CHARACTERIZATION OF SOME EARLY DEFENCE RESPONSES OF LEAF RUST-INFECTED WHEAT

by

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I prefer the errors of enthusiasm to the indifference of wisdom – Anatole France

To succeed….. you need to find something to hold on to, something to motivate you, something to inspire you – Tony Dorsett

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Table of Contents

Table of Contents

Table of Contents

Abbreviations

A

APX	Ascorbate peroxidases
AVG	Aminoethoxyvinylglycine
AVR	Avirulence

B

BTH	Benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester
bZIP	Basic-domain leucine zipper

C

CAT	Catalase
CC	Coiled-coil
CWA	Cell wall appositions
CR	Control resistant
CS	Control susceptible

D

DDRT-PCR	Differential display reverse transcription polymerase chain reaction
dCTP	Deoxycytosine triphosphate
DIBOA	2,4-dihydroxy-2H-1,4-benzoxazin-3(4H)-one
DIMBOA	2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one
dNTP's	Deoxynucleotide triphosphates
DTT	Dithiothreitol

E

ERF	Ethylene-responsive-element-binding factors

G

GLV	Green leaf volatiles

H

H_2O_2	Hydrogen peroxide
h.p.i	Hour post inoculation
HR	Hypersensitive response
HSF	Heat-shock factors
Hsp	Heat-shock proteins

I

IR	Infected resistant
IS	Infected susceptible
ISR	Induced systemic resistance

J

JA	Jasmonic acid

L

LAR	Localized acquired resistance
LIR	Later infected resistant
LIS	Later infected susceptible
LOX	Lipoxygenase
Lr	Leaf rust resistance
LRR	Leucine-rich repeats

M

MeJA	Methyl jasmonate
MeSA	Methyl salicylate

N

NBS	Nucleotide binding site
NO	Nitric oxide
NPR1	Non-expressor of *PR1*

P

PAL	Phenylalanine ammonia-lyase
PBD	Peptide-binding domain
PCD	Programmed cell death
PR	Pathogenesis related
PAHBAH	p-Hydroxybenzoic acid hydrazide

R

R	Resistance genes
RLK	Receptor-like protein kinases
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
RT	Reverse transcription

S

SA	Salicylic acid
SAR	Systemic acquired resistance
sHsp	Small Heat-shock proteins
SOD	Superoxide dismutase

T

TaHlp01	*Triticum aestivum* Heat shock-like protein 1
TIR	Toll/Interleukin-1 receptor
TMV	Tobacco mosaic virus
Tween™ 20	Polyoxyethylene sorbitan monolaurate

U

UR	Uninfected resistant
US	Uninfected susceptible

V

VOC	Volatile organic compounds

Tables and Figures

Chapter 1

Introduction

Chapter 1

Plants are exploited as a source of food and shelter by a wide range of organisms, including viruses, bacteria, fungi, nematodes, insects and even other plants (Odjakova and Hadjiivanova, 2001). The identification of potential pathogenic microbes by the plant leads to the activation of different defence responses which are designed to prevent further infection (Thatcher *et al.,* 2005). Disease resistance in plants is usually associated with the activation of a wide variety of defence responses that serve to prevent pathogen replication and/or movement.

In some plant-pathogen interactions, the ability of the host plant to recognize the pathogen and activate these responses is regulated in a gene-for-gene-specific manner by the direct or indirect interaction between the products of a plant disease resistance (*R*) gene and a pathogenic avirulence (*Avr*) gene (Flor, 1971; Marois *et al.,* 2002; Axtell and Staskawicz, 2003; Di Gaspero and Cipriani, 2003). When either the plant or the pathogen lacks its cognate gene, activation of the plant's defence responses either fails to occur or is delayed sufficiently so that pathogen colonization ensues (Flor, 1956; Dangl and McDowell, 2006). In contrast to this race/cultivar-specific form of resistance which is relatively rare, many plant species exhibit non-host resistance. Non-host resistance is characterized by the activation of many of the same defence responses as are associated with race/cultivar-specific resistance. However, it occurs in the absence of any known *R/Avr* gene combination (Heath 2001; Thordal-Christensen, 2003; Dodds *et al.,* 2006).

Resistance in plants is manifested by the inability of the pathogen to grow or multiply and spread and often takes the form of a hypersensitive response (HR) (Vranová, 2002). The hypersensitive response is characterized by localized cell death at the site of infection (Van Loon, 1997). As a result, the pathogen remains confined to necrotic lesions near the site of infection. A ring of cells surrounding the necrotic lesion becomes refractory to subsequent infection. This is known as localized acquired resistance (LAR) (Fritig *et al*., 1998; Ghannam *et al.,* 2005). These local responses trigger specific and nonspecific resistance throughout the plant which is known as systemic acquired resistance (SAR) and provides durable protection against infection by a broad range of pathogens (Scheel, 1998; Durrant and Dong, 2004).

One of the earliest responses activated after the host plant recognition of an Avr protein or a non-host specific elicitor is the oxidative burst, in which levels of reactive oxygen species (ROS) rapidly increase (Mittler *et al*., 2004; Neill *et al*., 2002a). Other rapid responses include the crosslinking of cell wall proteins (McLusky *et al*., 1999), the activation of protein kinases (Romeis, 2001) and the increased expression of various defence genes. Some of these genes encode peroxidases, glutathione S-transferases, proteinase inhibitors and various biosynthetic enzymes such as phenylalanine ammonia lyase (PAL) and pathogenesis-related (PR) proteins (Kessmann *et al.,* 1994; Klessig *et al*., 2000; Flors and Nonell, 2006).

Activation of signal transduction networks after pathogen recognition results in the reprogramming of cellular metabolism, involving large changes in gene transcriptional activity while basic incompatibility frequently results in the expression of defence related genes and localized host cell death (Yamamoto *et al.,* 2004).

Plants contain many defence related proteins. In addition to *R*-genes and genes encoding signal transduction proteins, they also possess downstream defence genes (Van Loon, 1997), enzymes involved in the generation of phytoalexins (Flors and Nonell, 2006), enzymes involved in oxidative stress protection (May *et al.,* 1998), lignification (Cano-Delgado *et al.,* 2003) and numerous others. Many of these genes are involved in the production of secondary metabolites such as those of the shikimate (Herrmann and Weaver, 1999) and phenylpropanoid pathways (Dixon *et al.,* 2002).

To establish a compatible interaction, biotrophic pathogens have to camouflage themselves against recognition, suppress the activation of plant defences or counter-defend activated defences by the detoxification of potentially harmful compounds. Additionally, they have to redirect the host's metabolic flow to their own benefit without killing the host (Panstruga, 2003; Glazebrook, 2005).

Rust diseases of wheat are amongst the oldest plant diseases known to man. Early literature on wheat cultivation mentions these devastating diseases and their ability to destroy entire wheat crops. Numerous studies have been conducted on the life cycle of rust pathogens and their management (Marsalis and Goldberg, 2006). Leaf rust on wheat (*Triticum aestivum* L.) is caused by *Puccinia triticina* Eriks and is found wherever wheat is grown (Marsalis and Goldberg, 2006). It is the most regularly occurring of the three wheat rusts namely leaf,

stem and yellow rust (Kolmer, 1996). Genetic resistance against rusts 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.

During SAR or induced systemic resistance (ISR), distal parts of a plant receive a signal from an infected part (Scheel, 1998). This signal allows the distal part of the plant to activate its own defence mechanisms as a preventative strategy. In a similar way, two neighbouring plants could communicate so that the uninfected plant could activate its defences based on an airborne signal coming from an infected neighbour (Huang *et al.,* 2005; Gómez and Stuefer, 2006).

Originally proposed as pheromonal sensitivity in red alders and willows (Rhoades, 1983) and further corroborated in a study using poplars and maples (Baldwin and Schultz, 1983), the idea that plants might warn each other about an imminent attack was quite exciting because of the clear advantage that forewarning would mean for plants. It would remove the window of vulnerability that plants would otherwise suffer due to the lag during an induced response.

An important point to consider is whether communication between plants must be seen as mutualistic. Perhaps neighbouring plants are merely opportunistically responding to volatile signals released by the wounded plants as indicators of imminent herbivore attack. However, the interplant signalling can be interpreted as a natural outcome of sensitivity to itself.

Based on this, the aim of this study was to investigate some of the early events occurring after the infection of wheat with leaf rust on molecular level. An attempt was first made to clone and identify putative genes involved in these early events in order to elucidate the early signalling events in this particular interaction. Secondly, a possible inter-plant communication event between infected and uninfected plants was investigated.

Chapter 2

Literature Review

2.1 INTRODUCTION

Plants are major targets for pathogenic microbes looking for a source of nutrition. A complex array of interactions between plants and microbes has thus evolved to reflect both the nutrient acquisition strategies of the microbes as well as the defence strategies of plants.

Penetration of fungi through the plant cell wall represents an Achilles heel for the plant. For biotrophic fungi, it initiates a transition from extra-cellular to invasive growth. Modification of the plant cell wall was recognized as a potential resistance mechanism against infection (Young, 1926; Franceschi *et al.,* 2005). The termination of fungal pathogenesis at the cell wall is commonly associated with cell wall thickenings and the formation of callosites in the paramural space (Matern *et al.,* 1995; McLusky *et al.,* 1999; Snyder and Nicholson, 1990). The formation of these cell wall appositions (CWA) is usually accompanied by the co-localized accumulation of phenolics and ROS (Bestwick *et al.,* 1998; Nicholson and Hammerschmidt, 1992; Thordal-Christensen *et al.,* 1997) aiming to deter invasion by the pathogen.

Specific host-pathogen interaction models describing induced defence responses in plants, have been influenced by the gene-for-gene concept reported by Flor (1956). In these specific host-pathogen interactions, resistance to a particular pathogen is conditional to the presence of a specific *Avr*-gene in the pathogen and a specific *R*-gene in the plant host.

2.2 PLANT-PATHOGEN INTERACTION

The biotrophic lifestyle of fungi is described as deriving energy from living plant cells (Mendgen and Hahn, 2002). Most parasitic biotrophs like mildews, rusts and smuts withdraw nutrients from shoot tissue and have no alternative energy source (Schulze-Lefert and Panstruga, 2003). A characteristic feature of many but not all biotrophic fungi is their ability to form a specialized infection structure, the haustorium. Formation of these intracellular fungal structures requires successful penetration of the host cell wall. This is a complex process that exposes the intruding fungus to cell wall-associated defence responses. Invasive growth of biotrophs after cell wall penetration leads to the invagination

of the plasma membrane that creates an interface between host and fungus consisting of the haustorial membrane, an extra-haustorial matrix and the host plasma membrane following the contours of the haustorial membrane (Schulze-Lefert and Panstruga, 2003).

To establish a compatible interaction, biotrophic pathogens have to camouflage themselves against recognition, suppress the activation of plant defences or counter-defend activated defences by the detoxification of potentially harmful compounds. Additionally, they have to redirect the host's metabolic flow to their own benefit without killing the host.

The most common response of resistant plants to the cellular invasion by fungal pathogens is a rapid cell death, which forms part of the HR. However, the HR is not an obligatory component of the plant's defence response since there are pathosystems in which resistance does not depend on the manifestation of HR (Heath, 2000).

2.2.1 Wheat Leaf Rust

Rust-causing fungi are obligatory biotrophic plant pathogens. Economically they are important biological agents that render damage to wheat plants. Leaf rust on wheat (*Triticum aestivum* L.) is caused by *Puccinia triticina* Eriks. and is found wherever wheat is grown. It is the most regularly occurring of the three wheat rusts, namely leaf rust, stem rust and yellow rust. Wheat cultivars that are susceptible to leaf rust suffer from yield reductions of between 5 to 30% or more, depending on the stage of crop development when the initial rust infection occurs (Kolmer, 1996). Wheat rust fungi spread in the form of clonally produced dikaryotic urediniospores, which can be blown by the wind for thousands of kilometers from initial infection sites (Roelfs, 1989). Thus, epidemics of wheat rusts can occur on a continental scale because of the widespread dispersal of urediniospores.

Wheat rust fungi are highly specific obligate parasites that interact with wheat in a gene-for-gene relationship (Person, 1959; Flor, 1971). This high degree of specificity has made durable rust resistance in wheat difficult to achieve, because the virulence of wheat rust fungi against wheat resistance genes is highly diverse, resulting in the existence of many different pathogenic races. Rust races that are virulent against cultivars containing resistance genes and are newly deployed in wheat can rapidly increase in frequency over

large geographic areas (Kolmer, 1999), thus rendering the resistance genes ineffective (Kolmer, 2005).

Genetic resistance is the most economical and preferred 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 leaf rust resistance genes allows for efficient incorporation of these genes into germplasm pools. Thus far, 52 different leaf rust resistance (*Lr*) genes conferring specific resistance to leaf rust, have been identified and assigned to specific chromosomes (Hiebert *et al.,* 2005).

2.3 PATHOGEN RECOGNITION

Plant responses to infection are usually initiated by the specific recognition of the pathogen and the transmission of the signal via plasma membrane-bound receptors. A surveillance system of receptors, some of which are encoded by *R*-genes, reacts by a similar mechanism to all classes of pathogens, irrespective of whether they are viruses, bacteria, fungi or nematodes (Di Gaspero and Cipriani, 2003).

Receptor-mediated recognition at the site of infection initiates cellular and systemic signalling processes that activate multi-component defence responses both at local and systemic levels, resulting in the rapid establishment of local resistance and delayed development of SAR (Scheel, 1998). The earliest reactions of plant cells include changes in plasma membrane permeability leading to calcium and proton influx (McDowell and Dangl, 2000). This in turn leads to the production of reactive oxygen intermediates (ROI) such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) catalyzed by plasma membrane-located NADPH oxidase and/or apoplastic peroxidases (Somssich and Hahlbrock, 1998).

These initial ion fluxes and production of ROI also trigger the localized production of secondary messengers for the initiation of HR and defence gene expression. Interactions between ROI, nitric oxide (NO) and salicylic acid (SA) have been postulated (Delledonne *et al*., 2001). Other interacting components might include specifically induced phospholipases which act on lipid-bound unsaturated fatty acids within the membrane resulting in the release of linolenic acid (Creelman and Mullet, 1997). Linolenic acid in turn acts as substrate

for the production of jasmonate (JA), methyl jasmonate (MeJA) and other related molecules (Hamberg and Gardner, 1992; Creelman and Mullet, 1997).

The expression of most of the inducible, defence-related genes are regulated by signal pathways involving one or more of the three key regulators namely jasmonate (Bell and Mullet, 1991; Creelman *et al.,* 1992), ethylene (Boller, 1995) and salicylic acid (Slaymaker *et al.,* 2002; Takahashi *et al.,* 2002*;* Kumar and Klessig, 2003; Van Wees and Glazebrook, 2003).

2.4 DEFENCE RESPONSES

Plants generally activate multiple defence responses upon pathogen attack, which leads to cellular reprogramming. The most effective defence response in plants is mediated by *R*-genes that are able to detect specific pathogenic races through recognition of pathogen encoded Avr proteins (Glazebrook, 1999).

2.4.1 SYSTEMIC RESISTANCE

In general, SAR may involve the activation of more than one biochemical pathway and is believed to be mediated by SA (Johal *et al.,* 1995). The central role of SA as a signal transducer of SAR was demonstrated in transgenic plants where SA could not accumulate. These plants failed to express SAR (Gaffney *et al.,* 1993). The formation of phenolic free-radicals, resulting from the interaction of SA with catalase or ascorbate peroxidases, has been proposed to be involved in the induction of SAR (Durner and Klessig, 1995). The discovery that the exogenous application of SA activate the same spectrum of disease resistance responses as the preliminary infection by a pathogen, led to the exploitation of SAR with the application of synthetic chemicals that act at, or just downstream, of SA but also producing a successful SAR (Gozzo, 2003). Correlating with the onset of SAR, plants express a set of PR-proteins (Kessmann *et al.,* 1994). Some of these PR proteins have been shown to have antimicrobial activity *in vitro* or to confer increased resistance when over-expressed in plants (Morrissey and Osbourn, 1999). Once established, SAR may last for a relatively long time (from weeks to months), during which any attempted invasion by certain pathogens is hampered.

