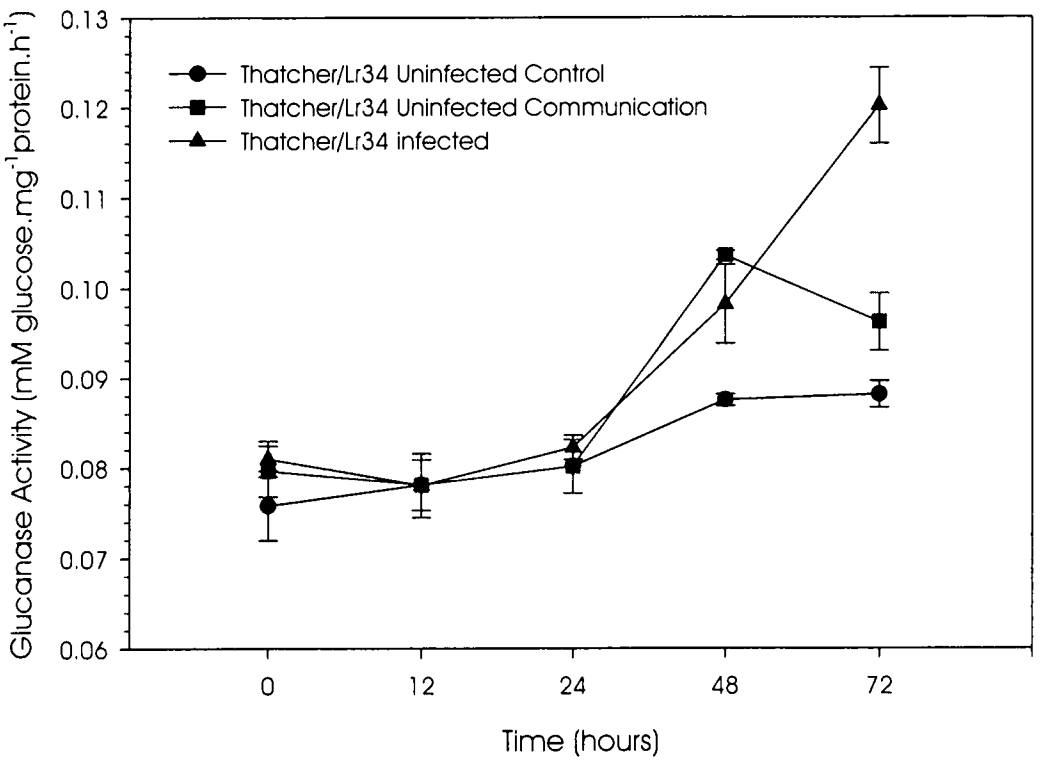


Figure 4.9: β-1.3-Glucanase activity in infected and uninfected *Thatcher/Lr34* plants involved in the preliminary study to determine interplant communication. Error bars indicate standard deviation and n=3.

4.3 Molecular Analysis

4.3.1 Optimization of RT-PCR

For this study we synthesized two primers specifically for the sub-domain VIb of the kinase domain of RLKs for both mono- and dicotyledonous plants. Sub- domain VIb of the kinase domain is highly conserved and specify for serine/threonine phosphorylation.

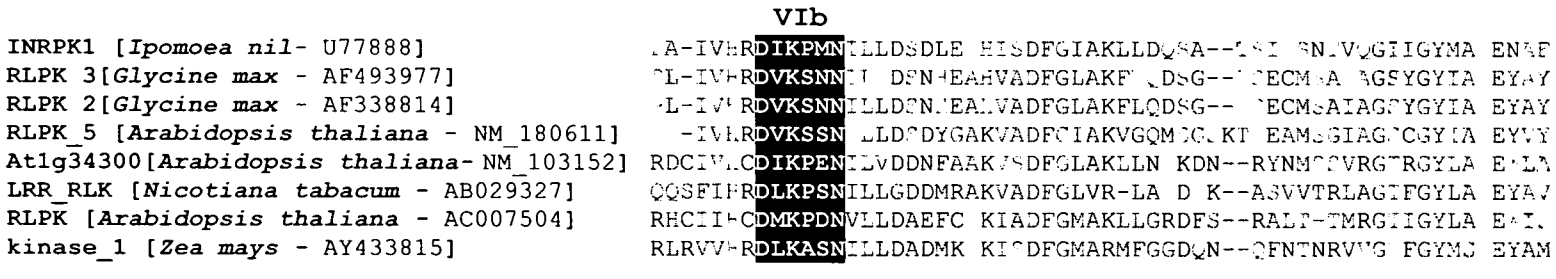
The protein sequences and alignments are shown in figure 4.10 a and b. As there was a difference of two amino acids between kinases from monocotyledonous and dicotyledonous plants. In monocots the consensus sequence was DIKP**H**N and for dicots it was D**L**KP**E**N (Hanks *et al.,* 1988). These sequences were thus used to synthesize two degenerate primers that will be able to recognize subdomain VIb of plant protein kinases during PCR

At first, total RNA were extracted from the leaves of infected *Th/Lr34* plants and separated on a agarose gel to confirm the quality of the RNA and to verify that there was no contaminating DNA (fig 4.11). The PCR reaction had to be optimized with the primers used. The annealing temperature had to be optimized and this was tested using a plasmid containing the *At-RLK3* gene as control (Czernic *et al.,* 1999). Since the *At-RLK3* gene contained the coding sequences for a kinase domain, an amplified DNA sequence would indicate optimal conditions for PCR.

The result of this optimization is indicated in figure 4.12. When two gene specific primers for *At-RLK3* (Bovis 13 and 14) were used, the 1000 bp kinase domain encoding cDNA fragment amplified at all three different temperatures (lanes 1 to 3).

Using the anchored oligo dT primer in combination with the forward *At-RLK3* gene specific primer, a single larger band 1300 bp in size was obtained at 30°C which decreased in intensity and specificity as the temperature increased (lanes 4 to 6). This fragment is larger than the previous one because the primers used in lanes 1 to

a.



b.

Figure 4.10: Sequence alignment of conserved sub domain VIb of kinases using different (a) dicot and (b) monocot plants

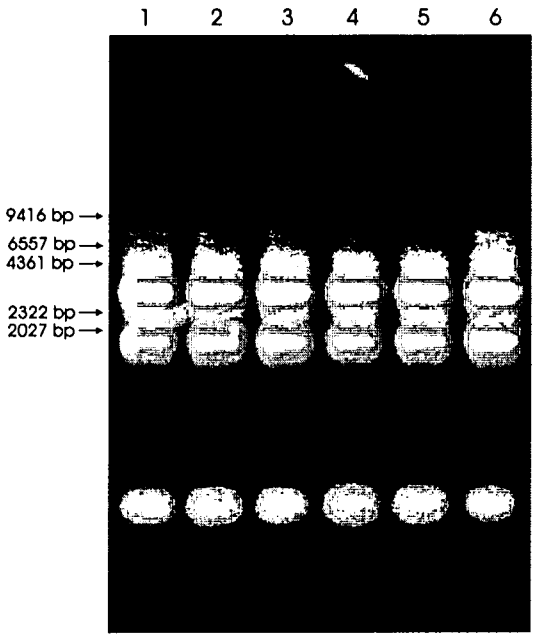


Figure 4.11: RNA concentrations after extraction showing good quality, uncontaminated RNA. Lane 1 is time zero, lane two is 3 h.p.i, lane three is 6 h.p.i, lane four is 9 h.p.i, lane five is 12 h.p.i, and lane six is 15 h.p.i.