A different form of systemic resistance was discovered in plants responding to non-pathogenic strains of *Pseudomonas fluorescens*. This has been referred to as ISR and is

effective against multiple pathogen types (Pieterse *et al.,* 1998). ISR is independent of SA and is not associated with the activation of pathogenesis-related (PR) protein 1 gene expression. Instead, ISR requires the signalling pathways responding to the plant growth hormones, JA and ethylene (Pieterse *et al.,* 1998).

2.4.2 Programmed Cell Death

Cell death is an essential process in the plant's life cycle (Vranová *et al.,* 2002). Two modes of cell death have been described in plants: programmed cell death (PCD) and necrosis. PCD is controlled genetically and shares features characteristic of the apoptotic cell death in animal cells, including cell shrinkage, cytoplasmic and nuclear condensation, chromatin condensation and DNA fragmentation (O'Brien *et al.,* 1998). Necrosis on the other hand results from severe and persistent trauma and is not considered to be genetically controlled (Vranová *et al.,* 2002).

Programmed cell death can be controlled by small cytotoxic molecules, ROS such as H_2O_2 and O_2^- and lipid peroxidases (Jabs, 1999). The execution stage of cell death is associated with an uncontrolled production of ROS that overwhelms the normal protective mechanisms of cells (Palma and Kermode, 2003). Another possibility is that ROS production is simply a consequence of cells undergoing death and plays no role in the initiation or execution phases of cell death.

One form of PCD is the HR that appears to be an integral part of plant defence mechanisms against pathogens (Palma and Kermode, 2003). Fungal infection can trigger the HR in plants, a process characterized by the rapid death of plant cells immediately surrounding the site of infection, which effectively prevents the spread of the pathogen (Ivanov *et al.,* 2005). The oxidative burst that precedes the HR is accompanied by the generation of ROS, mainly due to the activation of a plasma-membrane associated NAD(P)H oxidase (Jabs, 1999).

ROIs and reactive nitrogen intermediates (RNIs) are highly toxic and may directly offer protection against the pathogen, but they are the most non-discriminating defence molecules produced by the offended hosts (Veronese *et al.,* 2003). In animals, they are produced and accumulate only in specific self-sacrificing cells (Nathan and Shiloh, 2000).

The role of ROI in plant disease resistance is probably similar where the accumulation of ROI in self-sacrificing cells offers protection against the pathogen while limiting the damage to the host. ROIs and RNIs also participate in transcriptional reprogramming in and around the affected cell (Veronese *et al.,* 2003).

Expression of genes encoding enzymes that detoxify ROS e.g. catalase (CAT), superoxide dismutase (SOD) and ascorbate peroxidases (APX), correlates with the induction of the HR and may protect neighbouring cells from the uncontrolled diffusion of ROS (del Rio *et al.,* 1998; Heath, 2000).

2.4.3 H_2O_2

Until recently, H_2O_2 was viewed mainly as a toxic cellular metabolite (Neill *et al.,* 2002b). It is now clear that it can also function as a defence molecule that mediates responses to various stimuli in both plant and animal cells (Neill *et al.,* 2002a).

H_2O_2 is continually generated from various sources during normal metabolism and a wide range of steady-state H_2O_2 concentrations has been reported (Karpinski *et al.,* 1999; Veljovic-Jovanovic *et al.,* 2001). It can also be generated by specific enzymes during an oxidative burst, which results in rapidly increasing H_2O_2 synthesis and release into the apoplast (Orozco-Cárdenas, *et al.,* 2001). This oxidative burst is a common response to pathogens, elicitors, wounding, heat, ultra-violet light and ozone (Neill *et al*., 2002a). Knockout experiments demonstrated that the *AtrbohD* and *AtrbohF* genes encoding NADPH oxidase are required for H_2O_2 generation during fungal and bacterial challenges (Torres *et al.,* 2002). H_2O_2 generated after pathogen attack mediates cross-linking of cell wall proteins (Bradley *et al.,* 1992) and plant cell wall-bound phenolics (Grant and Loake, 2002).

H_2O_2 also regulates the expression of various genes, including genes encoding antioxidants as well as genes leading to the production of H_2O_2. This indicates the complex way in which intracellular H_2O_2 concentrations are monitored and maintained at a constant level (Neill *et al.,* 2002a). A microarray study showed that the expression of 1-2% of all *Arabidopsis* genes was altered in H_2O_2-treated cell cultures (Desikan *et al.,* 2001). Amongst these, genes encoding antioxidant proteins and proteins potentially involved in PCD, as well as defence proteins such as calmodulin, protein kinases and transcription factors, were upregulated.

2.4.4 SALICYLIC ACID

The involvement of SA as a signal molecule in local defences and SAR has been extensively studied because of its ability to induce protection against pathogens (Slaymaker *et al.,* 2002; Takahashi *et al.,* 2002*;* Kumar and Klessig, 2003; Van Wees and Glazebrook, 2003). Increases in endogenous levels of SA and its conjugates in pathogen-inoculated plants coincide with the elevated expression of genes encoding PR-proteins and the activation of disease resistance (Morrissey and Osbourn, 1999). As a result, the plant becomes more resistant to pathogen attack.

Plants that cannot accumulate SA due to the presence of transgenes that encodes salicylic acid-degrading enzymes, for example *NahG*, develop an HR response after a challenge with an avirulent pathogen, but do not exhibit systemic expression of defence genes and do not develop resistance to subsequent pathogen attacks (Glazebrook, 1999; Nawrath and Métraux, 1999; Van Wees and Glazebrook, 2003). In contrast, the exogenous application of SA or its synthetic functional analogue benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH) results in the activation of *PR*-gene expression and enhanced resistance to pathogens (Shah, 2003). The *Arabidopsis thaliana* non-expressor of *PR1* (*NPR1*) gene, also called non-inducible immunity 1 (*NIM1*), is an important component of SA defence. *Npr1* and *nim1* mutant plants are insensitive to SA and this compromises their disease resistance (Cao *et al., 1*994; Delaney *et al.,* 1995). An NPR1-independent, SA-mediated resistance mechanism also operates in *Arabidopsis* (Dong, 2001).

SA can specifically bind to a variety of plant proteins affecting their activity (Dempsey *et al.,* 1999; Slaymaker *et al.,* 2002). It can also activate gene expression by multiple mechanisms and at different steps in plant defence (Feys and Parker, 2000; Kunkel and Brooks, 2002).

2.4.5 JASMONIC ACID

The ubiquitous presence of JA and its methyl ester in all higher plants examined so far suggests a prominent role for these molecules in plant metabolism (Mason *et al.,* 1992). Jasmonates are derived from linolenic acid by a lipoxygenase (LOX)-mediated oxygenation

process and have structural similarities to mammalian eicosanoids, which are also derived from fatty acids through the action of LOX (Hamberg and Gardner, 1992).

In plants, jasmonates move easily in both the liquid and the vapour phases (Farmer and Ryan, 1990). Methyl jasmonate is especially volatile, suggesting that it might also act in the gaseous form in analogy to the plant hormone ethylene (Wasternack and Parthier, 1997). JA affect a variety of physiological processes, including root growth (Staswick *et al.,* 1992), tuber formation (Koda, 1992), tendril coiling (Weiler *et al.,* 1993), senescence of leaves and stomatal opening (Sembdner and Parthier, 1993). An additional role for jasmonates lies in the mediation of the plant responses to stresses such as pathogen and herbivore attack (Thaler *et al.,* 2002).

Low concentrations of JA regulate proteinase inhibitors like thionin (Epple *et al.,* 1995), osmotin (Xu *et al.,* 1994) and proline-rich cell wall protein (Creelman *et al.,* 1992) at transcription level. It can also induce the accumulation of different enzymes involved in plant defence reactions such as chalcone synthase (Creelman *et al.,* 1992), PAL (Gundlach *et al.,* 1992) and LOX (Bell and Mullet, 1991). The induction of these proteins suggests a role for JA in helping plants to contain the growth of microorganisms.

Reports on the induction of a resistant state in plants by JA are contradictory. Some authors (Kogel *et al.,* 1994; Schweizer *et al.,* 1993) found no evidence for the induction of resistance by jasmonate in the barley-*Erysiphe graminis* f.sp. *hordei* interaction, but Mitchell and Walters (1995) proved induced systemic protection in the same pathosystem by treating the first leaves of seedlings with methyl jasmonate.

2.4.6 ETHYLENE

Ethylene is a volatile plant hormone derived from methionine that is involved in numerous physiological processes (Kende, 1993). Ethylene is produced upon wounding or infection by pathogens, as well as by treatment with elicitors of defence responses (Boller, 1995). Exogenous application of ethylene to tobacco carrying the *N* gene for resistance to Tobacco Mosaic Virus (TMV) results in resistance to TMV marked by a decrease in the size of the necrosis (Van Loon and Antoniw, 1982).

Ethylene can induce the accumulation of some of the PR-proteins such as β-1,3-glucanase and chitinase (Abeles *et al.,* 1971; Yamamoto *et al.,* 2004). Structural reinforcement of the cell wall through lignification and the accumulation of hydroxyproline-rich cell wall proteins are also enhanced by ethylene (Boller, 1995). Although such results might suggest that ethylene is the signal involved in the induction of SAR, several experimental results indicate that ethylene might not be directly linked to the induction of SAR (Boller, 1995). The induction of chitinase and β-1,3-glucanase in pea pods by pathogens can take place in tissue treated with aminoethoxyvinylglycine (AVG), a potent inhibitor of ethylene biosynthesis. This indicates that ethylene synthesis after infection might be a symptom rather than a cause of the induction of defence reactions (Mauch *et al.,* 1984).

2.5 Changes in Gene Activity

Activation of signal transduction networks after pathogen recognition results in the reprogramming of cellular metabolism, involving large changes in gene transcriptional activity while basic incompatibility frequently results in the expression of defence related genes and localized host cell death (Yamamoto *et al.,* 2004).

Plants contain many defence related proteins. In addition to *R*-genes and genes encoding signal transduction proteins, they also possess downstream defence genes encoding PR-proteins (Van Loon, 1997), enzymes involved in the generation of phytoalexins (Flors and Nonell, 2006), enzymes involved in oxidative stress protection (May *et al.,* 1998), lignification (Cano-Delgado *et al.,* 2003) and numerous others. Many of these genes are involved in the production of secondary metabolites such as those of the shikimate (Herrmann and Weaver, 1999) and phenylpropanoid pathways (Dixon *et al.,* 2002).

2.5.1 Molecular Chaperones

Molecular chaperones are key components contributing to cellular homeostasis in cells under both optimal and abnormal growth conditions (Grene, 2002). They are responsible for the folding, assembly, translocation and degradation of proteins in a broad array of cellular processes. They also function in the stabilization of proteins and membranes and can assist in protein refolding under stress conditions (Wang *et al.,* 2004). Many molecular chaperones are stress proteins and most were originally identified as heat-shock proteins

(Hsp) (Lindquist, 1986; Lindquist and Craig, 1988) and thus the names of these molecular chaperones follow their early nomenclatures and are referred to as Hsps.

Except for the small Hsp (sHsp) family (Haslbeck, 2002), relatively little attention has been given to the role of the many other Hsps in plant responses to abiotic stress and the direct support for Hsp function during plant abiotic stress tolerance is limited. This, despite the fact that Hsps are known to be expressed in plants not only when they experience high temperature stress, but also in response to other environmental stresses such as water, salinity, osmotic, cold and oxidative stress (Boston *et al.,* 1996; Bukau and Horwich, 1998).

Hsp60, Hsp70 and Hsp90 interact with a wide variety of co-chaperone proteins that regulate their activity or aid in the folding of specific substrate proteins (Bukau and Horwich, 1998). Hsp70 is also involved in the modulation of signal transducers such as protein kinase A, protein kinase C and protein phosphatase (Ding *et al.,* 1998). Chaperones of the Hsp90 and Hsp70 families and their co-chaperones were also found to interact with a growing number of signal molecules, including nuclear hormone receptors, tyrosine- and serine/threonine kinases, cell-cycle and cell-death regulators, demonstrating that they could play a key role in cellular signal transduction networks (Nollen and Morimoto**,** 2002).

In mammalian cells, the sHsps are known to be involved not only in protection against stress, but also in the modulation of other cellular functions such as apoptosis and differentiation via their participation in the regulation of cellular redox states (Arrigo, 2002). Although most of the studies were carried out in organisms other than plants, similar cross-talk mechanisms might operate in plants. For example, heat-shock transcription-factor-dependent expression of the antioxidant ascorbate peroxidases in *Arabidopsis* (Panchuk *et al.,* 2002) suggests that heat-shock factors (HSFs) might not only be involved in Hsp synthesis but also in oxidative stress regulation of antioxidant gene expression.

Mehlen *et al.* (1996) and Garrido *et al.* (1999) demonstrated an interaction between sHsps and glutathione in mammalian cells that resulted in increased resistance to cell death induced by the tumour necrosis factor or by hydrogen peroxide. Resistance was dependent on both the increase in reduced glutathione and increased expression of sHsps and was shown to decrease the levels of cellular ROS. This interaction has not yet been investigated

in plant systems, but there is much evidence that suggests the importance of glutathione in the protection against ROS damage (May *et al.,* 1998; Noctor *et al.,* 1998).

The protective effects of Hsps can be attributed to the intra-cellular network of the chaperone machinery in which many chaperones interact (Wang *et al.,* 2004). During stress, many enzymes and structural proteins undergo functional changes. Therefore, maintaining proteins in their functional conformations, preventing aggregation of non-native proteins, refolding of denatured proteins to regain their function and the removal of non-functional, but potentially harmful polypeptides, are important for cell survival under stress.

Four distinct functions have been assigned to molecular chaperones (Grene, 2002). They can act as repair proteins, they can remove proteins that are irretrievably damaged and they can facilitate the import of newly synthesized proteins into the interior of organelles such as peroxisomes. The fourth function is as antioxidant molecules in conjunction with protein methionine-sulfoxide reductase.

Many molecular chaperones described so far are members of the Hsp60 and Hsp70 families. The various functions of Hsp70s rely on their ability to bind to unfolded segments of proteins in an ATP-dependent, reversible manner. All Hsp70s in prokaryotic and eukaryotic cells consist of an ATP-binding domain and a peptide-binding domain (PBD) (Hartl and Hayer-Hartl, 2002). The two domains cooperate and in doing so Hsp70s undergo substantial conformational changes. It is generally agreed upon that the PBD of Hsp70 in its ATP form has an open binding pocket which recognizes unfolded segments of polypeptides (Bukau and Horwich, 1998; Mayer *et al.*, 2001; Hartl and Hayer-Hartl, 2002). Upon hydrolysis of ATP by Hsp70, the peptide-binding pocket closes so that the ADP form holds the substrate tight. For their various functions Hsp70s require the cooperation with a set of co-chaperones which help in binding the polypeptide substrates and which support the ATP/ADP cycle (Bukau and Horwich, 1998; Kelley, 1998; Fan *et al.*, 2003).

Induction of host Hsp synthesis in response to an encounter with a pathogen has at least two major causes. Firstly, infected cells are confronted with antimicrobial mechanisms which they have activated themselves against the pathogen during infection (Buchmeier and Heffron, 1990). Effective protection against its own effecter molecules (e.g. reactive radicals) becomes vital to the host for survival and hence the need for Hsps. Second, once

inside a host, many microbes, especially those which persist in the host, interfere with intracellular host metabolism and thus, not surprisingly, many of these pathogens are potent inducers of plant Hsp synthesis (Zügel and Kaufmann, 1999). As soon as a pathogen enters its host, it is confronted with several changes, some of which are very stressful. Inside the host a pathogen is usually confronted with ROI and RNI's, attack by lysosomal enzymes and depletion of co-factors. To protect itself against its host, the pathogen thus activates various evasion mechanisms including the synthesis of its own Hsps (Buchmeier and Heffron, 1990).

2.5.2 TRANSCRIPTION FACTORS

Stress gene induction occurs primarily at the level of transcription and regulation of the expression patterns of specific stress related genes is an important part of the plant stress response (Rushton and Somssich, 1998). Plants devote larger portions of their genomes to genes encoding transcription factors compared to mammalian cells. The *Arabidopsis* genome, for example, contains more than 1500 transcription factor genes (Reichmann *et al.,* 2000) and these transcription factors belong to large gene families, some of which are unique to plants.

Transcription factors can be defined as regulatory proteins that bind to short stretches of DNA in a sequence-specific manner and mediate protein-protein interactions (Després and Fobert, 2006). These interactions facilitate or interfere with the recruitment of RNA polymerase and the basal transcriptional machinery. Three of the largest families include the ethylene-responsive-element-binding factors (ERF), basic-domain leucine zipper (bZIP) and WRKY proteins which are implicated in several stress responses.

ERF proteins form a subfamily of the APETALA2 (AP2)/ethylene-responsive-element-binding protein transcription factor family that is unique to plants. ERF proteins share a conserved 58-59 amino acid domain (the ERF domain) that can bind to two similar cis-elements: the GCC box, which is found in several *PR* gene promoters where it confers ethylene responsiveness and the C-repeat (CRT)/dehydration-responsive element motif, which is involved in the expression of dehydration- and low temperature responsive genes (Fujimoto *et al.,* 2000; Singh *et al.,* 2002). Several *Arabidopsis* ERF genes respond to pathogen

infection with different but overlapping kinetics and this may help to orchestrate the correct temporal defence response (Oñate-Sánchez and Singh, 2002).

bZIPs form a large family of transcription factors in plants with 75 members in *Arabidopsis* (Jakoby *et al.,* 2002). One class of bZIP proteins that is linked to stress responses comprises the TGA/octopine synthase (*ocs*)-element-binding factor proteins. These proteins bind to the *activation sequence-1* (*as-1)*/*ocs* element which regulates the expression of *PR1* and glutathione S-transferase genes (Lebel *et al.,* 1998; Chen and Singh, 1999). In *Arabidopsis* there are seven members of the TGA/OBF family which play roles in plant defence and development and have been shown to interact with the NPR1 protein of the SA-dependent defence pathway (Zhang *et al.,* 1999; Després *et al.,* 2000; Niggeweg *et al.,* 2000).

WRKY proteins form another family of transcription factors that are unique to plants (Eulgem *et al.,* 2000). WRKY proteins contain either one or two WRKY domains – a 60 amino acid region that contains the amino acid sequence WRKYGQK or a zinc finger like motif. Specific WRKY family members show enhanced expression and/or DNA-binding activity following induction by a range of pathogens, defence signals and wounding (Eulgem *et al.,* 2000).

2.5.3 R-GENES

Despite the great diversity in lifestyle and pathogenic mechanisms of disease-causing organisms, *R*-genes were found to encode proteins containing certain common motifs. The current classification of *R*-genes recognizes four classes, coding for (1) cytoplasmic serine/threonine kinases e.g. the *Pto* gene in tomato (Tang *et al.,* 1999); (2) proteins containing a nucleotide binding site (NBS) and leucine-rich repeats (LRRs) together with either a Toll/Interleukin-1 receptor (TIR) domain (e.g. the tobacco *N* gene (Liu *et al.,* 2002)) or a coiled-coil structure (CC) for example the *Arabidopsis RPS2* gene (Bent *et al.,* 1994); (3) extracellular LRRs anchored to a transmembrane domain (e.g. the tomato *Cf-9* gene (Jones *et al.,* 1994)) and (4) receptor-like protein kinases (RLKs) with extracellular LRRs and an intracellular serine/threonine kinase domain (e.g. the rice *Xa21* (Song *et al.,* 1995) and the wheat *Lrk10* genes (Feuillet *et al.,* 1997)).

R-gene products interact with proteins encoded by *Avr* genes in a race-specific recognition event or with general elicitors produced as a result of the infection. Once the receiver domain is activated by ligand binding, either the NBS or the serine/threonine kinase domain becomes accessible to the downstream components of the signal transduction pathway, ultimately triggering the defence response (Bendahmane *et al.,* 2002).

In addition to gene for gene recognition of a pathogen mediated by the *R* and *Avr* genes, nonhost resistance is achieved through communication of specific pathogen or plant cell wall derived signal molecules, called exogenous or endogenous elicitors respectively (Montesano *et al.,* 2003). These elicitors are often low-molecular-weight compounds that are either synthesized or liberated from polymeric precursors during infection (Somssich and Hahlbrock, 1998). The chemical structure of these elicitors varies greatly and includes glycoproteins, peptides and oligosaccharides (Boudart *et al.,* 1995; Montesano *et al.,* 2003). Some proteinaceous elicitors are directly produced by bacterial or fungal pathogens, whereas biologically active oligosaccharides are released from pathogen and plant cell walls by hydrolases secreted by the two organisms. Complex and largely unresolved perception systems exist for these elicitors on the plant cell surface which activate multiple intracellular defence pathways (Odjakova and Hadjiivanova, 2001).

The NBS-LRR class is by far the largest group of resistance proteins (Bai *et al.,* 2002). Two subgroups within the NBS-LRR class have been recognized by the presence or absence of a TIR domain and structural similarity to the cytoplasmic defence domains of the Toll and interleukin-1 receptor (Baker *et al.,* 1997; Parker *et al.,* 1997; Rock *et al.,* 1998).

The first subgroup (TIR-NBS-LRR) includes *N* (tobacco mosaic virus resistance (Holmes, 1938)), L6 (flax rust resistance (Flor, 1947)), *M* (flax rust resistance (Anderson *et al.,* 1997)) and *RPP5* (downy mildew resistance (Parker *et al.,* 1997)) which are all involved in defence processes. The second subgroup, which lacks the TIR region, includes the bacterial resistance proteins RPS2 (Bent *et al.,* 1994), RPM1 (Grant *et al.,* 1995), Xa1 (Yoshimura *et al.,* 1998), Prf (Salmeron *et al.,* 1996) and the fungal resistance protein I2 (Ori *et al.,* 1997).

Signalling through these two classes however is realized through different signal transduction pathways. In *Arabidopsis*, proteins in the TIR class signal via a pathway that

includes *EDS1* (Parker *et al.,* 1996) while proteins in the LZ class signal via a pathway that includes the *NDR1* gene (Century *et al.,* 1997).

Over the past few years, protein kinases have been identified for both race- and nonrace-specific elicitation of defence responses in plants. They often participate in the direct perception of elicitors of *Avr* products (Song *et al.,* 1995**;** Cervone *et al.,* 1997; Feuillet *et al.,* 1997; Thomas *et al.,* 1997*)*, mediate defence required for the production of defence mechanisms and function as regulators of defence responses (Romeis, 2001). The phosphorylation of proteins, probably initiated by a receptor, is thought to relay the defence signal to different downstream effectors (Ligterink *et al.,* 1997). 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).

2.6 INTERPLANT COMMUNICATION

SAR is the process whereby distal parts of a plant receive a signal from an infected part (Scheel, 1998). This signal allows the distal part to activate its own defence mechanisms as a preventative defence strategy. In a similar way two neighbouring plants could communicate so that the uninfected plant could activate its defences based on an airborne signal coming from an infected neighbour. Intra-plant communication and signal transduction have been described often, so the possibility of communication and signal transduction between plants, exists. If there are receptor proteins to facilitate intra-plant communication, there should also be ones able 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).

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).

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 a herbivore's physiology. An example is the accumulation of protease inhibitors in potato and soybean plants (Koiwa *et al.,* 1997). Plants may also indirectly defend themselves against herbivores by emitting specific blends of volatiles that attract natural carnivorous 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, while 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). There is a broad diversity of known inducible volatiles, including alkenes, alkanes (Preston *et al.,* 2001), two jasmonates (cis-jasmone (Preston *et al.,* 2001) and methyl jasmonate (Farmer and Ryan, 1990)) and methyl salicylate (MeSA) (Shulaev *et al.,* 1997) but the dominating compounds tend to be terpenes (Arimura *et al.,* 2001) and C6 green leaf volatiles (GLVs) (Holopainen, 2004; Van den Boom *et al.,* 2004).

Inducible volatiles can be divided into two classes. The first class, the GLVs is released immediately (0-5 min) after mechanical damage to leaves (Fall *et al.,* 1999) while the second class, which consists mainly of terpenes, is synthesized after damage but only released a few hours after the initial damage took place (Dudareva *et al.,* 2004). The compounds are also released from damaged and undamaged leaves (Holopainen, 2004).

Of all these proposed signals, only methyl jasmonate is detectable in volatile collections from sagebrush, where the chemical induced the accumulation of proteinase inhibitor 1 to higher levels than what could be induced by wounding (Karban *et al.,* 2000). It was also found that control plants, which were in the same cabinet, but not sprayed with methyl jasmonate, accumulated low levels of proteinase inhibitor 1.

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 increase methyl jasmonate production by up to 16 times and can also change the isomeric conformation of methyl jasmonate to the biologically more active *cis* isomer. It was 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 (Bate and Rothstein, 1998). Jasmonic acid and methyl jasmonate are known to induce various aspects of biochemically based defences within the plant or in tissue cultures, but the volatility of the methyl ester potentiates aerial activity (Pickett and Poppy, 2001). 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).

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).

Maize seedlings damaged by beet armyworm caterpillars release a specific cocktail of volatile terpenoids and indole that is recognized by parasitic wasps (Turlings *et al.,* 1991). Volicitin (*N*-(17-hydroxylinolenoyl)-L-glutamine) present in the saliva of beet armyworm caterpillars has been identified as the major active elicitor for the formation of volatiles in maize (Alborn *et al.,* 1997). Recently two genes, *Igl* and S*tc1,* whose expression is specifically induced by volicitin, have been isolated from maize. *Igl* encodes an indole-3-glycerol-phosphate lyase (IGL) (Frey *et al.,* 2000) and S*tc1* encodes a sesquiterpene cyclase (Shen *et al.,* 2000). IGL cleaves indole-3-glycerol-phosphate to form indole and glyceraldehyde-3-phosphate (Frey *et al.,* 2000). Indole in turn is further metabolized to form two benzoxazinoids namely 2,4-dihydroxy-2H-1,4-benzoxazin-3(4H)-one (DIBOA) and it's

methoxy derivative 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA). Benzoxazinoids are natural pesticides found predominantly in the Poaceae and serve as important factors in host-plant resistance to microbial diseases and insects (Gierl and Frey, 2001).

Emission of volatiles from pathogen-infected plants may serve as a direct defence against pathogen infections. Several lipid-derived volatiles, including (*Z*)-3-hexenol and (*E*)-2-hexenal are released from *Phaseolus vulgaris* leaves during an HR response to *Pseudomonas syringae* pv. *phaseolicola* (Croft *et al.,* 1993). Both (*Z*)-3-hexenol and (*E*)-2-hexenal are bacteriocidal but at different concentrations (Croft *et al.,* 1993). Maize-derived volatile compounds, hexanal and octanal, strongly inhibit growth of the fungus *Aspergillus parasiticus* on culture media (Zeringue *et al*., 1996). Peanut plants infected with white mould, *Sclerotium rolfsii,* emitted a mixture of lipoxygenase products, terpenoids, indole and MeSA which were both quantitatively and qualitatively different from volatiles collected from healthy plants (Huang *et al.,* 2003). Among these volatiles, (Z)-3-hexenyl acetate, linalool and MeSA significantly inhibited fungal growth on solid culture media.

While some studies found no evidence for the transfer of information between damaged and undamaged plants (Preston *et al.,* 2001), many others presented evidence supporting the hypothesis of information exchange between damaged and undamaged plants (Dicke *et al.,* 1990; Karban *et al.,* 2000; Arimura *et al.,* 2001). 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 neighbours (Dicke and Bruin, 2001). Once evidence for plant-to-plant communication has been found, it becomes feasible to investigate to what extent plants are informed about local conditions and what strategies they can follow (Karban *et al.,* 1999).

2.7 Concluding Remarks

Like animals, plants have evolved an elaborate system to protect them against pathogen infections. Numerous attempts have been made in the past to identify and clone the receptors facilitating the detection of pathogenic intruders and several have been

successful. The challenge now is to identify all the interacting mechanisms and to clarify the role of each in the defence response.

The aim of this study was to investigate, on molecular level, some early events following the infection of wheat with leaf rust. An attempt was made to identify putative genes involved in these early events. Once these genes were identified, their relevance in the biochemical defence response was investigated.

Chapter 3

Cloning of Differentially Expressed cDNA Fragments from Leaf Rust-Infected Wheat

3.1 INTRODUCTION

In nature, plants are continuously challenged by fungi, bacteria, viruses and nematodes. Few of these are successful in infecting a potential host. However, the cultivation of crops leads to large areas of genetically identical plants, containing relatively few in-bred resistance genes. Due to the genetic uniformity of these crops, pathogens can overcome these genes over time. Important components of a plant's surveillance system against such pathogens include resistance (*R*) genes. *R*-gene products recognize pathogenic avirulence (*Avr)* gene products directly or indirectly. When recognized and bound by specific receptors, they activate defence pathways which in turn lead to the activation of the defence response. This leads to the activation of a diverse array of defence mechanisms inside the plant. When corresponding *R* and *Avr* genes are present, disease resistance occurs. If either one is inactive or absent, the result is disease (Flor, 1971).

Various defence responses are induced in infected plants to deter invading pathogens. Both endogenous and exogenous signal compounds, called elicitors, include amongst others, proteins, glycoproteins, oligosaccharides and lipids (Morris and Walker, 2003) which are crucial components of defence in plants.

This activation leads to immediate reactions directly at the site of infection. Included are the production of reactive oxygen species (ROS) and nitric oxide (NO) (Pastori and Foyer, 2002), the activation of the hypersensitive response (HR) (Johal *et al.,* 1995; Ladyzhenskaya and Protsenko, 2002), the accumulation of phenolics and other secondary metabolites as well as cell wall reinforcements (Bradley *et al.,* 1992). There are also local tissue responses such as the synthesis of pathogenesis-related (PR) proteins and the accumulation of salicylic acid (SA), ethylene and jasmonic acid (JA) (Stintzi *et al.,* 1993; Xu *et al.*, 1994). In addition to this, systemic responses that prime uninfected parts of the plant against the potential pathogen attack, are also activated (Ward *et al.,* 1991; Luan, 1998; Tang *et al.,* 1999; Sessa and Martin, 2000).

The most common response of resistant plants to cellular invasion by fungal pathogens is rapid cell death, which forms part of the HR. The death of cells in infected areas blocks the

pathogen's development and spread. The HR is, however, not an obligatory component of the plant's defence response (Heath, 1997).