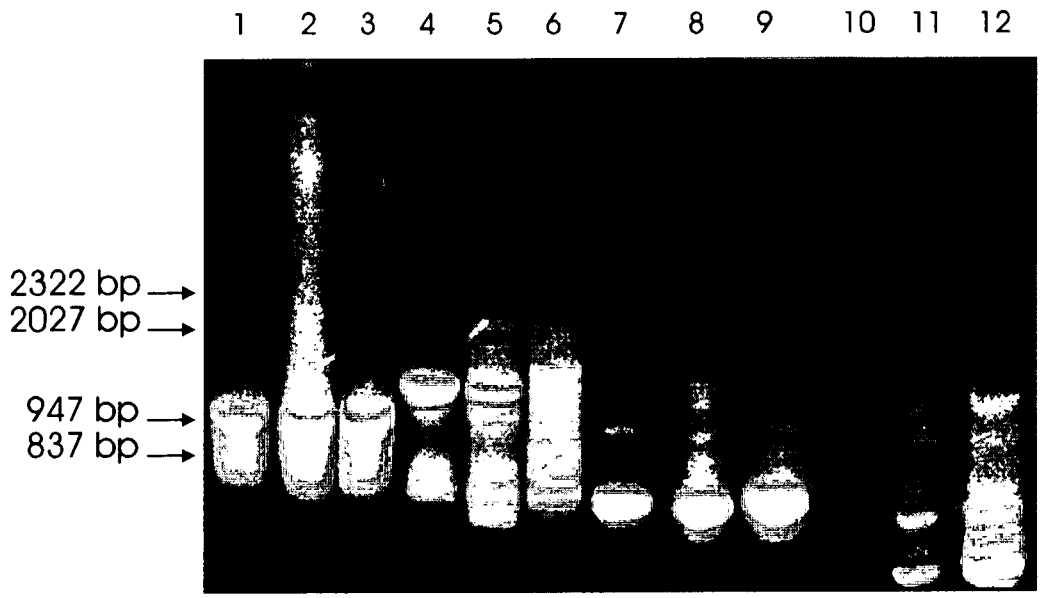


Figure 4.12: PCR optimization reactions. Reactions in lane 1 to 3 were performed with two specific primers (Bovis 13 and 14) for *At-RLK3* at 30, 35, and 40°C respectively. Reactions in lanes 4 to 6 were performed with the forward *At-RLK3* specific primer (Bovis 13) and an anchored oligo-dT as reverse primer (Bovis 21) at 30, 35, and 40°C respectively. Reactions in lanes 7 to 9 were performed with the monocot primer (Bovis 22) as forward primer and an anchored oligo-dT primer (Bovis 21) as reverse primer at 30, 35, and 40°C respectively. Reactions in lanes 10 to 12 were performed with the dicot primer (Bovis 23) as forward primer and an anchored oligo-dT primer (Bovis 21) as reverse primer at 30, 35, and 40°C respectively.

3 amplified only the kinase domain, while those used in lanes 4 to 6 amplified the kinase domain as well as the 3' untranslated region.

Using the anchored oligo dT primer in combination with the monocot specific primer, a fragment amplified at all three temperatures (lanes 7 to 9). Using the anchored oligo dT primer in combination with the dicot specific primer the amplification was nonspecific at 30°C with the amplified fragment only just visible. Random amplification however increased as the annealing temperature increased (lanes 10 to 12). A RT temperature of 37°C and annealing temperature of 35°C were therefore chosen for all subsequent RT-PCR reactions.

4.2.3 Differential Display

DD RT-PCR was used to identify differentially expressed putative protein kinase genes from infected resistant *Th/Lr34* plants. In both experiments using the monocot and dicot primers respectively, the background radiation of time 0 was very high. This was even though some of the amplified fragments present within showed constitutive expression. Differentially expressed fragments were therefore still easy to identify. With the monocot primer set, six differentially expressed putative protein kinase genes were identified (fig 4.13) and with the dicot primer set another three (fig 4.14). The differentially expressed genes were called 03jjam1-6 for the monocot primer and 03jjad1-3 for the dicot primer.

M1, M3 and M6 was first expressed at 3 h.p.i, being absent at 0.h.p.i, reaching maximum expression 6 h.p.i where after the expression decreased. The expression of M2 is first notable 12 h.p.i at a very high level. M4 was first expressed at 3 h.p.i also being absent at 0.h.p.i, and expressed at low levels until further increased expression occurred at 12 h.p.i. The expression of M2 and M5 was first notable 12 h.p.i at a very high level, being absent up to that time. D1 was first expressed at 3 h.p.i, being absent at 0.h.p.i, reaching maximum expression at 6 h.p.i where after its expression decreased. D2 was first expressed at 9 h.p.i. D3 was first expressed 3 h.p.i, being absent at 0.h.p.i where after its expression decreased only to increase again at 12 h.p.i.

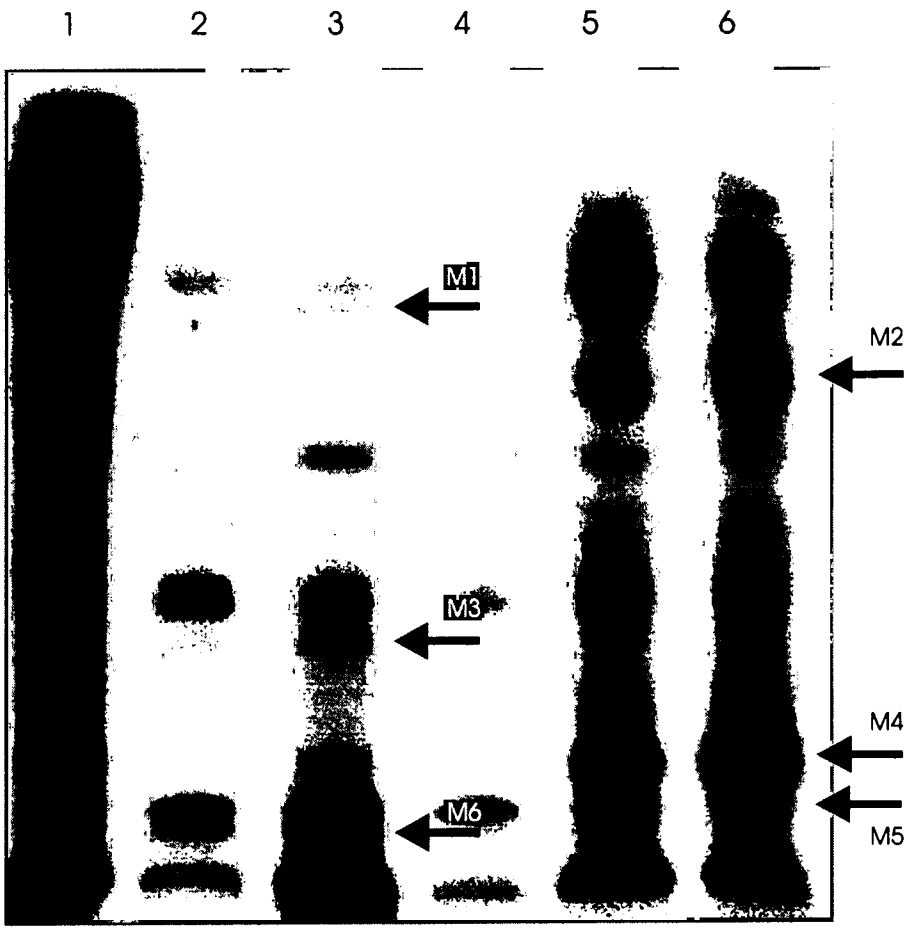


Figure 4.13: Differential display of infected *Thatcher/Lr34* with the monocot primer as the forward primer and an anchored oligo dT primer as the downstream primer. Lane 1 is time zero, lane two, 3 h.p.i, lane three, 6 h.p.i., lane four, 9 h.p.i., lane five, 12 h.p.i., and lane six, 15 h.p.i. Arrows indicate possible differentially expressed kinases.