In most plant/pathogen interactions, phosphorylation and dephosphorylation play key roles during the defence events (Xing *et al.,* 2002). Thus, several protein kinases are implicated to be involved during the defence response. Examples of such protein kinases include Lrk10 (Feuillet *et al.,* 1997), Xa21 (Song *et al.,* 1995) and Pto (Tang *et al.,* 1999). Since protein kinases are very conserved (Hanks *et al.,* 1988), degenerate primers specific for subdomain VIb could be used to clone protein kinase genes expressed during the early plant/pathogen interaction. Subdomain VIb is the most conserved subdomain of protein kinases in both monocot and dicot plants and differs by only two amino acids.

The aim of the study was therefore to clone and identify putative protein kinase genes using degenerate primers for subdomain VIb with the emphasis on genes expressed shortly after the infection of wheat with leaf rust.

3.2 Materials and Methods

3.2.1 Plant Material

Triticum aestivum cv Thatcher and line RL6058 (Thatcher*+Lr34*) was grown in a growth 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 (1 part nitrogen: 2 parts phosphorous: 3 parts potassium). Thereafter, fertilizer was administered weekly until plants started developing flag leaves.

3.2.2 Leaf Rust Inoculation

Adult Thatcher and Thatcher+*Lr34* plants were sprayed with fresh spores (approx 65000 spores/ml) of *Puccinia triticina* pathotype UVPrt9 that were suspended in distilled water containing a drop of Polyoxyethylene sorbitan monolaurate (Tween™ 20). Control plants were sprayed with distilled water containing Tween™ 20. The plants were left to dry and then placed in a dark dew-simulation cabinet at 18 to 20°C for 16 h to facilitate spore germination where after it was transferred to the greenhouse. Flag leaf material was collected at 3 h intervals for 15 h with the first time point taken directly after inoculation. All

samples were quick-frozen in liquid nitrogen and stored at -70°C. Tissue was ground to a fine powder in liquid nitrogen. Some of the infected plants were left to confirm the success of the infection.

3.2.3 Differential Display Reverse Transcription Polymerase Chain Reaction (DDRT-PCR)

Total RNA was extracted from infected wheat tissue according to the method of Chomczynski and Sacchi (1987). The quantity and quality of the extracted RNA was confirmed spectrophotometrically as described by Sambrook *et al.* (1989).

DDRT-PCR of mRNA was performed according to the method of Liang and Pardee (1992) with minor modifications. Total RNA (10 ng) from infected resistant plants was reverse transcribed and amplified using the Titan One-Tube RT-PCR system (Roche) in the presence of 2.5 µM of an anchored oligo-dT primer (Bovis 32) (Table 3.1), 2.5 µM of a degenerate subdomain VIb specific primer (Bovis 22 for monocot or Bovis 23 for dicot protein kinases respectively (Hanks *et al.,* 1988)), 2.5 µM of an anchor specific primer (Bovis 39), 25 µM deoxynucleotide triphosphates (dNTP's), 10 µCi [α-^{32}P]-deoxycytosinetriphosphate (dCTP) and 5 mM dithiothreitol (DTT). The amplification conditions were according to the Titan kit specifications, with the temperature for the reverse transcription (RT) step being 42°C and the PCR annealing temperature 45.3°C. A diagrammatical representation of the DDRT-PCR strategy is indicated in Figure 3.1. PCR products were analyzed on a 6% (w/v) non-denaturing Long Ranger™ (FMC Bioproducts) gel using 1x TBE (89 mM Tris pH 8.0, 89 mM boric acid, 2 mM EDTA) as running buffer. The gel was dried at 80°C for 2 h and exposed to radiographic film for 4 days.

Differentially expressed cDNA bands were cut from the gel, eluted in 200 µl water at 95°C for 15 min and re-amplified using the same conditions as described above. Re-amplified cDNA fragments were analyzed on 1% (w/v) agarose gels (Sambrook *et al.,* 1989) and fragments with the expected sizes were purified and cloned into the pGemT-Easy vector (Promega) according to the manufacturer's instructions. Plasmids containing inserts were then used in reverse Northern blots.

Chapter 3

Table 3.1: Names and sequences of primers used during DDRT-PCR. Bases indicated in bold show the difference between the monocot and dicot specific primers.

Primer name	Nucleotide sequence
Bovis 22	5' – GAY **A**TH AAR CCN **C**AY AAY – 3'
Bovis 23	5' – GAY **G**TN AAR CCN **G**AR AAY – 3'
Bovis 32	5' – GAA GAA TTC TCG AGC GGC CGC T_{19}VN – 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

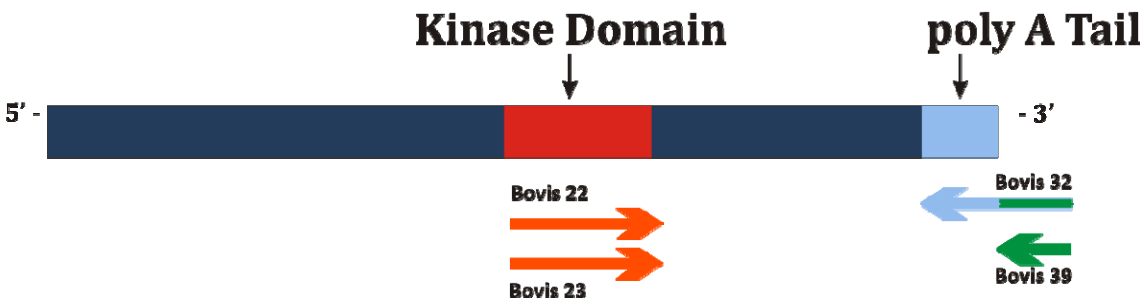

Figure 3.1: The amplification strategy used for the DDRT-PCR. While Bovis 32 was used for the reverse transcription step, Bovis 39 was used in combination with Bovis 22 or 23 for the PCR step.

3.2.4 Reverse Northern Blot Analysis

Nylon membranes were prepared by blotting purified recombinant plasmids (500 ng) onto the membranes (Hybond N^+, Amersham Biosciences) using a Hoefer slot blot system according to the manufacturers' instructions. As control, a cloned actin gene fragment was transferred onto the nylon membrane. These membranes were then used to perform a reverse Northern blot as described by Dilks *et al.* (2003). As probes, 10 µg total RNA isolated from leaf tissue at each time interval was reverse transcribed in the presence of 10 µCi [α-^{32}P]-dCTP using SuperScript™ III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. After hybridization, each individual hybridized clone was cut from the membrane, transferred to a sample vial and counted on a liquid scintillation counter using the Cherenkov channel. The expression levels of the individual cDNA fragments at the different time intervals were determined by first expressing the obtained values relative to that of actin and then the normalized values relative to time zero.

3.2.5 Sequencing of Cloned cDNA Fragments

cDNA fragments showing a marked increase in expression after infection were sequenced. Sequencing was done at Inqaba Biotech (South Africa). The resulting sequences were used to do a BLAST analysis on the NCBI website (http://www.ncbi.nlm.nih.gov/Genbank) to search for similarities to known genes.

3.3 Results

3.3.1 Leaf Rust Infection

Infection of wheat by *P. triticina* leads to leaf rust disease (Fig 3.2). The susceptible Thatcher cultivar showed brown-red pustules on the surface of the leaves one week after infection. The resistant *Lr34* cultivar on the other hand showed mainly necrotic flecks, indicative of an active defence response within the plants.

3.3.2 Identification of Differentially Expressed Genes

Following DDRT-PCR, a total of 29 differentially expressed cDNA fragments were cloned from leaf rust-infected resistant wheat (results not shown).

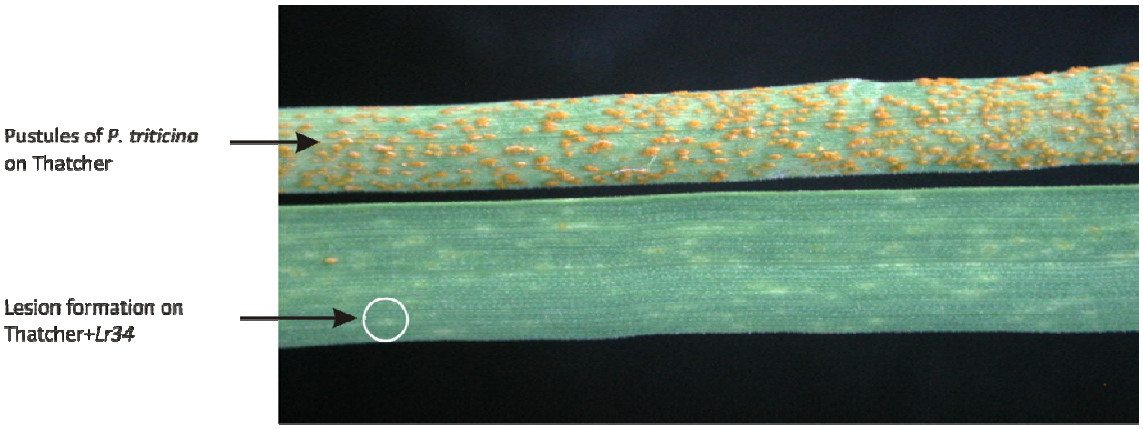

Figure 3.2: Flag leaves of adult Thatcher and Thatcher+*Lr34* plants one week after infection with *P. triticina.* The top leaf indicates infected susceptible Thatcher and the bottom leaf infected resistant Thatcher+*Lr34* leaves.

Of these, 20 were amplified using the monocot/anchor specific primer combination and nine using the dicot/anchor specific primer combination. The cloned inserts were the predicted sizes and were used in the reverse Northern blot.

3.3.3 Confirmation of Differential Gene Expression

To confirm that the cloned cDNA fragments were not artefacts of DDRT-PCR, but differentially expressed gene fragments, reverse Northern blots were performed (Fig 3.3). Four of the 29 cloned cDNA fragments showed a significant increase in expression, while one showed a dramatic decrease of expression in infected resistant (IR) plants. In contrast, none showed a change in expression in infected susceptible (IS) plants within the allocated time interval. The other 24 cDNA fragments showed very little variation in gene expression in both the IR and IS plants. They were consequently discarded and not studied any further. The cDNA fragments that showed differential expression were sequenced and compared with sequences included in GenBank.

Clone M8 showed a 3.6 fold induction in expression 6 hours post inoculation (h.p.i.). When sequenced, the cDNA insert was 625 bases in length (Fig 3.4). Two open reading frames coding for two polypeptides of 115 and 130 amino acids respectively, were found. On amino acid level, only the second polypeptide shared sequence homology to an *Oryza sativa* monosaccharide transporter 3 (GenBank accession nr NP919214.1, $E=7e^{-63}$). The polypeptide also contained a pfam00083 conserved sugar transporter domain.

Clone M9 showed a 1.5 fold increase in expression 3 h.p.i. and had a nucleotide sequence 832 bp in length (Fig 3.5). Its longest open reading frame coded for a polypeptide of 222 amino acids. On amino acid level it shared sequence similarity with an *O. sativa* cell wall invertase 1 (GenBank accession nr AY342319, $E = 2e^{-59}$).

Clone M27 showed a 58 fold increase in expression 9 h.p.i. and had a nucleotide sequence of 236 base pairs (Fig 3.6). The longest open reading frame of 12 amino acids contained a conserved pfam00012 heat shock domain. On nucleotide level it shared sequence similarity to an *O. sativa hsp70* gene for heat shock protein (Hsp) 70 (GenBank accession nr X67711, $E = 1e^{-09}$). This region of homology was just upstream of the proposed stop codon, while the

proposed 3' untranslated region downstream of the stop codon differed substantially. Unfortunately the encoded polypeptide was too short to show any real similarity.

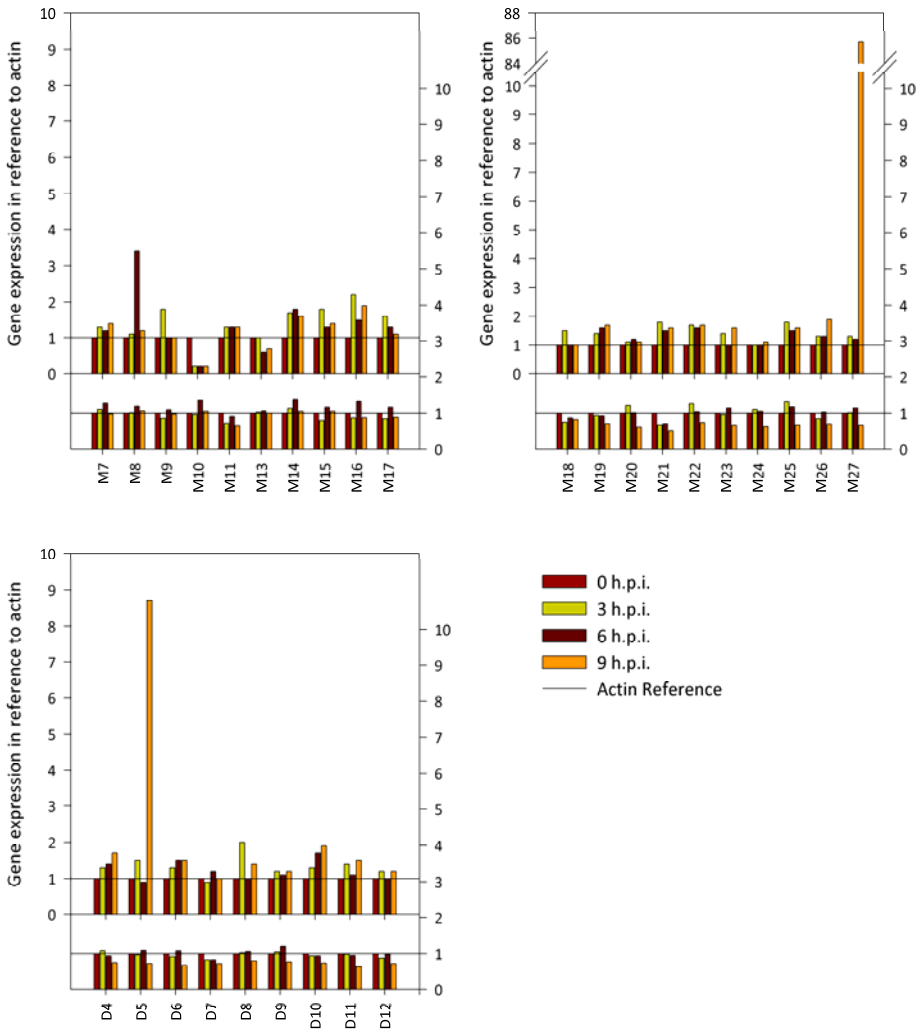

Figure 3.3: Reverse Northern blot analysis of cloned cDNA fragments. The expression of each cDNA fragment was expressed relative to a constitutively expressed actin gene fragment. Actin expression is represented by 1 and indicated with a solid line across the graph. The expression of each fragment in infected resistant (top panel) and infected susceptible wheat (bottom panel) is indicated in each graph.

a. G G G G A L H L P L C G R V R V V V G A P G V A G A Q R D L P A G D Q A G G A E H Q R V G
E H A L H L R H R A G V P H H A L P H E V R P L L L L R R L G G D H D R L H R A L P A G D Q E
R A H R G D G A R L E G T L V L A Q V H R R R **Stop**

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

```
c. MST3        MAGGAVVSTGAGKDYPGKLTLFVFFTCVVAATGGLIFGYDIGISGGVTSMDPFLRKFFPE
   M8          ------------------------------------------------------------

   MST3        VYRKKQMADKNNQYCKYDNQLLQTFTSSLYLAALVSSFFAATVTRVLGRKWSMFAGGLTF
   M8          ------------------------------------------------------------

   MST3        LIGAALNGAAENVAMLIVGRILLGVGVGFANQSVPVYLSEMAPARLRGMLNIGFQLMITI
   M8          ------------------------------------------------------------

   MST3        GILAAELINYGTAKIKAGWGWRVSLALAAVPAAIITLGSLFLPDTPNSLIDRGHPEAAER
   M8          ------------------------------------------------------------

   MST3        MLRRIRGSDVDVSEEYADLVAASEESKLVQHPWRNILRRKYRAQLTMAICIPFFQQLTGI
   M8          ------------------------------------------------------------

   MST3        NVIMFYAPVLFDTLGFKSDASLMSAVITGLVNVFATLVSIFTVDRLGRRKLFLQGGAQMV
   M8          ------------------------------------------------------------

   MST3        VCQVVVGTLIAVKFGTSGIGDIPKGYAAVVVLFICMYVAGFAWSWGPLGWLVPSEIFPLE
   M8          ---------------------------AAVVLFICLYVAGFAWSWGPLGWLVPSEIFPLE
                                           *.******:************************

   MST3        IRPAGQSINVSVNMLFTFVIAQAFLTMLCHMKFGLFYFFAGWVVIMTVFIALFLPETKNV
   M8          IRPAGQSINVSVNMLFTFVIAQAFLTMLCHMKFGLFYFFAGWVVIMTVFIALFLPETKNV
               ************************************************************

   MST3        PIEEMVLVWKSHWFWRRFIGDHDVHVGANHVSNNKLQP
   M8          PIEEMVLVWKGHWFWRRYIGDADVHVGANNGKGAAIA-
               **********.******:*** *******: ..  :
```

Figure 3.4: Sequence analysis of clone M8. In a + b the two longest open reading frames are indicated. In c, the longest polypeptide is aligned with Monosaccharide transporter 3 from *O. sativa* (GenBank accession nr NP 919214.1). Consensus sequences are indicated with an (*) while (:) indicates conservative substitutions and (.) semiconservative substitutions.

a.