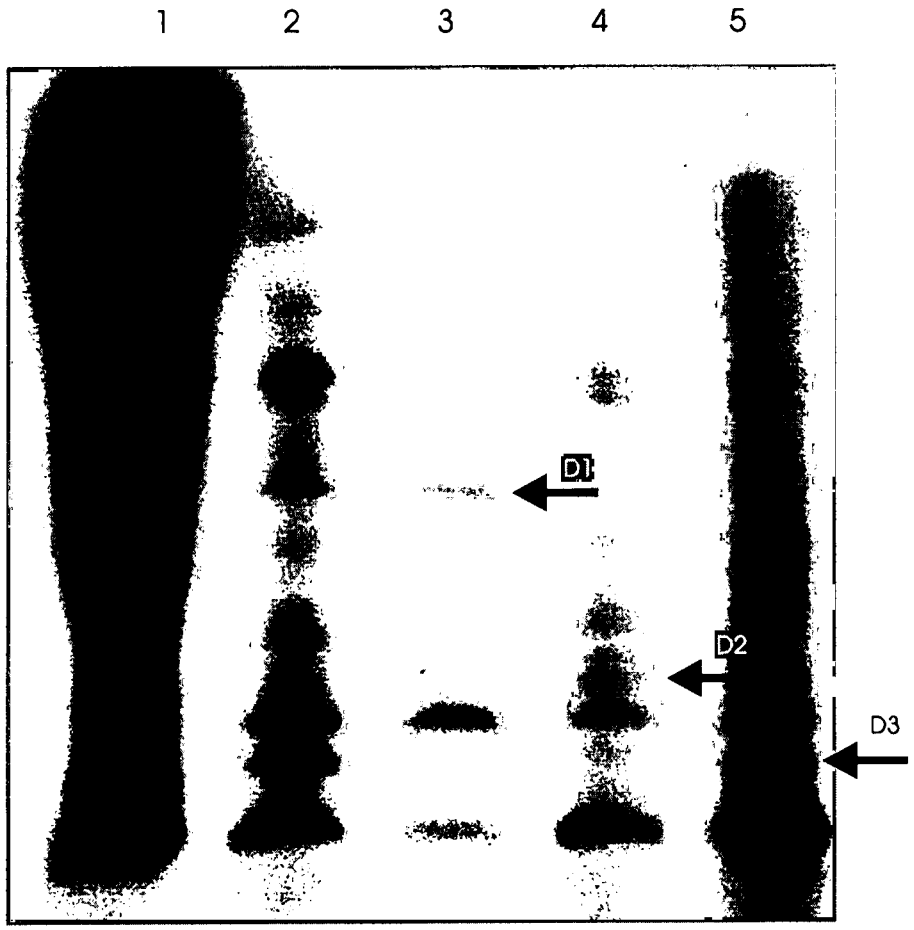


Figure 4.14: Differential display of infected *Thatcher/Lr34* with the dicot primer as the forward primer and an anchored oligo dT primer as the downstream primer. Lane 1 is time zero, lane two, 3 h.p.i, lane three, 6 h.p.i., lane four, 9 h.p.i., lane five, 12 h.p.i., Arrows indicate putative differentially expressed protein kinases.

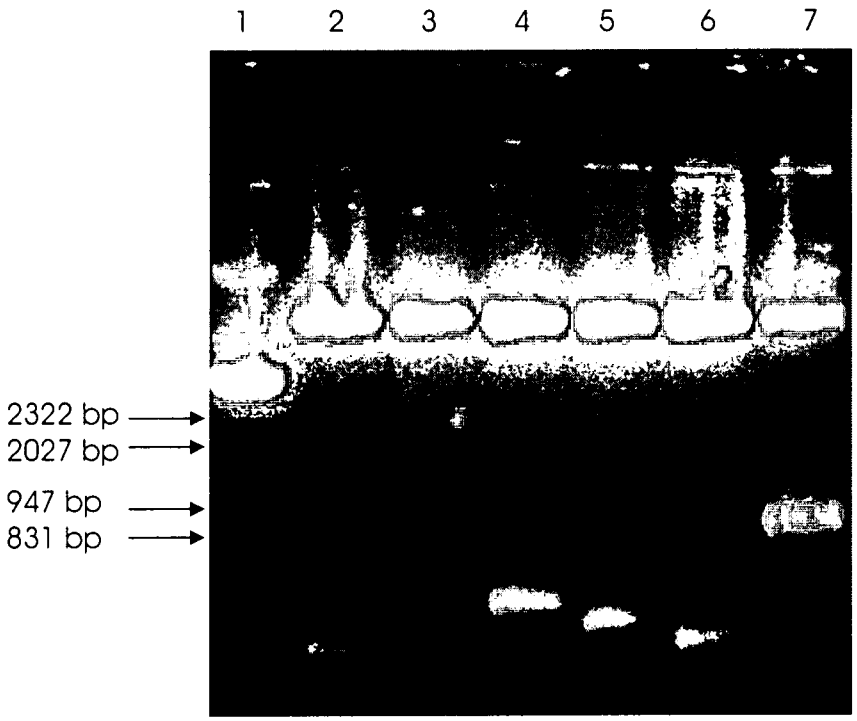


Figure 4.15: Restriction digestion of plasmid DNA extracted from transformed JM109 *E. coli* cells. Lane 1 is undigested pGemT plasmid, lane 2 to 7 is the digested plasmids containing 03jjam1 in lane 2, 03jjam6 in lane 4, 03jjad1 in lane 5, 03jjad2 in lane 6 and 03jjad3 in lane 7. The plasmid in lane 3 contained no insert.

4.3.2 Re-amplification of differentially expressed kinases

The putative genes obtained, were cloned into pGemT-Easy where after the recombinant plasmids were digested to verify the presence of the inserts (fig 4. 15). As can be seen in fig 4.15, six different recombinant plasmids were tested for the presence of an insert. It was only clone 3 that did not contain an insert. All the others contained the correct size insert and were thus sequenced.

Once the sequence data of these five inserts were analyzed, the cloning and sequencing of the other DD RT-PCR fragments were abandoned, since it was clear that the isolated gene fragments would not satisfy our needs for this particular project. The sequences obtained from the five clones were compared to known genes present in the GenBank database and the alignments are shown in fig. 4.16 to 4.20. Sequence identities and E-values are indicated in Table 4.1.

The first clone, 03jjam1, contained a sequence of 308 bases. There were no significant similarities with any other known gene (fig. 4.16). The longest open reading frame coded for a polypeptide of 41 amino acids which contained a site corresponding to subdomain VIb of the kinase domain of serine/threonine protein kinases.

Clone 03jjam6 had a base sequence of 466 bases and its longest open reading frame coded for a polypeptide of 104 amino acids (fig. 4.17). On nucleotide level, it shared sequence similarities to a *Hordeum vulgare* (GenBank accession nr AF307145.1, 94% identity) and a *T. aestivum* (GenBank accession nr BT009245, 98% identity) glutamine-dependent asparagine synthetase (AS). It also contained a Subdomain VIb corresponding site.

Clone 03jjad1 had a base sequence of 399 bases and its two longest open reading frames coded for two possible polypeptides (fig. 4.18), one of 67 amino acids and another of 132 amino acids. It shared nucleotide sequence similarities to an *O. sativa* asparaginyl endopeptidase (GenBank accession nr AB025310, 88%.

Table 4.1. Identities and E-values of equivalent genes

Clone	Similar gene	Identities	E-value
03jjam6	BT009245.1 Triticum aestivum	296/299 (98%)	e-161
03jjad1	AF307145.1 Hordeum vulgare subsp. vulgare glutamine-dependent asparagine synthetase 1	284/299 (94%)	e-133
	AB025310.1 Oryza sativa mRNA for asparaginyl endopeptidase	144/163 (88%)	2e-40
03jjad2	AJ131718.1 Zea mays mRNA for legumain-like protease	154/185 (83%)	5e-25
	K01229.1 Wheat mitochondrial small subunit (18S) rRNA gene	303/308 (98%)	e-160
03jjad3	AY494981.1Triticum turgidum A genome HMW glutenin	49/51 (96%)	2e-14

```
5' - GAC ATT AAG CCT CAT AAC CGA AGT GCT ACT AAC AAT CCG AAG CTA ACA
      D   I   K   P   H   N   R   S   A   T   N   N   P   K   L   T

     GCG ACC AAT GTG GAA AGA ATG AAC CAA TCA AAG AAA ATT ACA TAC GGG GAT
      A   T   N   V   E   R   M   N   Q   S   K   K   I   T   Y   G   D

     AAT GTA CAA GCA AGC ACC AAT TGA ACA GTT GAC ATC GTG TGT TTT TGG CTT
      N   V   Q   A   S   T   N STOP

     CAG AGC GAG AGA CCT GCA CGG CAT GAG ACT TCC ATT CGT GAA TGC TCA GAG
     CAT TCT GAA AGG ATC TAC GGA TAG TAG CTT CCG GTT CT GCG CAG GAG GTG CAG
     GAG GCG CCA GTC CCG GGA TGT CTG GGA TAC TCT TGT TGT GTG GCT TAA TGT CA
     - 3'
```

Figure 4.16: (a) Sequence of clone 03jjam1and the predicted polypeptide

a.

```
D I K P H N T P T T K E X X C Y R M I F E R F F P Q N S A I L T V P G
G P S V A C S T A K A V E W D A Q W S G N L D P S G R A A L G V H L S
A Y E Q E H L P A T I M A G T S K K P R M I E V A A P G V A I E S Stop
```

b.