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

b.

```
CIN1        MGTRLLALAPWLLLLLLQLAGASHVVHRSLEAEQAPSSVPASIVSPLLRTGYHFQPPMNW
M9          ------------------------------------------------------------

CIN1        INDPNGPLYYKGWYHLFYQYNPKGAVWGNIVWAHSVSQDLINWIALEPAIKPDIPSDQYG
M9          ------------------------------------------------------------

CIN1        CWSGSATILPDGTPAILYTGIDRPNINYQVQNIAFPKNASDPLLREWVKPAYNPVATPEP
M9          ------------------------------------------------------------

CIN1        GMNATQFRDPTTAWYADGHWRMLVGGLKGARLGLAYLYRSRDFKTWVRAKHPLHSALTGM
M9          ------------------------------------------------------------

CIN1        WECPDFFPLQAPGLQAGLDTSVPSSKYVLKNSLDLTRYDYYTVGIYNKVTERYVPDNPAG
M9          ------------------------------------------------------------

CIN1        DYHRLRYDYGNFYASKTFFDPVKHRRILLGWANESDSVTYDKAKGWAGIHAIPRKVWLDP
M9          -------------------------------------------EEFS-------------
                                                        : ::

CIN1        SGKQLLQWPIEELETLRGKSVSVFDKVVKPGEHFQVTGLGTYQADVEVSLEVSGLEKAEA
M9          SGKQLLQWPVEELDQLRGKAVSVGDKVVKPGQHFEVTGLQSYQSDVEVSFEVPSLDKAEP
            *********:***: ****:*** *******:**:**** :**:*****:**..*:***.

CIN1        LDPAFGDDAERLCGAKGADVRGGV-VFGLWVLASAGLEEKTAVFFRVFKPAGHGAKPVVL
M9          FDPAYANDAQKLCGMKNADVKGGVGPFGLWVLASSNLAEKTAVFFRVFK-DGHG-KPLVL
            :***:.:**::*** *.***:***  ********:.* ***********  *** **:**

CIN1        MCTDPTKSSLSPDLYKPTFAGFVDTDISSGKISLRSLIDRSVVESFGAGGKTCILSRVYP
M9          MCSDPTKSSLTPGLYKPTFAGFVDTDISSGKISLRSLIDRSVAESFGAGGKTCILSRVYP
            **:*******:*.*****************************.*****************

CIN1        SMAIGDKAHLYVFNNGEADIKISHLKAWEMKKPLMNGA
M9          SMAIGTDAHLYVFNNGDTDIKVSKLTAWEMKKPMMNGA
            ***** .*********::***:*:*.*******:****
```

Figure 3.5: Sequence analysis of clone M9. In a, the longest open reading frame is indicated. In b, the polypeptide is aligned with Cell wall invertase 1 from *O. sativa* (GenBank accession nr AY342319). Consensus sequences are indicated with an (*) while (:) indicates conservative substitutions and (.) semiconservative substitutions.

a. S G G A G P K I E E V D **Stop**

b.

```
M27         ------------------------------------------------------------
X67711      GGCTGGACGGCAACCAGCTCGCTGAGGCTGATGAGTTCGATGACAAGATGAAGGAGCTGG

M27         ------------------------------------------------------------
X67711      AGGGCATCTGCAACCCCATCATCGCCAAGATGTACCAGGGCGCTGGCGCGGACATGGCCG

M27         -----------------------------------GCGGCGGTGCTGGCCCCAAGATCG
X67711      GCGGCATGGACGAGGACGATGCTCCCCCGGCTGGCGGCAGCGGTGCTGGCCCCAAGATCG
                                               ** ********************

M27         AGGAGGTCGACTAAGTTAGTCGCTT--TTATATAGTGAGAAATACGCTATACACCATTTT
X67711      AGGAGGTCGACTAAGCGCCAAATTTGGTTAAAAACTTGGGCATGAGTTCGAAACCTACAG
            ***************          **  *** * * *  *  **  * *  * ***

M27         ATTCAGTCCTGGGTGCCAATCATATCACCTGGGCTGGAAGTTTTGTTGTACTTTTTGGTC
X67711      ATTTGGGGCTGAACTTTGGTTAGGTGATCCGCGCTTCAAGTTATCTTAT-----TGCATT
            ***  *  ***         * *  * * * * * ***  ***** * ** *     *   *

M27         TGTAGCATCACGAGACTTGTGTGTGTCGTGAACAATGCAATGATGCA-TGTTTTGGCTCA
X67711      ATCAGTGTCTCTTTATTAGT-TGTGTTAAAAACTTGGGGATTATGTGGTGCTATGGTACC
               **  ** *   * * ** *****    ***   *  ** ***   ** * ***  *

M27         TTGTTGATTTCAGCTTAATGCTTATTATTTG--------------------
X67711      TT-TTGGTCT--GTTTGG-GCAGTCTATTTGAAGAACTTCGTTGGGAGCTC
            ** *** * *  * **   **    ******
```

Figure 3.6: Sequence analysis of clone M27. In a, the longest open reading frame is indicated. In b, the nucleotide alignment of M27 with the gene encoding the Heat shock protein 70 from *O. sativa* (GenBank accession nr X67711) is shown. Consensus sequences are indicated with an asterisk. The putative stop codon is indicated in bold.

Clone D5 showed an eight fold increase in expression within 9 h.p.i. and had a sequence of 801 bases with the longest open reading frame coding for a polypeptide of 170 amino acids (Fig 3.7). On amino acid level it shared sequence similarity with a *Hordeum lechleri* indole-3-glycerol phosphate lyase (GenBank accession nr AY462226, E = $2e^{-43}$).

Despite several attempts to sequence the cloned M10 cDNA insert, no reliable sequence information was obtained. This fragment was therefore also discarded.

3.4 DISCUSSION

DDRT-PCR was used to identify differentially expressed genes from resistant Thatcher+*Lr34* wheat infected with leaf rust using primers specific for subdomain VIb of protein kinases.

Twenty nine cDNA fragments that were putatively differentially expressed within the first 15 hours after inoculation were cloned. In order to eliminate false positives generated during the differential display, a reverse Northern blot was performed. Of the 29 cloned cDNA fragments, only three showed markedly higher gene expression compared to time zero. These three, and a fourth that showed only a moderate increase in expression level, were sequenced. The high number of false positives is similar to that of other DDRT-PCR studies (Dilks *et al.,* 2003).

Clone M9 showed sequence similarity to an *O. sativa* cell wall invertase 1. Extracellular invertase is a cell-wall-bound enzyme that catalyses the irreversible cleavage of sucrose released into the apoplast via sucrose transporters. The resulting hexose monomers are then imported into the sink cell by monosaccharide transporters (Hall and Williams, 2000; Roitsch *et al.,* 2003).

Infection of plants by pathogens may result in a general increase in apoplastic glucose levels due to the activity of the cell wall invertases which catalyze the cleavage of sucrose to glucose and fructose (Hall and Williams, 2000). There is also evidence that glucose may play a key role in biotrophic pathogen/plant interactions since glucose appears to be the major carbon energy source transferred from the host to cereal and pea powdery mildew (Mendgen and Nass, 1988) although the route and mechanisms by which this occurs are not

clear.

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

b.

```
IGL         GTRAPVPVPVAAGDRGLSVSQAMSKVMEKGKTAFIPYITAGDPDLATTAAALRLLDALGA
D5          ----------------------------------------------------LRILE----
                                                                 **:*:

IGL         DVVELGMPFSDASADGAVIKASAARALAAGATVDAIMAMLKEVTPELSCPVVIFSYFSPI
D5          ------------------------------------------------------------

IGL         AQRGTASFAAAVKEAGVKGLIVPDLPYAETSAFRDEAIKNELELVLLTTPSTPPERMKEI
D5          -RRGTGSFTAAAREAGVRGLIVPDLPYTEASVLNIEAKKSEIELVLLTTPTTKAERMNEI
             :***.**:**.:****:*********:*:*.:. ** *.*:********:*  .***:**

IGL         TEASGGFVYLVSVDGVRGARATVNPRVESLLKKIKQVTDKAVAVGFGISTPDHVKQIAEW
D5          TSASEGFVYLVSINGLTGARPSVNPHVKDLLREIKQATDKAVAVGFGISTPDHVRQVAQW
            *.** *******::*: ***.:***:*:.**::***.*****************:*:*:*

IGL         GADGVIIGSAMVKQLGEAASPEEGLKRLEVYARSLKDALP------
D5          GADGVIIGSAMVKQLGEANSPREGLKRLEVYARSLKDALHAVICTI
            ****************** **.****************
```

Figure 3.7: Sequence analysis of clone D5. In a, the longest open reading frame is indicated. In b, the putative polypeptide is aligned with Indole-3-glycerol phosphate lyase from *Hordeum lechleri* (GenBank accession nr AY462226). Consensus sequences are indicated with an (*) while (:) indicates conservative substitutions and (.) semiconservative substitutions.

3.5

The second clone, M8 showed sequence similarity to an *O. sativa* monosaccharide transporter 3. Monosaccharide transporters normally play a role in the uptake of sucrose from the phloem or apoplast into sink cells after the hydrolysis of these monosaccharides by cell wall invertases (Büttner and Sauer, 2001).

Increased transport activity may be due to enhanced activity of existing transporters or synthesis of new transporters possibly in the epidermal cells, thereby increasing the level of glucose available to the invading fungal haustoria. Alternatively, the increased activity levels may be a retrieval mechanism of the plant to support the increased demand for carbon in defence mechanisms (Fotopoulos *et al.,* 2003). The fact that these two genes showed differential expression, indicate the possibility of sugar defence during the defence response (Fotopoulos *et al.,* 2003).

Clone M27 had sequence similarity to an *O. sativa* Hsp70 gene (OSHSC70A) even though the sequenced fragment was very short. Heat shock protein 70 is a molecular chaperone and is constitutively expressed during normal cellular conditions, but is inducibly expressed under stress conditions, especially thermal and oxidative stresses (Wang *et al.,* 2004). Hsp70 normally functions by preventing aggregation and assist with the refolding of non-native proteins. Hsps are also involved in protein import and translocation (Wang *et al.,* 2004). Unfortunately, information about Hsp70 function in plants is limited, but they are highly conserved between plants and mammals, so their function could be similar. Hsp70 in mammals is involved in the modulation of signal transducers such as protein kinase A, protein kinase C and protein phosphatase (Ding *et al.,* 1998). In this respect, the Hsp70 chaperones might play a role by participating in modulating the expression of many downstream genes in signal transduction pathways both during stress and under normal conditions.

Clone D5 shared sequence similarity with a *Hordeum lechleri* indole-3-glycerol phosphate lyase (IGL). Frey *et al.* (2000) have demonstrated that maize seedlings damaged by beet armyworm caterpillars release a specific blend of terpenoids and indole that is attractive to *Cortesia marginiventris*, a parasitic wasp that attacks larvae of several species of Lepidoptera. Volicitin [*N*-(17-hydroxylinolenoyl)-L-glutamine] was identified as the major

active elicitor in the oral secretion of these larvae. IGL catalyzes the formation of free indole and is selectively activated by volicitin. IGL's enzymatic properties are similar to BX1, a maize enzyme that serves as the entry point to the secondary defence metabolites 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIBOA) and 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA). *Igl* and *Bx1* are evolutionarily related to the tryptophan synthase alpha subunit gene (Gierl and Frey, 2001).

Even though the original aim was to clone and identify protein kinase genes involved in the wheat/leaf rust interaction, none of the identified clones were protein kinases. The highly conserved subdomain VIb of protein kinases encodes a serine/threonine phosphorylation region and thus primers designed for subdomain VIb bound randomly to serine/threonine phosphorylation sites. The result was that any induced gene that contained a serine/threonine phosphorylation site was amplified.

To conclude, four differentially expressed cDNA fragments were cloned and sequenced. All four share homology with genes that were previously shown to be involved in plant defence. Clones M8 and M9 will be the subject of another study to determine whether a sugar signal transduction event is active during leaf rust infection. During this current study, the putative role of clone M27 during the response of resistant wheat infected with leaf rust will be investigated. Of special interest will be to investigate whether this gene is selectively linked to the resistance response of Thatcher+*Lr34* during leaf rust infection. In addition the cloning of IGL indicates a possible volatile defence event between the wheat plants. Both these aspects will be covered in this study.