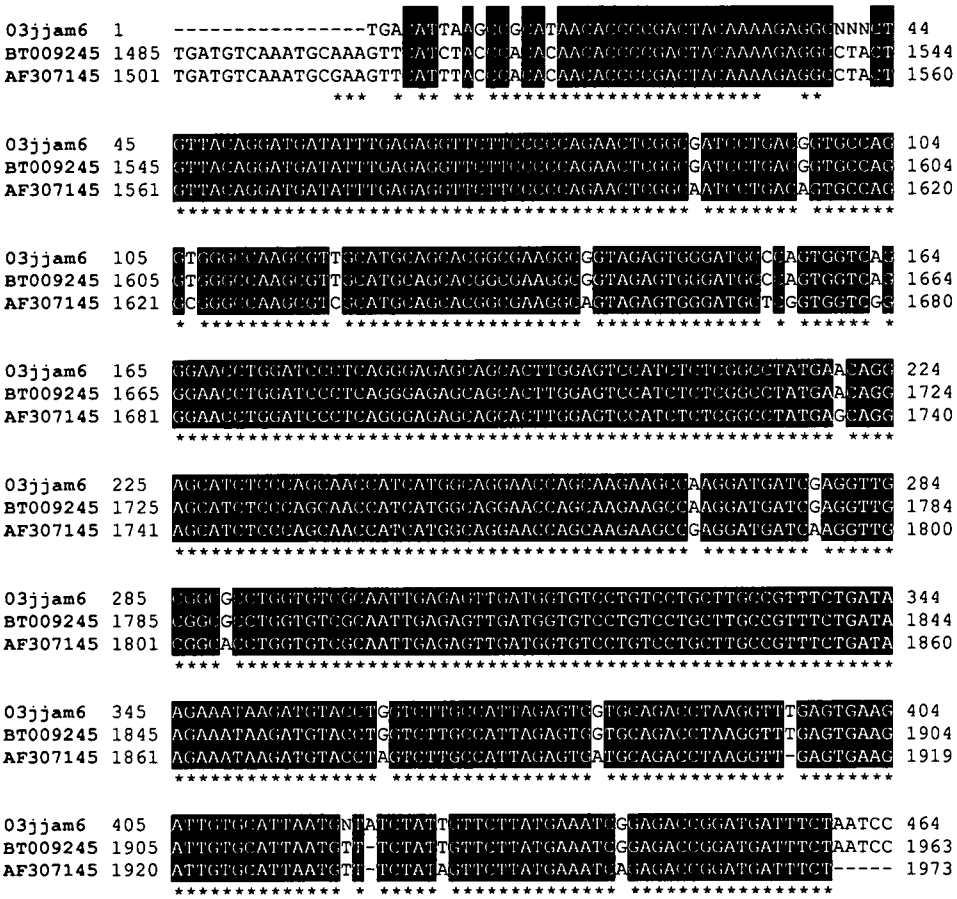


Figure 4.17: (a) The polypeptide encoded by clone 03jjam6, and (b) the nucleotide alignment of clone 03jjam6 to known genes. BT009245 = *T. aestivum* glutamine-dependent asparagine synthetase. AF307145 = *Hordeum vulgare* glutamine-dependent asparagine synthetase. Consensus sequences are indicated with an asterisk.

a. **M** L N I C S R T L G Q T L L T R T L H L L K I T H C H H S Q E
L L I R G **M** L I L F I S G R S T G N W L R A P L R K R **M** L G S
N C L K **Stop**

b. **M** L N Q R T E S L K Q Q Y N L V K K R T A A Q D P Y S Y G S H
V **M** Q Y G S L D L N A E H L F S Y I G S N P A N E N T T F V E
D N A L P S F S R A V N Q R D A D L V Y F W Q K Y R K L A E S
S P E K K D A R K Q L L E **M** **M** G H R S H I D N S V E L I G N L
L F G S Q A Y V

c.

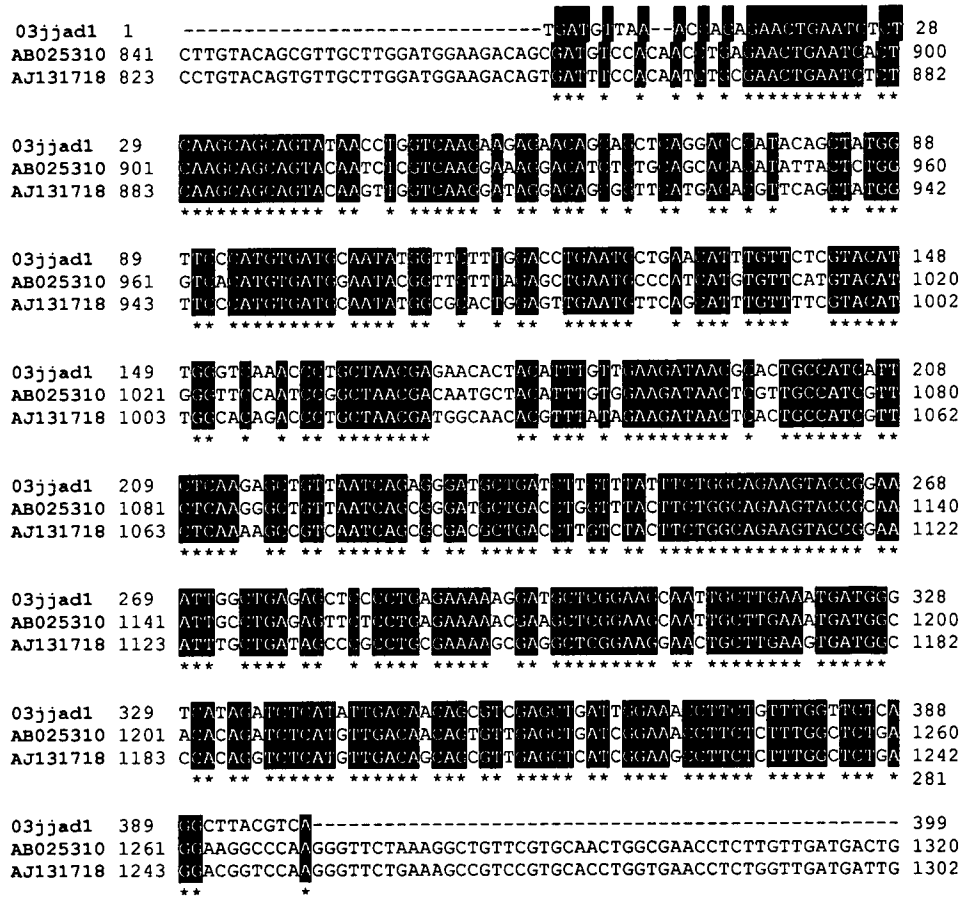


Figure 4.18: (a and b) Possible polypeptides encoded by clone 03jjad1, and (c) the nucleotide alignment of clone 03jjad1 to known genes. AB025310 = *O. sativa* asparaginyl endopeptidase and AJ131718 = *Zea mays* legumain-like protease. Consensus sequences are indicated with an asterisk.

a.

D V K P E N G V E R S G N S T R P A P K Q R K R C V P H S R G T
A S E I L E E G G D D V K S A W P L W T G P R T Y Y N G N D N G
K Q G C K A E R I R K D C L S S D C S L Q L G N **Met** K L K S L V
I A D Q H A A V N **Met** F S G L

b.

```
03jjad2 1     ----------------------------------------------------TGATGTTAA 9
K01229  1381  GTTTGGTTGAAATTGGTTACGACGACGTCGAGTTGGCGGCGGAGGAAGACTCGGCATGAA 1440
                                                                  *   * **

03jjad2 10    G-GCAGAGAACGGTGTGGAACGTAGTGGTAATAGTACGCGCCCCGCTCCGAAACAAAGAA 68
K01229  1441  GGCCAGAAAATGGTGTGGAACGTAGTGGTAATAGTACGCGCCCCGCTCCGAAACAAAGAA 1500
              * ***** ** **************************************************

03jjad2 69    AAAGGTGCGTGCCGCACTCACGAGGGACTGCCAGTGAGATACTGGAGGAAGGTGGGGATG 128
K01229  1501  AAAGGTGCGTGCCGCACTCACGAGGGACTGCCAGTGAGATACTGGAGGAAGGTGGGGATG 1560
              ************************************************************

03jjad2 129   ACGTCAAGTCCGCATGGCCCTTATGCACTGGGCCAGCGCACGTACTACAATGGCAATGACA 188
K01229  1561  ACGTCAAGTCCGCATGGCCCTTATGCGCTGGGCCAGCACACGTGCTACAATGGCAATGACA 1620
              ************************* ********* ***** ******************

03jjad2 189   ATGGGAAGCAAGGCTGTAAGGCGGAGCGAATCCGGAAAGATTGCCTCAGTTCGGATTGTT 248
K01229  1621  ATGGGAAGCAAGGCTGTAAGGCGGAGCGAATCCGGAAAGATTGCCTCAGTTCGGATTGTT 1680
              ************************************************************

03jjad2 249   CTCTGCAACTCGGGAACATGAAGTTGAAATCGCTAGTAATCGCGGATCAGCATGCCGCGG 308
K01229  1681  CTCTGCAACTCGGGAACATGAAGTTGAAATCGCTAGTAATCGCGGATCAGCATGCCGCGG 1740
              ************************************************************

03jjad2 309   TGAATATGTTCTCAGGCT-TCAA---------------------------------- 330
K01229  1741  TGAATATGTACCCGGGCCCTGTACACACCGCCCGTCACACCCTGGGAATTGGTTTCGCCC 1800
              ********* * * ***  ** *
```

Figure 4.19: (a) Possible polypeptide encoded by clone 03jjad2 and (b) the nucleotide alignment of clone 03jjad2 to known genes. K01229 = *T. aestivum* mitochondrial small subunit (18S) rRNA gene. Consensus sequences are indicated with an asterisk.

a. C S X X L P E G Q I X P R W P X S E G I V P X S G A E G **Met** Y P
V A **Met** C L I S L S L S L V F L I W H D L D V P R A L L L **Stop**

b.

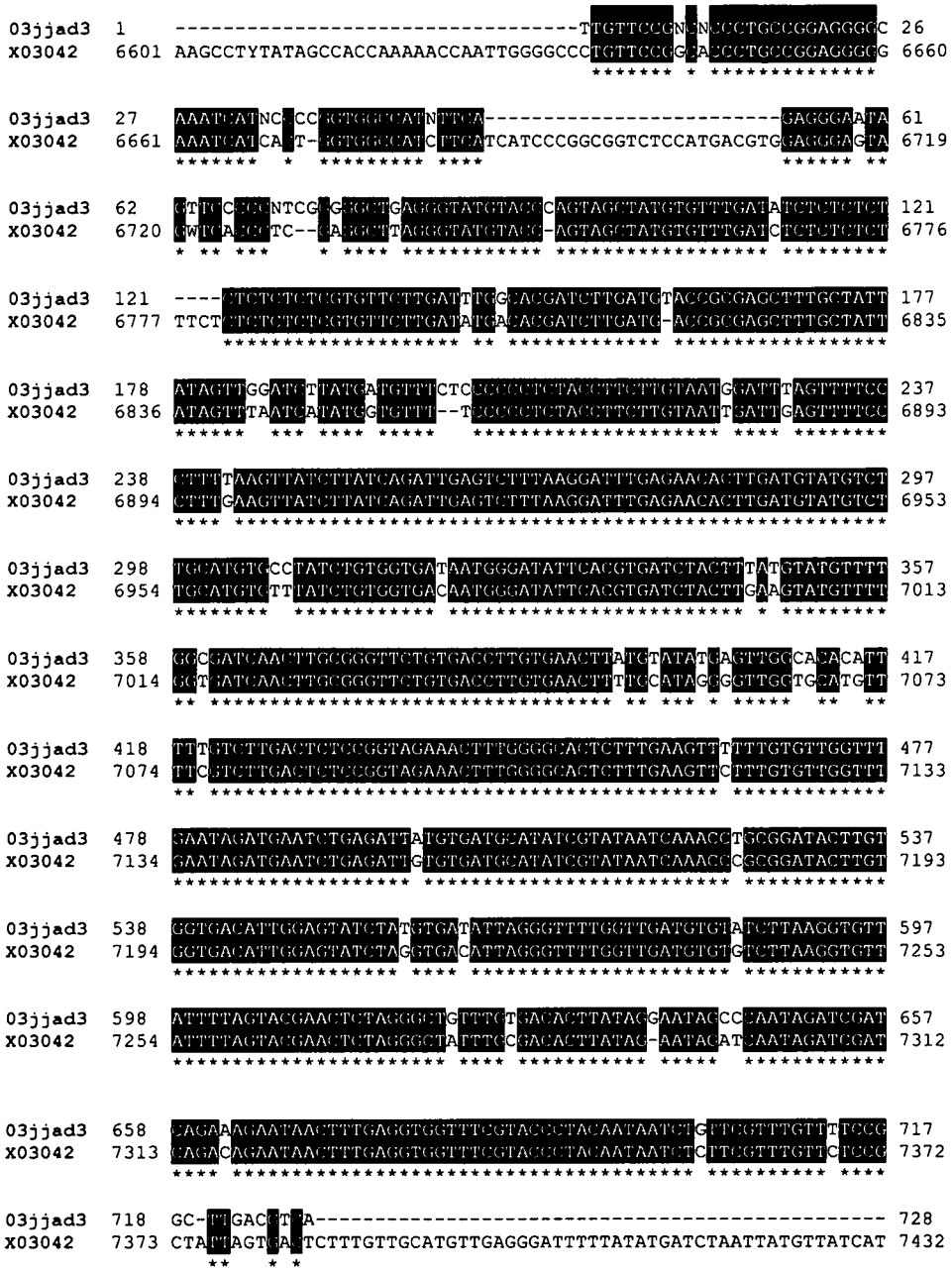


Figure 4.20: (a) Possible polypeptide encoded by clone 03jjad3 and (b) the nucleotide alignment of clone 03jjad3 to known genes. X03042 = *T. aestivum* gene for high-molecular-weight glutenin subunit. Consensus sequences are indicated with an asterisk.

identity) and a *Zea mays* legumain-like protease (GenBank accession nr AJ131718, 83% identity) respectively.

Clone 03jjad2 had a sequence of 330 bases and its longest open reading frame coded for a polypeptide of 109 amino acids (fig. 4.19). It shared sequence similarities to a *T. aestivum* mitochondrial 18S rDNA gene (GenBank accession nr K01229, 98% identity). It also contained a Subdomain VIb corresponding site

Clone 03jjad3 had a sequence of 728 bases and its longest open reading frame coded for a polypeptide of 60 amino acids (fig. 4.20). It shared sequence similarities to a *T. aestivum* gene for a high-molecular-weight glutenin subunit (GenBank accession nr X03042, 94% identity).

4.3.3 High specificity DD RT-PCR

It was quite evident from the sequenced DNA fragments that the stringency of the DD RT-PCR reactions was not high enough and that the primer annealing during the PCR reaction was non-specific. As a result of this it was decided to repeat the DD RT-PCR reactions with a new oligo dT primer containing a unique 5' tail. This allowed the use of a new primer that would attach to this sequence during the PCR step, thereby allowing us to use higher annealing temperatures during PCR.

With the repeat DD RT-PCR, we found 20 putative differentially expressed genes with the monocot primer and 10 putative differentially expressed genes with the dicot and oligo dT primer combination (fig 4.21). Due to the large format of the differential display gel (A3), it is only possible to show part of the gel. Differentially expressed putative protein kinase genes are indicated with arrows and in future these putative genes will also be sequenced and characterized.