3.5 REFERENCES

Bradley D., Kjellbom P. and Lamb C. 1992. **Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defence response.** *Cell* 70: 21-30

Büttner M. and Sauer N. 2001. **Monosaccharide transporters in plants: structure, function and physiology.** *Biochimica et Biophysica Acta* 1465: 263-274

Chomczynski P. and Sacchi N. 1987. **Single step method of RNA isolation by acid Guanidine thiocyanate – Phenol Chloroform extraction.** *Analytical Biochemistry* 162: 156-159

Dilks D.W., Ring R.H., Khawaja X.Z., Novak T.J. and Aston C. 2003. **High-throughput confirmation of differential display PCR results using reverse Northern blotting.** *Journal of Neuroscience Methods* 123: 47-54

Ding X.Z., Tsokos G.C. and Kiang J.G. 1998. **Overexpression of HSP-70 inhibits the phosphorylation of HSF1 by inactivating protein phosphatase and inhibiting protein kinase C activity.** *FASEB Journal* 12: 451-459

Feuillet C., Shachermayr G. and Keller B. 1997. **Molecular cloning of a new receptor-like kinase gene encoded at the Lr10 disease resistance locus of wheat.** *The Plant Journal* 11: 45-52

Flor H.H. 1971. **Current status of the gene-for-gene concept.** *Annual Review of Phytopathology* 9: 275-296

Fotopoulos V., Gilbert M.J., Pittman J.K., Marvier A.C., Buchanan A.J., Sauer S., Hall J.L. and Williams L. 2003. **The monosaccharide transporter gene, *AtSTP4*, and the cell-wall invertase, *At_fruct1*, are induced in *Arabidopsis* during infection with the fungal biotroph *Erysiphe cichoracearum*.** *Plant Physiology* 132: 821-829

Frey M., Stettner C., Pare P.W., Schmelz E.A., Tumlinson J.H. and Gierl A. 2000. **An herbivore elicitor activates the gene for indole emission in maize.** *Proceedings of the National Academy of Sciences USA* 97: 14801–14806

Gierl A. and Frey M. 2001. **Evolution of benzoxazinone biosynthesis and indole production in maize.** *Planta* 213: 493-498

Hall J.L. and Williams L.E. 2000. **Assimilate transport and partitioning in fungal biotrophic interactions**. *Australian Journal of Plant Physiology* 27**:** 549–560

Hanks S.K., Quinn A.M. and Hunter T. 1988. **The protein kinase family: conserved features and deduced phylogeny of the catalytic domains.** *Science* 241: 42-52

Heath M. 1997. **Signalling between pathogenic rust fungi and resistant or susceptible host plants.** *Annals of Botany* 80: 713-720

Johal G.S., Gray J., Gruis D. and Briggs S.P. 1995. **Convergent insights into mechanisms determining disease and resistance response in plant-fungal interactions.** *Canadian Journal of Botany* 73: S468-S474

Ladyzhenskaya E.P. and Protsenko M.A. 2002. **Plasma membrane-bound mechanisms of signal transduction in the control of plant dormancy and resistance.** *Biochemistry Moscow* 67: 151-160

Liang P. and Pardee A.B. 1992. **Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction.** *Science* 257: 967-971

Luan S. 1998. **Protein phosphatases and defence cascades in higher plants.** *Trends in Plant Science* 3: 271-275

Mendgen K. and Nass P. 1988. **The activity of powdery-mildew haustoria after feeding the host cells with different sugars, as measured by potentiometric cyanine dye**. *Planta* 174**:** 283–288

Morris E.R. and Walker J.C. 2003. **Receptor-like protein kinases: the keys to response.** *Current Opinion in Plant Biology* 6: 1-4

Pastori G.M. and Foyer C.H. 2002. **Common components, networks and pathways of cross-tolerance to stress. The central role of "Redox" and Abscisic acid-mediated controls.** *Plant Physiology* 129: 460-468

Roitsch T., Balibrea M.E., Hofmann M., Proels R. and Sinha A.K. 2003. **Extracellular invertase: key metabolic enzyme and PR protein.** *Journal of Experimental Botany* 54: 513-524

Sambrook J., Fritsch E.F. and Maniatis T. 1989. **Molecular cloning: a laboratory manual.** 2nd ed. USA: *Cold Spring Harbour press*

Sessa G. and Martin G.B. 2000. **Protein kinases in the plant defence response.** *In Advances in Botanical Research Vol. 32,* Academic Press, 379-404

Song W-Y., Wang G-L. and Chen L-L. 1995. **A receptor kinase-like protein encoded by the rice disease resistance gene, *Xa-21.*** *Science* 270: 1804-1806

Stintzi A., Heitz T. and Prasad V. 1993. **Plant 'pathogenesis-related' proteins and their role in defence against pathogens.** *Biochimie* 75: 687-706

Tang X., Xie M., Kim Y.J., Zhou J., Klessig D.F. and Martin G.B. 1999. **Overexpression of *Pto* activates defence responses and confers broad resistance.** *The Plant Cell* 11: 15-29

Wang W., Vinocur B., Shoseyov O. and Altman A. 2004. **Role of heat-shock proteins and molecular chaperones in the abiotic stress response.** *Trends in Plant Science* 9: 244-252

Ward E.R., Uknes S.J., Williams S.C., Dincher S.S., Wiederhold D.L., Alexander D.C., Ahl-Goy P., Métraux J-P. and Ryals J.A. 1991. **Coordinate gene activity in response to agents that induce systemic acquired resistance.** *The Plant Cell* 3: 1085-1094

Xing T., Quellet Th-R-S.E. and Miki B.L. 2002. **Towards genomic and proteomic studies of protein phosphorylation in plant-pathogen interactions.** *Trends in Plant Science* 7: 224-230

Xu Y., Chang P.F.L., Liu D., Narasimhan M.L., Raghothama K.G., Hasegawa P.M. and Bressan R.A. 1994. **Plant defence genes are synergistically induced by ethylene and methyl jasmonate**. *The Plant Cell* 6: 1077-1085

Chapter 4

Molecular characterization of *TaHlp01,* a putative heat shock-like protein in wheat induced upon infection with leaf rust

4.1 INTRODUCTION

Plants need to defend themselves against attacks from viruses, microbes, invertebrates and even other plants. Because plants lack a circulatory system, each plant cell must possess preformed and/or inducible defence capabilities (Thatcher *et al.,* 2005). In higher plants, the systemic induction of defence responses by mechanical, herbivorous and pathogenic damage is well characterized (Martin *et al.,* 2003). These responses are elicited by a variety of defence molecules including jasmonic acid (JA) (Turner *et al.,* 2002) and salicylic acid (SA) (Klessig *et al.,* 2000).

Attempted infection by avirulent pathogens elicits the activation of a battery of defences in resistant plants that are often accompanied by the collapse of challenged plant cells during the hypersensitive response (HR) (Staskawicz *et al.,* 1995; Ren *et al.,* 2006). The HR is the result of ligand/receptor interactions specified by paired plant resistance (*R*) and pathogenic avirulence (*Avr*) genes. This results in a restricted lesion at the site of attack, clearly delimited from surrounding healthy tissue. The death of the infected cell prevents the pathogen from spreading further within the plant. Systemic acquired resistance (SAR) then gradually develops throughout the rest of the plant, providing resistance to subsequent attacks by virulent pathogens (Raskin, 1992; Ryals *et al.,* 1994).

Heat shock proteins (Hsps) are a group of highly conserved proteins induced in pro- and eukaryotes in response to elevated temperatures and a variety of other stresses, including oxidative stress (Boston *et al.,* 1996; Bukau and Horwich, 1998). Hsps thus appear to be general stress proteins that are involved in maintaining cell function and survival during stress or facilitating recovery from stress (Fernandez *et al.,* 2004; Kanzaki *et al*., 2003; Nürnberger and Lipka, 2005). Amongst the most prominent Hsps (28, 60, 70, 90 and 110 kDa), the 70 kDa species appears as a doublet (approximately 72 and 73 kDa) consisting of Hsp70 (a major inducible homologue in most organisms) and Hsc70 (cognate or constitutive), respectively. While both of these Hsp70s are cytoplasmic, other Hsp70 family members are found in every major sub-cellular compartment of eukaryotic cells (Henics, 2003). A primary amino acid sequence homology of over 60% identifies these proteins as some of the most conserved gene products (Hunt and Morimoto, 1985).

The Hsp70 class of proteins is believed to act as molecular chaperones and is found in the cytosol and most organelles (Guy and Li, 1998). Constitutively expressed Hsp70s assist in protein folding and translocation (Dobson, 2003; Mayer and Bukau, 2005). During heat stress, Hsp70s may be involved in the refolding of denatured proteins or protecting proteins from stress-induced damage (Larkindale and Knight 2002; Nollen and Morimoto, 2002). In non-stressed cells, Hsps are present in low concentrations, while in stressed cells they accumulate to high levels. In *Escherichia coli*, for example, the Hsp60 homolog GroEL, which was first described by Hendrix (1979), represents 1 to 2% of the total protein content under normal conditions. Under stress conditions, however, its concentration is increased four to five fold (Shinnick, 1991). Although Hsp70s do not accumulate to such high levels in eukaryotes, their concentration is also increased after heat shock (Schett *et al.,* 2003).

There is evidence that oxidative stress in plants induces the expression of Hsps and that it provides a protective function (Grene, 2002; Larkindale and Knight, 2002). In tomato and rice, both mitochondrial Hsp22 and chloroplastic Hsp26 expression are induced by H_2O_2 (Banzet *et al*., 1998; Lee *et al*., 2000)**.**

The aim of this study was to characterize a gene fragment named *Triticum aestivum Heat shock-like protein 01 (TaHlp01)* that was identified using DDRT-PCR from leaf rust infected wheat, describe its expression pattern and propose a possible role for the protein in the defence response.

4.2 Materials and Methods

4.2.1 Biological Material

Triticum aestivum seed was germinated and grown in pots containing a sterilized 1:1 soil: peat mixture in a glasshouse at 25^{o}C with a 16 h day/8 h night cycle. For leaf rust infection studies, Thatcher and Thatcher*+Lr34* cultivars were grown to flag leaf stage. For the other tests, Thatcher*+Lr34* plants were grown to 3 leaf stage in a growth cabinet where cool-white fluorescent tubes emitting 120 µE.m^{-2}.s^{-1} light, provided a 16 h day length. For the Southern blot analysis, Thatcher+*Lr1*, *Lr2a*, *Lr3a*, *Lr3ka*, *Lr10*, *Lr15*, *Lr16*, *Lr26* and *Lr34* cultivars were grown to 3 leaf stage. For the yellow rust infections, Avoset R and Avoset S wheat cultivars were grown until 3 leaf stage in the glasshouse before infection.

Puccinia triticina pathotype UVPrt9 was multiplied on susceptible Karee seedlings while *P. striiformis* pathotype 6E22 was multiplied on Avoset S seedlings. Freshly collected spores were used for inoculation purposes.

4.2.2 Rust Inoculation Of Wheat

Adult Thatcher and Thatcher+*Lr34* plants were sprayed with fresh *P. triticina* spores (approx 65000 spores/ml) that were suspended in distilled water containing a drop of Tween™ 20. The plants were left to dry and then placed in a dark dew-simulation cabinet at 18 to 20°C for 16 h to facilitate spore germination, where after it was transferred to the greenhouse. Flag leaf material was collected at 3 h intervals for 36 h with the first time point being taken directly after inoculation.

Avoset R and Avoset S seedlings were inoculated with fresh *P. striiformis* spores that were suspended in kerosene oil. Plants were left to dry for approximately 2 h at room temperature where after cool sterile water was sprayed on the plants to simulate high humidity conditions. Finally, the plants were incubated for 48 h in the dark at 8°C under high humidity to allow infection to occur. Plant tissue was collected in 6 h intervals for 72 h starting immediately after infection. All samples were quick-frozen in liquid nitrogen and stored at -70°C. Frozen tissue was ground to a fine powder in liquid nitrogen.

4.2.3 Differential Display of Leaf Rust Infected Wheat RNA

Total RNA was extracted from infected wheat tissue according to the method of Chomczynski and Sacchi (1987). The quantity and quality of the extracted RNA was confirmed spectrophotometrically as described by Sambrook *et al.* (1989).

Differential display reverse transcription PCR (DDRT-PCR) of mRNA was performed according to the method of Liang and Pardee (1992) with minor modifications. Total RNA (10 ng) from infected resistant plants was reverse transcribed and amplified using the Titan One-Tube RT-PCR system (Roche) in the presence of 2.5 µM of an anchored oligo-dT primer (5' – GAA GAA TTC TCG AGC GGC CGC T_{19}VN – 3'), 2.5 µM of a degenerate kinase subdomain VIb specific primer (5' – GAY ATH AAR CCN CAY AAY – 3'), 2.5 µM of an anchor specific primer (5' – GAA GAA TTC TCG AGC GGC – 3'), 25 µM dNTP's, 10 µCi [α-^{32}P]-dCTP and 5 mM DTT. The amplification conditions were according to the Titan kit specifications, with the

temperature for the reverse transcription (RT) step being 42^{o}C and the PCR annealing temperature 45.3^{o}C. PCR products were analyzed on a 6% (w/v) non-denaturing Long Ranger™ (FMC Bioproducts) gel using 1x TBE (89 mM Tris-HCl pH 8.0, 89 mM boric acid, 2 mM EDTA) as running buffer. The gel was dried at 80^{o}C for 2 h and exposed to radiographic films for 4 days.

Differentially expressed cDNA bands were cut from the gel and eluted in 200 µl water at 95^{o}C for 15 min and re-amplified using the same conditions as described above. Re-amplified cDNA fragments were analyzed on 1% (w/v) agarose gels (Sambrook *et al.,* 1989). Fragments with the expected sizes were purified and cloned into the pGemT-Easy vector (Promega) according to the manufacturer's instructions.

4.2.4 Sequencing and DNA Analysis

Sequencing was done at Inqaba Biotech (South Africa). The resulting DNA sequences were used to do a BLAST search on both the NCBI (http://www.ncbi.nlm.nih.gov/Genbank) and the GrainGenes websites (http://wheat.pw.usda.gov/GG2) to search for similarities to known genes. Where applicable, overlapping expressed sequence tags (ESTs) were combined and used to search for known genes or contigs. A motif and pattern scan was performed on the amino acid sequences according to Falquet *et al*. (2002) on the PROSITE database.

4.2.5 Southern and Northern Blot Analysis

For Southern blots, genomic DNA was extracted from wheat leaves as described by Sambrook *et al*. (1989) and 20 µg aliquots were digested with *Eco*R1 or *Bam*H1 respectively. The digested DNA was then separated on a 0.8% (w/v) agarose gel (Sambrook *et al*., 1989). For the Northern blot, total RNA was extracted from wheat leaves (4.2.3) and 20 µg total RNA was separated on 1% (w/v) formaldehyde containing agarose gel (Sambrook *et al*., 1989). The digested DNA or RNA was transferred onto a Hybond N^{+} nylon membrane (GE Health Sciences) by downward capillary action as described by Chomczynski and Mackey (1994). As probe, a [α-^{32}P] dCTP-labelled, PCR amplified 236 bp *TaHlp01* (previously M27) fragment was used. Hybridization was performed as described by Chomczynski (1992) but

the membranes were exposed to a phosphor storage screen (Kodak) for 1 day. The screens were scanned on a Personal Molecular Imager (Bio-Rad).

4.2.6 Expression Analysis Using RT-PCR

RT-PCR assays was performed with 10 ng total RNA using the RobusT II RT-PCR kit (Finnzymes) in the presence of 2.5 µM sequence specific primer, 25 µM dNTP's and 1.5 mM $MgCl_2$. The amplification conditions were according to the RobusT kit specifications, with the temperature for the RT-step being 48^{o}C while the PCR reaction consisted of 94^{o}C for 2 min, 30 cycles of 94^{o}C for 30 sec, annealing temperature based on the particular primer combination for 30 sec, 72^{o}C for 3 minutes, followed by a final extension step of 72^{o}C for 5 min. PCR products were analyzed on a 1% (w/v) agarose gel using 0.5x TAE (20 mM Tris pH 8.0, 0.5 mM EDTA and 0.28% (v/v) acetic acid) as running buffer (Sambrook *et al.,* 1989).

To test the expression of *TaHlp01*, specific primers (5'- CAG CAG CTG CGC AAA GGC TC -3' and 5'- TCA CAC GGG TAG GGT AGT CC -3'; 2200 bp product) with an annealing temperature of 58^{o}C were used. These primers were designed to amplify the coding region of *TaHlp01* after Blast analysis revealed it's similarity to a known contig. To confirm that equal amounts of total RNA were used for the RT-PCR reactions, the 18S rDNA gene was amplified using specific primers (5'- CAA CTT TCG ATG GTA GGA TAG -3' and 5'- CTC GTT AAG GGA TTT AGA TTG -3'; 226 bp product) with an annealing temperature of 58^{o}C. Gradient PCR's were first performed with both primer sets to obtain the optimum annealing temperature.

4.2.7 Western Blot Analysis

Total cellular protein was extracted from the ground tissue in an extraction buffer (50 mM Tris-HCl pH 7.5, 2.0 mM EDTA, 2.0 mM PMSF, 10 mM β-mercaptoethanol). After centrifugation at 10000 *g* for 10 min at 4^{o}C, the concentration was determined according to Bradford (1976) on a microplate reader (Bio-Rad Model 3550) according to the method described by Rybutt and Parish (1982).