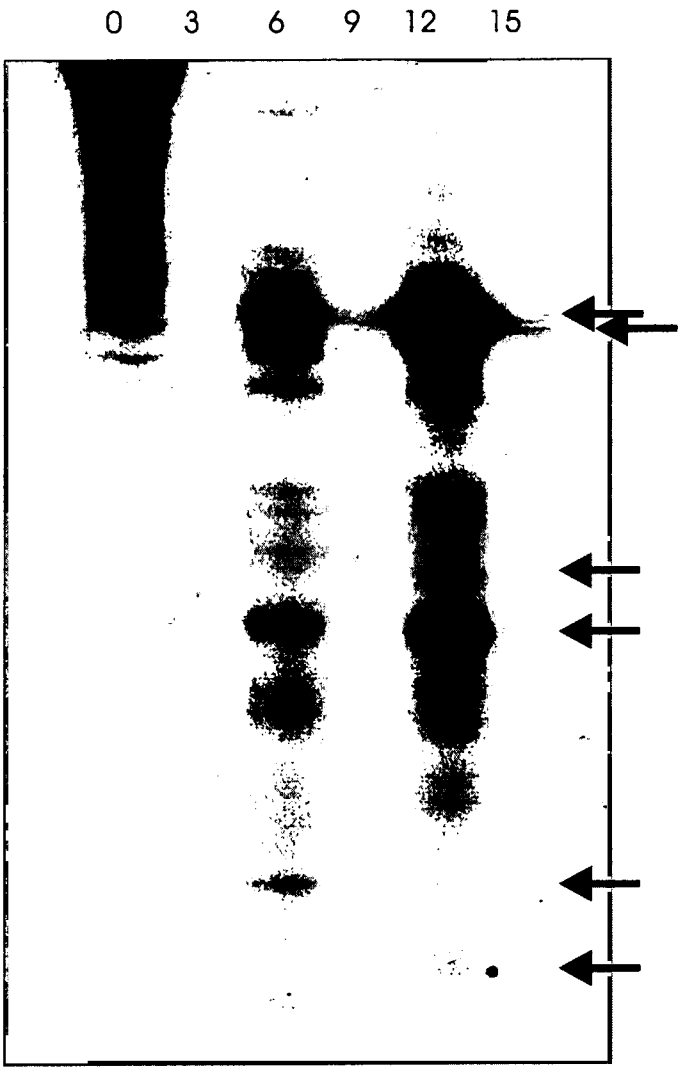


Figure 4.21: Repeat differential display of infected *Thatcher/Lr34* with the monocot primer as the forward primer and the new oligo dT primer as the downstream primer. Lane 1 is time zero, lane two, 3 h.p.i, lane three, 6 h.p.i., lane four, 9 h.p.i., lane five, 12 h.p.i., and lane six, 15 h.p.i. Arrows indicate possible differentially expressed kinases.

Chapter 5:

Discussion

Plants are continually subjected to attack by potentially pathogenic micro-organisms. Although the development of disease symptoms is the exception rather than the rule, the exceptions can be costly, particularly when agriculturally important plants surrender to pathogen infection. Communication between the cytoplasm and the cell wall is thus necessary since pathogen infection, amongst others, lead to altered biosynthesis and modification of cell wall components and downstream cytoplasmic events such as SAR.

Pathogen induced accumulation of β-1,3-glucanases has been reported in wheat (Anguelova *et al.,* 1999; Caruso *et al.,* 1999), sunflower (Jung *et al.,* 1993) and tomato (Oldroyd and Staskawicz, 1998) to name a few. This suggests that this enzyme play a direct role in plant defence mechanisms against pathogens. β-1,3-Glucanase is a PR protein which can be used as an indicator of the activation of the defence response in wheat after infection by leaf rust (Anguelova *et al.,* 1999). More specifically an increase in β-1,3-glucanase activity as well an increase in β-1,3-glucanase protein levels would indicate the positive activation of defence responses.

The activation of β-1,3-glucanases was found as early as 12 h after inoculation in infected resistant *Th/Lr34* (fig 4.2b) as well as in infected susceptible *Thatcher* plants (fig 4.2a). This was followed by a later increase in β-1,3-glucanase activity 24 h after inoculation in the resistant infected plants and 54 h after inoculation in the infected susceptible plants. Although the basal β-1,3-glucanase levels between the resistant *Th/Lr34* and the susceptible *Thatcher* plants differed, the final activated level was almost the same. The earlier reaction of the *Thatcher* plants were also more pronounced than that of the *Th/Lr34* plants.

The detection of β-1.3-glucanase polypeptides through Western blots confirmed the activation of the enzymes in the infected plants. A comparison of the polypeptide levels with the activity profiles of the infected *Thatcher* plants indicated that the early increased β-1.3-glucanase activity occurred without an increase in β-1.3-glucanase protein levels. A later *de novo* increase in β-1.3-glucanase proteins at

24 h.p.i was followed by an increase in β-1.3-glucanase activity at 54 h.p.i. This suggests that there was an initial activation of present β-1.3-glucanases followed by the synthesis of new polypeptides at a later stage. Such activation was probably the result of a signal transduction event where the signal transferred to the nucleus led to enhanced transcription of defence related genes, as well as the activation of existing defence mechanisms

A further comparison of the polypeptide levels of infected *Th*/Lr34 with its activity profiles indicated that the early increased β-1.3-glucanase activity (12 h.p.i) occurred without a corresponding increase in β-1.3-glucanase protein levels. In contrast to infected *Thatcher* plants, an increase in β-1.3-glucanase proteins was visible within 18 h.p.i, 6 hours earlier than that of the susceptible plants. Protein levels remained high during of the activation of the β-1.3-glucanases followed by a decrease in both protein levels and enzyme activity at 54 h.p.i. This is in contrast to the infected *Thatcher* plants where it remained elevated for only a very short time. This indicates that either the recognition or the signal transduction mechanism in the resistant plant was much more efficient in reacting to the infection than in the susceptible plants.

Anguelova *et al.*, (1999) found an increase of glucanase activity within 48 h.p.i in adult *Thatcher/Lr35* wheat plants infected with leaf rust (*P. triticina*) while Kemp *et al.* (1999) found an increase within 12 h.p.i in the wheat cultivars Palmiet/Lr29 and Palmiet/Lr34 infected with leaf rust. Anguelova *et al.*, (1999) also speculated that the increase in β-1.3-glucanase activity in the susceptible plants was an attempt to survive the infection. In another study using *A. thaliana* plants were infected by *Peronospora parasitica* it was found that β-1,3-glucanase expression increased within a day after inoculation (Rairdan and Delaney, 2002). Hanselle and Barz (2001) found that there were increases in extracellular β-1,3-glucanase activity two days after inoculation of chickpeas with *Ascochyta rabiei*, which was one day earlier than an similar increase in intracellular β-1,3-glucanase activity.

In contrast to previous studies, we have found that a sudden and as yet unexplained decline in β-1,3-glucanase activity in both the *Thatcher* and *Th/Lr34* infected plants occurred at 78 h.p.i. Furthermore this decrease in activity correlated with a decrease in protein levels. The uninfected plants showed a similar, albeit later decrease in the case of *Th/Lr34*. This decrease however was not the result of decreasing protein levels.

Thus, leaf rust infection led to the activation of β-1,3-glucanases in infected *Thatcher and Thatcher/Lr34* plants. This was accompanied by increased polypeptide levels.

Since we were interested in a protein kinase gene involved in the initial recognition of the pathogen, it was necessary to reduce the time intervals of the study and focus on the first 18 h.p.i since it was around this time interval that the first positive activation of defence responses took place. This time frame was chosen because several protein kinases are implicated in the early signal transduction events. The increases in β-1,3-glucanase protein levels around this time implicated that such a signal transduction event already took place within 18 h.p.i.

In the shorter time study, the infected *Thatcher* plants showed a transient increase in activity as early as 6 h.p.i (fig 4.4a) while a similar reaction was absent in the resistant plants (fig 4.4b). This initial activation was followed by another increase in activity 15 h.p.i. The resistant plants showed a single transient increase in β-1,3-glucanase activity, 12 h.p.i which correlated with the results obtained from the longer time study (fig 4.2b). Again, the uninfected control plants showed very similar activity profiles to the infected plants. It must be stressed that the longer and shorter time experiments were done on separate sets of plants and infections. It is thus possible that the occurrence of certain events did not correlate accurately between the two time studies, leading to slightly different results.

Western blots were again performed and we found that the early activation (6 h.p.i) in the infected susceptible plants was again due to the activation of already

present β-1,3-glucanase proteins since the polypeptide levels were unchanged. The later activation was however due to more synthesized proteins (fig 4.5b). The infected *Th/Lr34* plants showed the same tendency where the activation of the β-1,3-glucanases correlated with an previous increase in β-1,3-glucanase proteins (fig 4.5d). As seen in the longer time interval study, the increase in polypeptide levels was again earlier in the resistant than in the susceptible plants. In the uninfected plants, increases in β-1,3-glucanase polypeptide levels were also found. These increases were 3 hrs later than in the infected plants. A second increase at 6 h.p.i, in the uninfected *Thatcher* plants was also found. This increase can however not be explained at this stage.

As previously shown, the tendency of the uninfected control plants to show similar defence responses as that of the infected plants, led us to hypothesize that there might be some sort of communication event taking place between the infected and uninfected plants. This could also be due to the close proximity of the plants in the growth chamber.

Karban (2001) showed that wild tobacco plants (*Nicotiana attenuata*) became more resistant to herbivores when grown in the proximity of artificially damaged sagebrush (*Artemisia tridentata*) through air contact. The *N. attenuata* plants also exhibited increased levels of a defensive enzyme, polyphenol oxidase, compared to plants growing next to undamaged sagebrush plants. Another study on *Phaseolus vulgaris* showed that undamaged plants exposed to volatiles from a herbivore-infested neighbor had elevated expressions of several genes involved in defence mechanisms (Arimura *et al.*, 2001).

To determine if interplant communication took place, plants were grown in enclosed bell jars and β-1.3-glucanase activity of uninfected resistant plants were compared to uninfected plants exposed to infected plants (fig 4.9). Until 24 h.p.i there was no real difference in the β-1.3-glucanase activity between the infected and uninfected plants. When the plants were isolated in bell jars, there was a slight increase in glucanase activity, possibly because of the restricted environment.

The infected plants showed a gradual increase in glucanase activity, starting from 24 h.p.i to a high at 72 h.p.i, indicating positive infection. The uninfected control plants exposed to the infected plants showed a decrease in activity at 72 h.p.i (fig 4.9). When comparing the β-1.3-glucanase activities of this experiment with the previous ones, it was found that the levels were several fold lower. The observed repression at 72 h.p.i could therefore represent normal fluctuations in β-1.3-glucanase activity observed in uninfected plants. Despite the fact that the glucanase activity of the infected plants rose after 48 h.p.i to 72 h.p.i, it probably meant that threshold levels of a possible airborne signal were not reached and the defenses in the uninfected plants were not activated. The low activity levels were most probably due to a very poor infection of the plants by the leaf rust. This was concluded since other plants infected at the same time with the same batch of rust spores, developed poor disease symptoms. In future this experiment will be repeated with freshly prepared leaf rust spores.

To further confirm this early activation of the defence response, we determined the H_2O_2 concentrations at the different time intervals. Hydrogen peroxide production is one of the earliest known events of the plant defence reaction (Baker and Orlandi, 1999). It was thus used as a biochemical marker to confirm the β-1.3-glucanase studies.

The infected *Thatcher* plants showed an induction of H_2O_2 levels at 9 h.p.i (fig 4.7 a) which followed the activation of the β-1,3-glucanases at 6 h.p.i (fig 4.3 a). In the infected *Th/Lr34* plants (fig 4.7 b), H_2O_2 levels increased 9 h.p.i, preceding the activation of β-1,3-glucanases at 12 h.p.i.

The inoculation of potato leaf discs with zoospores of compatible and incompatible races of *Phytophthora infestans* resulted in the production of H_2O_2 approximately 1 h.p.i which lasted 3-4 h. This was followed by another increase in peroxide levels, 4 h.p.i and lasted for 10 h (Chai and Doke, 1987). Similar results were obtained by Keppler *et al.,* (1989) on tobacco inoculated with *Pseudomonas* pathovars. In these studies, it was found that H_2O_2 induction occurred in two stages – the first

stage was very early after infection (1 h.p.i) followed by a decrease, and then the second stage that consisted of a more sustained increase in H_2O_2 levels. In relation to our study, the increases in H_2O_2 levels that were found (fig 4.7), might be due to this second stage H_2O_2 burst.

Generation of H_2O_2 as well as other ROS may be a part of the defence response but since the oxidative burst takes place prior to the activation of transcription-dependent defenses, it is possible that ROS function as second messengers in the elicitor transduction pathway (Cervone *et al.*, 1997).

Peroxidase enzymes use H_2O_2 in a range of oxidations and their activity can be correlated to defence responses (Bowles, 1990). Peroxidase utilizes H_2O_2 to synthesize lignin to strengthen cell walls in the case of a pathogen attack (Baker and Orlandi, 1999). An increase in H_2O_2 would normally lead to an increase in peroxidase activity. Peroxidase activity was therefore determined to support the H_2O_2 levels.

Increases in peroxidase activity did coincide with increased H_2O_2 levels in the infected susceptible plants (fig 4.8 a). The infected resistant plants showed an increase in peroxidase activity 6 h.p.i that correlated with the increases in H_2O_2 levels starting at 6 h.p.i and continue to increase to 15 h.p.i. It is thus clear that increased H_2O_2 levels were accompanied by increased peroxidase levels. Again, as was the case of the β-1.3-glucanase activity, the profiles of peroxidase activity of the uninfected plants were quite similar to that of the infected plants. It was only at 6 h.p.i in the uninfected *Thatcher/Lr34* plants, that the peroxidase activity was markedly different from the infected plants. This coincided with an increase in the H_2O_2 levels in the infected plants, while the H_2O_2 levels of the uninfected plants decreased to its lowest level. These results again strengthened our theory of an airborne signal that might be involved in a possible interplant communication event.

Mohase and van der Westhuizen (2002) reported on an increase in peroxidase activity 48 hours after infestation of Tugela *DN* with the Russian wheat aphid

confirming the findings of Nandakumar *et al.,* (2001). In this study it was found that there was an increase in peroxidase activity that correlated with a decrease in H_2O_2 levels. This suggested the need for cell wall lignification in order to stop the penetration of the cell walls by the fungal hyphae. This was confirmed by the β-1,3-glucanase activity.

DD RT-PCR was used to identify possible differentially expressed protein kinase genes from total RNA extracted from tissue of the short time interval study. The expressed mRNA in the plant was transcribed to cDNA, PCR amplified and sequenced. It was decided to use the resistant infected plants for the isolation of putative protein kinase genes. The presence and induction of such genes would then also be determined within the infected susceptible plants at a later stage.

Nine putative differentially expressed protein kinase genes were shown to be inducibly expressed during the first 15 hours after inoculation (fig 4.12 and 4.13). The induction of these genes shortly after inoculation with the pathogen confirmed the biochemical results, in that there was recognition and signaling events occurring very early after infection.

The first clone, 03jjam1 (fig 4.15) displayed no sequence similarity at nucleotide or amino acid level to any other gene present in GenBank. This gene is expressed at 3 h.p.i that might indicate an involvement in the signal transduction event. The putative polypeptide contains a domain homologous to the subdomain VIb of protein kinases and might possibly be a protein kinase.

Clone 03jjam6 (fig 4.16) had sequence similarity to a *T. aestivum* and *H. vulgare* glutamine-dependent asparagine synthetase and encodes a polypeptide with a possible serine/threonine phosphorylation site. Glutamine-dependent asparagine synthetases play a role in nitrogen transport and catalyses the transfer of an amide group from glutamine to aspartate in an ATP-dependent reaction. Under normal growth conditions, the C/N ratio in the plant is the most important determining factor for whether asparagine or glutamine is selected as the major nitrogen

transport compound (Møller *et al.*, 2003). Under conditions favoring a high C/N ratio (light, sucrose addition) the expression of glutamine synthetase is induced and asparagine synthesis is repressed. In contrast, under a low C/N ratio (darkness, nitrogen addition) asparagine biosynthesis is activated and glutamine synthesis is repressed (Lam *et al.*, 1994). Lam *et al.* (1998) and Møller *et al.* (2003) found that plant asparagine synthetase gene expression is repressed by light. In *Arabidopsis* (Lam *et al.* 1998) the response to light can be seen after 2 h and in barley within 10 h after dark-treatment (Møller *et al.*, 2003).

Clone 03jjad1 (fig 4.17) shared DNA sequence similarity to an *O. sativa* asparaginyl endopeptidase and a *Z. mays* legumain-like protease. During normal plant growth, asparaginyl endopeptidase and legumain-like proteases are involved in the degradation of storage proteins. Both types of enzymes have an asparagine substrate specificity and is normally localized in the vacuoles of developing seeds (Correa *et al.*, 2001; Kato *et al.*, 2003), hypocotyls, roots and mature leaves. This suggests that they might be key enzymes functioning in vacuolar metabolism (Hara-Nishimura *et al.*, 1998).

The cloning of two genes possibly involved in nitrogen metabolism was possibly due to the infection procedure. After the wheat was grown in continuous light for a month, it was infected with the rust and then incubated in a dark dew cabinet for 16 hours to allow the rust to germinate. The induction of the expression of these genes was therefore most probably the result of the dark incubation and not the specific infection of leaf rust.

Clone 01jjad2 had sequence similarity to a *T. aestivum* mitochondrial small subunit 18S rRNA gene. Yao *et al.* (2002) found that a mitochondrial oxidative burst is involved in the apoptotic response in oats, where the mitochondria are responsible for the activation of several ROS. This may suggest that the pathogen was recognized and this gene was inducibly expressed to provide more ribosomal subunits in the mitochondria to facilitate a larger ROS related enzyme production.