Twenty microgram total cellular protein was separated on a 12% (w/v) SDS-Page gel according to Laemmli (1970) for 1 h at 150 V and constant current. The gels were either stained with Coomassie (0.1% (w/v) Coomassie Blue G-250, 10% (v/v) acetic acid, 50% (v/v) methanol) or transferred to PVDF membranes (GE Health Sciences) using a Mini Trans-Blot

cell (Bio-Rad) for 1 h at 350 mA according to the manufacturer's specifications. The membranes were probed with a rabbit-anti-HSP70/HSC70 polyclonal antibody (StressGen Biotechnologies), diluted 1:10000 in TBST (Burnett, 1981). A 1:3000 diluted alkaline phosphatase conjugated goat-anti-rabbit-IgG (Sigma) was used as secondary antibody. Detection was performed by epitope staining as described by Blake *et al.* (1984).

4.2.8 Chemical Treatments of Wheat

Thatcher+*Lr34* seedlings were sprayed with 20 mM hydrogen peroxide (H_2O_2) (Neill *et al.,* 2002), 100 µM methyl jasmonate (MeJA) (Wang and Wu, 2005), 5 mM methyl salicylate (MeSA), 200 µM Menadione (Borges *et al.,* 2003) or water as control respectively. For heat stress, Thatcher and Thatcher*+Lr34* seedlings were incubated at 42^{o}C for 8 h. Each sample was quick-frozen in liquid nitrogen and stored at -70^{o}C. Tissue was ground to a fine powder in liquid nitrogen and gene expression was determined using RT-PCR as described.

4.3 Results

4.3.1 Identification of *TaHlp01*

TaHlp01 was identified using a DDRT-PCR reaction performed on resistant wheat infected with leaf rust. Using a reverse Northern blot, it was shown that the gene fragment was inducibly expressed in the infected resistant (IR) plants. In contrast, expression within the infected susceptible (IS) plants was constitutive (Figure 3.3).

When the first BLAST search with *TaHlp01* was performed, it showed homology to a longer *T. aestivum* EST (GenBank accession nr CA501682, E=0.0). This EST was used to do another BLAST search and homology was found with a *T. aestivum* contig (GrainGenes accession nr NSFT03P2_Contig18805, E=0.0) which in turn showed homology to a *Triticum aestivum* gene for heat shock protein 70 (*TaHSP70d*) (GenBank accession nr AF005993, E=0.0). Contig 18805 aligned 100% with the coding sequence of *TaHlp01* and was thus used to make two primers for RT-PCR analysis.

During RT-PCR of infected resistant wheat, two different sized fragments were repeatedly amplified (Fig 4.1a). The larger fragment was approximately 2200 bp in size and the smaller one approximately 600 bp.

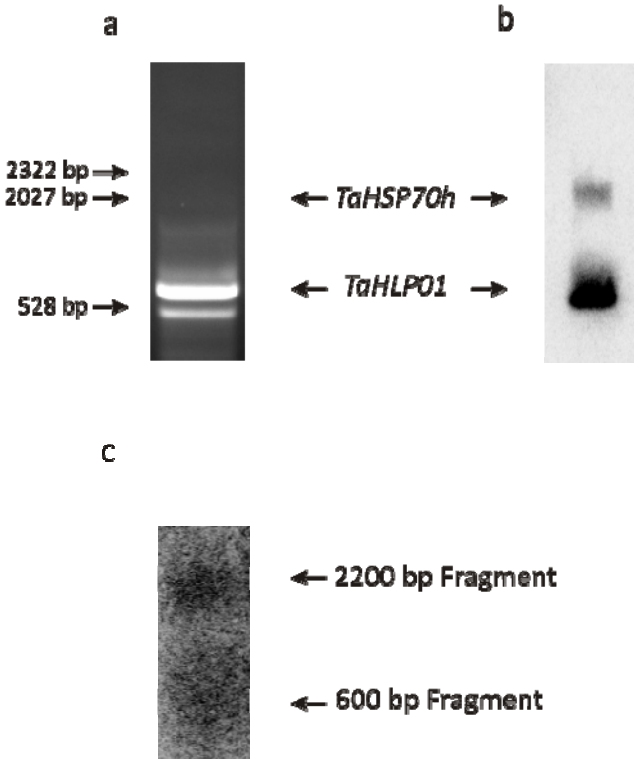

Figure 4.1: RT-PCR amplification of *TaHlp01* and *TaHsp70h*. In (a) the RT-PCR result is indicated, in (b) the resulting southern blot and in (c) a Northern blot showing the expression of two different sized cDNA fragments.

While both fragments were amplified, the smaller was amplified to much higher levels. In order to determine if both amplified fragments represented the same gene or possible artefacts, the two fragments were hybridized with the cloned *TaHlp01* cDNA fragment as probe (Fig 4.1b). Both fragments hybridized with the probe under high stringency conditions indicating that they were related to each other. This was further confirmed when a Northern blot was performed on total RNA extracted from infected resistant wheat (Fig 4.1c). Even though the resulting blot was of poor quality, two hybridizing fragments were visible. This indicated that *TaHlp01* shared extensive homology with two different mRNA species that are expressed within wheat. The two fragments amplified with RT-PCR were therefore cloned and sequenced.

The smaller of the two fragments had a nucleotide sequence of 640 base pairs and coded for a polypeptide of 188 amino acids, 20.4 kDa in size (Fig 4.2a). On amino acid level, it shared sequence similarity to the *Oryza sativa* heat shock cognate 70 protein (GenBank accession nr AAO65876 $E=1e^{-95}$) (Fig 4.2b). Due to the fact that the fragment was similar to the *O. sativa* heat shock protein, the fragment was called *Triticum aestivum* Heat Shock Like Protein 01 (*TaHlp01*). A motif and pattern scan on the PROSITE database showed two motifs present in the amino acid sequence of *TaHlp01.* The first was a PF00012 HSP70 motif spanning amino acids 1-183 and the second one was a SSF53067 Actin-like ATPase domain spanning amino acids 8-183.

The larger of the two cloned cDNA fragments was initially partially sequenced from both ends. When aligned with Contig18805, both sequences showed 100% sequence similarity on amino acid level and it was concluded that the cloned cDNA sequence was identical to the contig (Fig 4.3). This larger fragment or contig had a nucleotide sequence of 2247 bp and coded for a polypeptide of 648 amino acids (Fig 4.4a). On amino acid level it shared sequence similarity with Heat shock cognate 70 protein from *O. sativa* (GenBank accession nr ABA95500, E=0.0). Due to the high similarity of this fragment with *O. sativa* HSP70*,* the cDNA fragment was called *T. aestivum* Heat Shock Protein 70 homolog (*TaHsp70h*). A motif and pattern scan on the PROSITE database showed three motifs present in the amino acid

sequence of *TaHsp70h.* The first motif was a PF00012 HSP70 motif spanning amino acids 1-534.

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

b.

```
TaHlp01         MAPTKGEGPAIGIDLGTTYSCVGVWQHDRVEIIANDQGNRTTPSYVAFTDSERLIGDAAK 60
AAO65876        MAPTKGEGPAIGIDLGTTYSCVGVWQHDRVEIIANDQGNRTTPSYVGFTDTERLIGDAAK 60
                ****************************************.***.*********

TaHlp01         NQVAMNPINTVFDAKRLIGRRFADAPVQSDIKMWPYKVIPGPADKPMIVVQYKGEDKQFS 120
AAO65876        NQVAMNPINTVFDAKRLIGRRFSDASVQSDIKMWPYKVIPGPGDKPMIVVQYKGEEKQFS 120
                **********************:**.***************.************:****

TaHlp01         AEEISSMVLIKMREIAEAYLGVTIKNAVVTVPAYFNDSQRQATKDAGVIAGLNVMRIINE 180
AAO65876        AEEISSMVLIKMREIAEAYLGSTVKNAVVTVPAYFNDSQRQATKDAGVIAGLNVMRIINE 180
                ********************.*:**********************************

TaHlp01         PFAQLL---------------------------------------------------- 186
AAO65876        PTAAAIAYGLDKKATSVGEKNVLIFDLGGGTFDVSLLTIEEGIFEVKATAGDTHLGGEDF 240
                * *  :
```

Figure 4.2: Sequence analysis of *TaHlp01*. In (a) the open reading frame is indicated. In (b), the amino acid alignment with Heat Shock Protein 70 cognate from *O. sativa* (GenBank accession nr AAO65876) is indicated. Consensus sequences are indicated with an (*) while (:) indicates conservative substitutions and (.) semiconservative substitutions.

```
Contig18805       MAPTKGEGPAIGIDLGTTYSCVGVWQHDRVEIIANDQGNRTTPSYVAFTDSERLIGDAAK 60
TaHsp70h          MAPTKGEGPAIGIDLGTTYSCVGVWQHDRVEIIANDQGNRTTPSYVAFTDSERLIGDAAK 60
                  ************************************************************

Contig18805       NQVAMNPINTVFDAKRLIGRRFADAPVQSDIKMWPYKVIPGPADKPMIVVQYKGEDKQFS 120
TaHsp70h          NQVAMNPINTVFDAKRLIGRRFADAPVQSDIKMWPYKVIPGPADKPMIV---------- 109
                  ******************************************

Contig18805       AEEISSMVLIKMREIAEAYLGVTIKNAVVTVPAYFNDSQRQATKDAGVIAGLNVMRIINE 180
TaHsp70h          ------------------------------------------------------------

Contig18805       PTAAAIAYGLDKKATSVGEKNVLIFDLGGGTFDVSLLTIEEGIFEVKATAGDTHLGGEDF 240
TaHsp70h          ------------------------------------------------------------

Contig18805       DNRMVNHFVQEFKRKHKKDISGNPRSLRRLRTSCERAKRTLSSTAQTTIEIDSLFEGVDF 300
TaHsp70h          ------------------------------------------------------------

Contig18805       YSTITRARFEELNMDLFRKCMEPVEKCLRDAKMDKSTIHDVVLVGGSTRIPRVQQLLQDF 360
TaHsp70h          ------------------------------------------------------------

Contig18805       FNGKELCKSINPDEAVAYGAAVQAAILSGEGNEKVQDLLLLDVTPLSLGLETAGGVMTVL 420
TaHsp70h          ------------------------------------------------------------

Contig18805       ITRNTTIPTKKEQVFSTYSDNQPGVLIQVFEGERTRTRDNNLLGKFELSGIPPAPRGVPQ 480
TaHsp70h          ------------------------------------------------------------

Contig18805       ITVCFDIDANGILNVSAEDKTTGQKNKITITNDKGRLSKDDIEKMVQDAEKYKSEDEEHK 540
TaHsp70h          DTVCFDIDANGILNVSAEDKTTGQKNKITITNDKGRLSKDDIEKMVQDAEKYKSEDEEHK 168
                   ************************************************************

Contig18805       KKVDAKNSLENYAYNMRNTIQDEKIASKLPADDKKKIEDAVDAAIQWLDANQLGEVDEFE 600
TaHsp70h          KKVDAKNSLENYAYNMRNTIQDEKIASKLPADDKKKIEDAVDAAIQWLDANQLGEVDEFE 228
                  ************************************************************

Contig18805       DKMKELEGLCNPIIAKMYQGAGADMPGGMDEDAPAASGGAGPKIEEVD 648
TaHsp70h          DKMKELEGLCNPIIAKMYQGAGADMPGGMDEDAPAASGGAGPKIEEVD 276
                  *********************************************
```

Figure 4.3: Amino acid sequence alignment of Contig 18805 and TaHsp70h. Consensus sequences are indicated with an (*) while (:) indicates conservative substitutions and (.) semiconservative substitutions

a.

```
MAPTKGEGPAIGIDLGTTYSCVGVWQHDRVEIIANDQGNRTTPSYVAFTDSERLIG
DAAKNQVAMNPINTVFDAKRLIGRRFADAPVQSDIKMWPYKVIPGPADKPMIVVQ
YKGEDKQFSAEEISSMVLIKMREIAEAYLGVTIKNAVVTVPAYFNDSQRQATKDAG
VIAGLNVMRIINEPTAAAIAYGLDKKATSVGEKNVLIFDLGGGTFDVSLLTIEEGIFE
VKATAGDTHLGGEDFDNRMVNHFVQEFKRKHKKDISGNPRSLRRLRTSCERAKRTL
SSTAQTTIEIDSLFEGVDFYSTITRARFEELNMDLFRKCMEPVEKCLRDAKMDKSTIH
DVVLVGGSTRIPRVQQLLQDFFNGKELCKSINPDEAVAYGAAVQAAILSGEGNEKV
QDLLLLDVTPLSLGLETAGGVMTVLITRNTTIPTKKEQVFSTYSDNQPGVLIQVFEG
ERTRTRDNNLLGKFELSGIPPAPRGVPQITVCFDIDANGILNVSAEDKTTGQKNKITI
TNDKGRLSKDDIEKMVQDAEKYKSEDEEHKKKVDAKNSLENYAYNMRNTIQDEKIA
SKLPADDKKKIEDAVDAAIQWLDANQLGEVDEFEDKMKELEGLCNPIIAKMYQGA
GADMPGGMDEDAPAASGGAGPKIEEVD
```

b.

```
TaHsp70h    MAPTKGEGPAIGIDLGTTYSCVGVWQHDRVEIIANDQGNRTTPSYVAFTDSERLIGDAAK 60
ABA95500    MAG-KGEGPAIGIDLGTTYSCVGVWQHDRVEIIANDQGNRTTPSYVGFTDSERLIGDAAK 59
            **  ****************************************.************

TaHsp70h    NQVAMNPINTVFDAKRLIGRRFADAPVQSDIKMWPYKVIPGPADKPMIVVQYKGEDKQFS 120
ABA95500    NQVAMNPINTVFDAKRLIGRRFSDASVQSDIKLWPFKVIAGPGDKPMIVVQYKGEEKQFA 119
            **********************:**.******:**:***.**.************:***:

TaHsp70h    AEEISSMVLIKMREIAEAYLGVTIKNAVVTVPAYFNDSQRQATKDAGVIAGLNVMRIINE 180
ABA95500    AEEISSMVLIKMREIAEAYLGTTIKNAVVTVPAYFNDSQRQATKDAGVIAGLNVMRIINE 179
            **********************.****************************************

TaHsp70h    PTAAAIAYGLDKKATSVGEKNVLIFDLGGGTFDVSLLTIEEGIFEVKATAGDTHLGGEDF 240
ABA95500    PTAAAIAYGLDKKATSVGEKNVLIFDLGGGTFDVSLLTIEEGIFEVKATAGDTHLGGEDF 239
            ************************************************************

TaHsp70h    DNRMVNHFVQEFKRKHKKDISGNPRSLRRLRTSCERAKRTLSSTAQTTIEIDSLFEGVDF 300
ABA95500    DNRMVNHFVQEFKRKNKKDITGNPRALRRLRTACERAKRTLSSTAQTTIEIDSLYEGIDF 299
            ***************:****:****:******:**********************:**:**

TaHsp70h    YSTITRARFEELNMDLFRKCMEPVEKCLRDAKMDKSTIHDVVLVGGSTRIPRVQQLLQDF 360
ABA95500    YSTITRARFEELNMDLFRKCMEPVEKCLRDAKMDKSSVHDVVLVGGSTRIPRVQQLLQDF 359
            ***********************************::**********************

TaHsp70h    FNGKELCKSINPDEAVAYGAAVQAAILSGEGNEKVQDLLLLDVTPLSLGLETAGGVMTVL 420
ABA95500    FNGKELCKNINPDEAVAYGAAVQAAILSGEGNEKVQDLLLLDVTPLSLGLETAGGVMTVL 419
            ********.***************************************************

TaHsp70h    ITRNTTIPTKKEQVFSTYSDNQPGVLIQVFEGERTRTRDNNLLGKFELSGIPPAPRGVPQ 480
ABA95500    IPRNTTIPTKKEQVFSTYSDNQPGVLIQVYEGERTRTRDNNLLGKFELSGIPPAPRGVPQ 479
            *.*************************:****************************

TaHsp70h    ITVCFDIDANGILNVSAEDKTTGQKNKITITNDKGRLSKDDIEKMVQDAEKYKSEDEEHK 540
ABA95500    ITVCFDIDANGILNVSAEDKTTGQKNKITITNDKGRLSKEEIEKMVQEAEKYKSEDEEHK 539
            ************************************::******:************

TaHsp70h    KKVDAKNSLENYAYNMRNTIQDEKIASKLPADDKKKIEDAVDAAIQWLDANQLGEVDEFE 600
ABA95500    KKVESKNALENYAYNMRNTIKDEKIASKLPAADKKKIEDAIDQAIQWLDGNQLAEADEFD 599
            ***::**:************:********** ********:* ******.***.*.***:

TaHsp70h    DKMKELEGLCNPIIAKMYQGAGADMPGGMDED-APAASG-GAGPKIEEVD 648
ABA95500    DKMKELEGICNPIIAKMYQGAGADMAGGMDEDDAPPAGGSGAGPKIEEVD 649
            ********:****************.****** **.*.* **********
```

Figure 4.4: Sequence analysis of *TaHsp70h*. In (a) the longest open reading frame is indicated. In (b) the polypeptide is aligned with Heat Shock Protein 70 from *Oryza sativa* (GenBank accession nr ABA95500). Consensus sequences are indicated with an (*) while (:)

indicates conservative substitutions and (.) semiconservative substitutions.