The nucleotide sequence of clone 01jjad3 was similar to that of a *T. aestivum* gene for a high-molecular-weight glutenin subunit. Glutenin is normally used in the storage of reserve metabolites (Forde *et al.*, 1985) but it is usually not associated with leaf tissue. It is therefore possible that DNA contamination could have led to its amplification.

Therefore the use of these current primer combinations failed to amplify putative protein kinase genes, possibly due to the low annealing temperatures used. The fact that three of the five clones contained a conserved phosphorylation motive at the beginning of the cloned insert, confirmed this. An increase in the annealing temperature was not successful due to the low melting temperature of the original oligo dT primer. As a result of this it was decided to repeat the DD RT-PCR reactions with an oligo dT primer containing an additional 5' tail. By using a second primer with the sequence for the 5' tail of the oligo dT primer, the stringency of the PCR reaction was increased.

Using this modified procedure, the RT-PCR was repeated and a total of 20 inducibly expressed putative protein kinase genes were identified using the monocot and 10 using the dicot primer. However, due to a time constraint, these genes could not be characterized.

To conclude, this study has demonstrated that in the interaction between wheat and leaf rust, a signaling event is initiated very early after infection. This leads to the activation of plant defense, including the activation of PR proteins as well as the generation of H_2O_2 and the expression of genes involved in plant signaling and defence. Several differentially expressed genes were isolated of which the first few were not protein kinase genes, but whose products are implicated in nitrogen metabolism. Following this, 30 more genes were identified but their identity as protein kinase genes still has to be verified.

Chapter 6:

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Summaries

Summary

The aim of this study was to isolate a gene encoding a protein kinase that is involved in the specific recognition of the leaf rust fungus by wheat. It was determined that the susceptible *Thatcher* and resistant *Thatcher/Lr34* plants reacted within 12 h.p.i to the presence of the leaf rust, by activating the β-1,3-glucanase enzyme. A second increase in β-1,3-glucanase activity was found 24 h.p.i in the resistant plants and 54 h.p.i in the susceptible plants. The second increase occurred after an increase in β-1,3-glucanase protein levels, which occurred at 18 h.p.i in the resistant plants and 24 h.p.i in the susceptible plants. The initial activation could have been the result of the activation of already existing β-1,3-glucanase proteins within the cell, while the second was due to elevated protein levels.

The time study was refined by concentrating on the very early events after infection. β-1,3-Glucanase activity increased within 12 hours in the resistant and at 15 h.p.i in the susceptible plants. The latter also showed an increase at 6 h.p.i. This correlated with increased β-1,3-glucanase polypeptide levels. In both the resistant and susceptible infected plants there was an increase in H_2O_2 levels within 6 hours that was accompanied by a simultaneous increase in peroxidase activity.

A possible communication event between infected and uninfected resistant *Thatcher/Lr34* plants was investigated since the biochemical results suggested the occurrence of such an event. At this stage no concrete evidence supporting possible interplant communication could however be presented.

DD RT-PCR was used to isolate potential differentially expressed protein kinase genes from the resistant plants during the first 18 h after infection. Nine cDNA sequences were cloned. The first five encoded a putative glutamine-dependent asparaginyl synthetase normally involved in nitrogen metabolism, an asparagine endopeptidase/legumain-like protease normally involved in the degradation of storage peptides, a mitochondrial 18S rDNA subunit, a high-molecular-weight

glutenin subunit normally involved in storage of reserve metabolites, while the last clone did not show any similarity with any known gene. The DD RT-PCR was repeated at more stringent conditions and another 30 putative protein kinase genes that were differentially expressed, were isolated. These cDNAs are currently being characterized.

Keywords: *Triticum aestivum, Puccinia triticina,* Plant defence, Signal transduction, Protein kinase

Opsomming

Die doel van hierdie studie was om 'n geen wat kodeer vir 'n proteïen kinase betrokke by die herkenning van blaarroes deur koring, te isoleer. Verhoogde β-1,3-glukanase-aktiwiteit is binne 12 uur na infeksie in beide vatbare *Thatcher* en weerstandbiedende *Thatcher/Lr34* lyne gevind. 'n Tweede toename in β-1,3-glukanase aktiwiteit was 24 uur na infeksie in die weerstandbiedende en 54 uur na infeksie in die vatbare plante, waargeneem. Die latere toename was die gevolg van verhoogde β-1,3-glukanaseproteïenvlakke. Die vroeë toename in aktiwiteit kan waarskynlik toegeskryf word aan die aktivering van reeds bestaande β-1,3-glukanaseproteïene in die sel, terwyl die tweede die gevolg van verhoogde transkripsie was.

Die tydstudie was verder verfyn deur die eerste 15 uur van besmetting te bestudeer. β-1,3-Glukanase aktiwiteit het binne 12 uur in die weerstandbiedende plante toegeneem, terwyl die vatbare plante 'n toename eers na 15 uur getoon het. Laasgenoemde plante het egter 'n tweede verhoging in β-1,3-glukanase aktiwiteit na 6 uur getoon. Hierdie toenames was weereens die gevolg van verhoogde β-1,3-glukanasevlakke. In beide die weerstandbiedende en vatbare plante was daar 'n toename in H_2O_2-vlakke binne 6 ure na besmetting. 'n Gelyktydige toename in peroksidase-aktiwiteit was ook gevind.

Die moontlike kommunikasie tussen geïnfekteerde en ongeïnfekteerde weerstandbiedende *Thatcher/L34* plante was ondersoek na aanleiding van die biochemiese resultate wat op so 'n moontlikheid gedui het. Daar is egter geen konkrete resultate verkry wat dit kon bevestig nie.

DD RT-PCR was gebruik om differensieël uitgedrukte proteïenkinase-gene te kloneer. Nege cDNA volgordes wat binne die eerste 15 uur na infektering differensiële uiting getoon het, was geïsoleer. Die eerste vyf het onderskeidelik kodeer vir 'n glutamien-afhanklike asparaginielsintetase wat normaalweg by stikstof metabolisme betrokke is, 'n asparagienendopeptidase/protease wat normaalweg by die afbraak van

bergingsproteïene betrokke is, 'n mitochondriale 18S rDNA subeenheid, 'n hoë molekulêre-massa gluteniensubeenheid wat 'n bergingproteïen is, terwyl die laaste een geen homologie met enige bekende geen gehad het nie. Die DD RT-PCR was toe teen 'n hoër strengheid herhaal en 'n verdere 30 cDNA fragmente wat moontlik vir proteïnkinases kodeer, was gevind. Hierdie fragmente word tans gekarakteriseer.

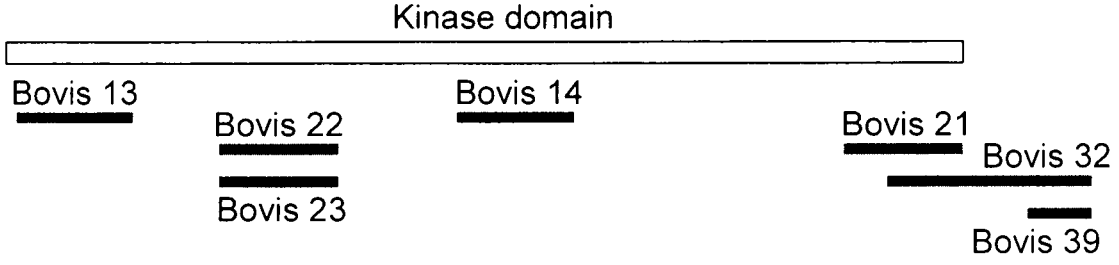
Sleutelwoorde: *Triticum aestivum, Puccinia triticina,* plant verdediging, seintransduksie, proteïen kinase

Appendix A

Primers

Nucleotide sequences of the primers used

Primer name	Nucleotide sequence
Bovis 13	5' - CTA CAG ATT CCA TGG TAT ACG ATT TTA AGA G – 3'
Bovis 14	5' – GAC TTG CTT GTC GAC TGA TCA GAC – 3'
Bovis 21	5' – TTT TTT TTT TTT TTT TVN – 3'
Bovis 22	5' – GAY ATH AAR CCN CAY AAY – 3'
Bovis 23	5' – GAY GTN AAR CCN GAR AAY – 3'
Bovis 32	5' – GAA GAA TTC TCG AGC GGC CGC TTT TTT TTT TTT TTT TTT TVN – 3'
Bovis 39	5' – GAA GAA TTC TCG AGC GGC – 3'
V = G/C/A	N = G/C/T/A Y = C/T H = C/T/A R = G/A



Attachment sites of the primers used