The second was a SSF53067 Actin-like ATPase domain spanning amino acids 8-193 and 199-416 and the third was a SSF56778 Heat shock protein 70 (HSP70), C-terminal substrate-binding fragment spanning amino acids 398-618. Upon alignment of the two cloned cDNA fragments, *TaHlp01* aligned only with the first part of *TaHsp70h* (Fig 4.5) indicating that *TaHlp01* could be formed through differential transcription or could be the product of another gene similar to *TaHsp70h*. Despite the high similarity in the first part of the amino acid sequence, it seems as if *TaHlp01* is a truncated version with perhaps a different function than *TaHsp70h*. Both PCR primer binding sites could be found in the coding region of *TaHsp70h* while the forward primer was located in the coding region of *TaHlp01* and the reverse primer location was found in the predicted 3' untranslated region of *TaHlp01*.

4.3.2 Genomic Presence of *TaHlp01*

In order to determine the copy number of *TaHlp01* and its presence in the resistant and/or susceptible plants, a southern blot was performed on Thatcher and Thatcher*+Lr34* genomic DNA (Fig 4.6). Two cross hybridizing fragments were observed in Thatcher and *Thatcher+Lr34* genomic DNA digested with *Eco*R1 while only one fragment was visible in the *Bam*H1 digested DNA. The hybridization pattern of the two plants was identical, indicating that no polymorphisms were present.

The presence of *TaHlp01* in other Thatcher cultivars containing different *Lr*-genes was also investigated to determine if *TaHlp01* is coupled to the Lr34 resistance locus or inherited from the Thatcher parent. *Bam*H1 digested genomic DNA of these 10 different cultivars was probed using the 640 bp *TaHlp01* as probe (Fig 4.7). In all cases, a single hybridizing fragment was present, indicating the presence of *TaHlp01* in all the cultivars including again the susceptible Thatcher cultivar.

4.3.3 Expression Analysis of *TaHlp01*

To determine the expression pattern of *TaHlp01* and *TaHsp70h*, RT-PCR was used. The expression of *TaHsp70h* remained constant in both the IR and IS plants for the duration of the study, indicating that its expression is unaffected by leaf rust infection (Fig 4.8).

TaHlp01 on the other hand was differentially expressed in the IR plants but constitutively in the IS plants (Fig 4.8). In the IR plants, the expression of *TaHlp01* increased rapidly 6 h.p.i

```
TaHlp01         MAPTKGEGPAIGIDLGTTYSCVGVWQHDRVEIIANDQGNRTTPSYVAFTDSERLIGDAAK  60
TaHsp70h        MAPTKGEGPAIGIDLGTTYSCVGVWQHDRVEIIANDQGNRTTPSYVAFTDSERLIGDAAK  60
                ************************************************************

TaHlp01         NQVAMNPINTVFDAKRLIGRRFADAPVQSDIKMWPYKVIPGPADKPMIVVQYKGEDKQFS  120
TaHsp70h        NQVAMNPINTVFDAKRLIGRRFADAPVQSDIKMWPYKVIPGPADKPMIVVQYKGEDKQFS  120
                ************************************************************

TaHlp01         AEEISSMVLIKMREIAEAYLGVTIKNAVVTVPAYFNDSQRQATKDAGVIAGLNVMRIINE  180
TaHsp70h        AEEISSMVLIKMREIAEAYLGVTIKNAVVTVPAYFNDSQRQATKDAGVIAGLNVMRIINE  180
                ************************************************************

TaHlp01         PFAQLL------------------------------------------------------  186
TaHsp70h        PTAAAIAYGLDKKATSVGEKNVLIFDLGGGTFDVSLLTIEEGIFEVKATAGDTHLGGEDF  240
                * *  :

TaHlp01         ------------------------------------------------------------
TaHsp70h        DNRMVNHFVQEFKRKHKKDISGNPRSLRRLRTSCERAKRTLSSTAQTTIEIDSLFEGVDF  300

TaHlp01         ------------------------------------------------------------
TaHsp70h        YSTITRARFEELNMDLFRKCMEPVEKCLRDAKMDKSTIHDVVLVGGSTRIPRVQQLLQDF  360

TaHlp01         ------------------------------------------------------------
TaHsp70h        FNGKELCKSINPDEAVAYGAAVQAAILSGEGNEKVQDLLLLDVTPLSLGLETAGGVMTVL  420

TaHlp01         ------------------------------------------------------------
TaHsp70h        ITRNTTIPTKKEQVFSTYSDNQPGVLIQVFEGERTRTRDNNLLGKFELSGIPPAPRGVPQ  480

TaHlp01         ------------------------------------------------------------
TaHsp70h        ITVCFDIDANGILNVSAEDKTTGQKNKITITNDKGRLSKDDIEKMVQDAEKYKSEDEEHK  540

TaHlp01         ------------------------------------------------------------
TaHsp70h        KKVDAKNSLENYAYNMRNTIQDEKIASKLPADDKKKIEDAVDAAIQWLDANQLGEVDEFE  600

TaHlp01         ---------------------------------------------
TaHsp70h        DKMKELEGLCNPIIAKMYQGAGADMPGGMDEDAPAASGGAGPKIEEVD             648
```

Figure 4.5: Amino acid sequence alignment of *TaHlp01* and *TaHsp70h*. Consensus sequences are indicated with an (*) while (:) indicates conservative substitutions and (.) semiconservative substitutions.

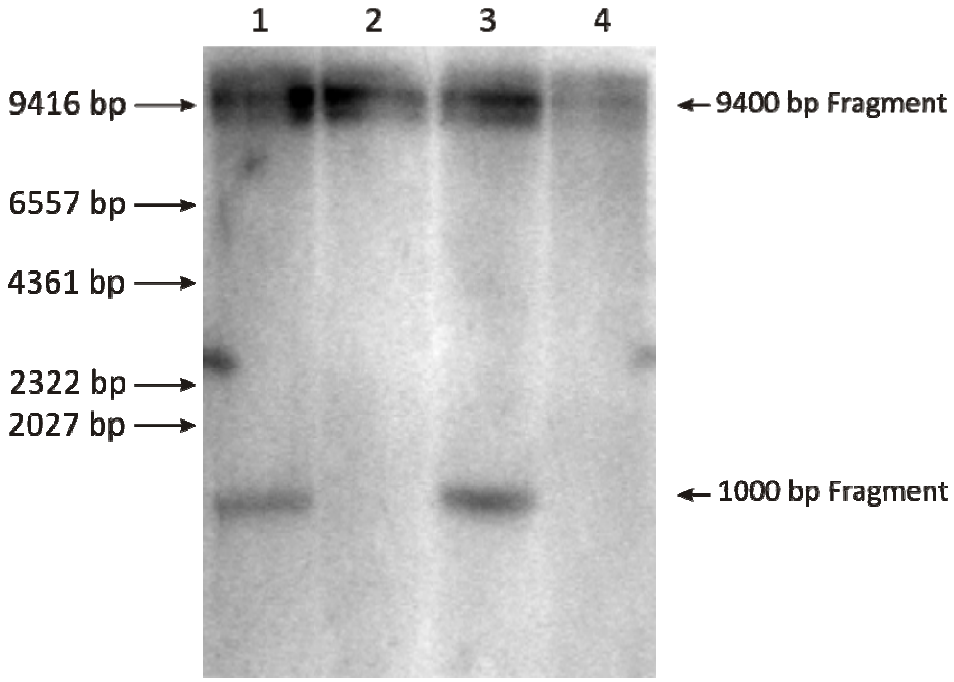

Figure 4.6: Southern blot analysis of Thatcher and Thatcher*+Lr34* genomic DNA probed with *TaHlp01*. Lanes 1 and 2 indicate *Eco*R1 and *Bam*H1 digested Thatcher DNA and lanes 3 and 4 *Eco*R1 and *Bam*H1 digested Thatcher*+Lr34* DNA respectively.

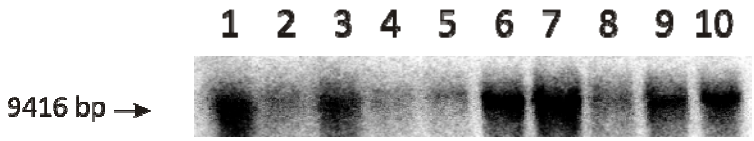

Figure 4.7: Southern blot analysis of different leaf rust resistant wheat cultivars within the Thatcher background probed with *TaHlp01*. Lane 1 contained Thatcher, lane 2 Thatcher+*Lr1*, lane 3 Thatcher+*Lr2a*, lane 4 Thatcher+*Lr3a*, lane 5 Thatcher+*Lr3ka*, lane 6 Thatcher+*Lr10*, lane 7 Thatcher+*Lr15*, lane 8 Thatcher+*Lr16*, lane 9 Thatcher+*Lr26* and lane 10 *Thatcher+Lr34* genomic DNA digested with *Bam*H1.

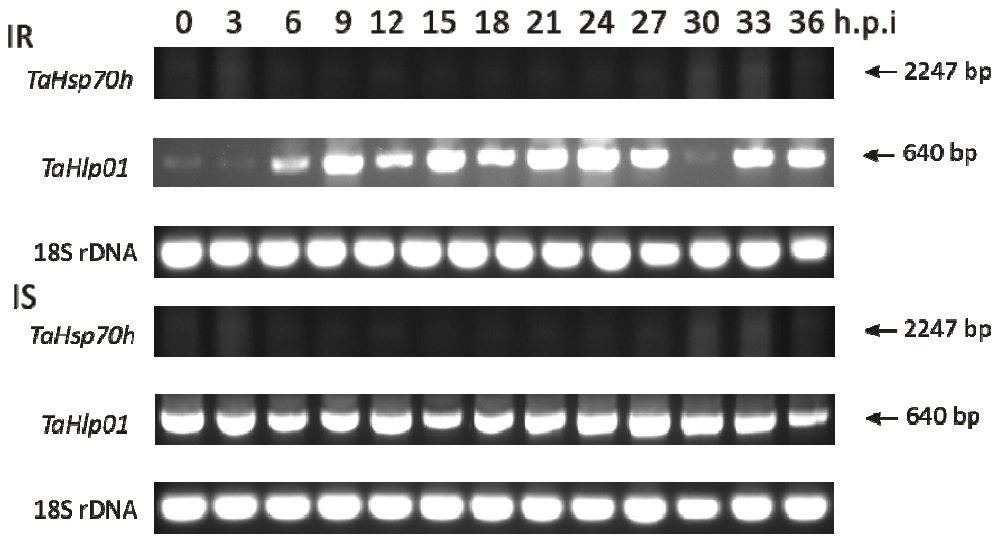

Figure 4.8: Expression analysis of *TaHlp01* and *TaHsp70h* in adult Thatcher and Thatcher+*Lr34* wheat infected with *P. triticina*. IR indicates the expression in infected resistant and IS the expression in infected susceptible wheat.

and stayed at the same high, elevated levels for the duration of the experiment except at 30 h.p.i when expression was completely shut down. In the IS plants, on the other hand, the expression levels of *TaHlp01* remained the same. The initial expression level of *TaHlp01* in the IS plants was however much higher than that of the IR plants at the start of the time trial.

To determine if the increases in RNA levels for *TaHlp01* indeed led to increased protein synthesis, a Western blot was performed on IR and IS plants and probed with an anti-HSP70 antibody. Three polypeptides were expected since the polyclonal antibody recognizes both the differentially expressed Hsp70 as well as the constitutive Hsc70 polypeptides. In addition it should also detect TaHlp01 due to its similarity to Hsp70. In the resulting Western blots (Fig 4.9), only one constitutively expressed polypeptide was observed in both the IR and IS plants. The detected polypeptide was in the order of 70 kDA and was present at very low levels. The antibody did not detect any smaller polypeptides that would represent the *TaHlp01* encoded gene product.

In order to determine whether the huge increase in *TaHlp01* expression was due to the *Lr34* resistance locus in the Thatcher*+Lr34* plants, the RT-PCR was repeated on wheat that was infected with *P. striiformis* (Fig 4.10). In the IR plants, *TaHlp01* showed increased expression from 12 h.p.i onwards. A total repression of gene expression occurred between 36 to 42 h.p.i. The decreased expression was not due to technical error since the 18S rDNA showed constant expression. Even after several repeats, the same decrease in expression was found.

In the IS plants, the expression pattern of *TaHlp01* stayed constant. The same total repression of *TaHlp01* expression as noticed in the IR plants occurred at 42 h.p.i in the IS plants. As was seen during the leaf rust wheat interaction, the expression of *TaHlp01* in the IS plants was much higher during the initial stages of yellow rust infection compared to that of the IR plants. It thus appears as if *TaHlp01* expression in susceptible plants occurs at very high constitutive levels, while in the resistant plants it is switched off. The expression is however strongly induced in the resistant plants upon infection.

4.3.4 Chemical Induction of *TaHlp01 Expression*

In order to elucidate the relevant pathway through which the expression of *TaHlp01* is regulated, Thatcher*+Lr34* plants were treated with MeJA or MeSA.

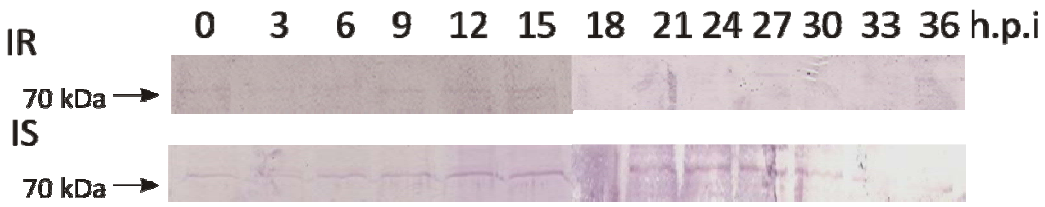

Figure 4.9: Immunological detection of expressed polypeptides by means of a polyclonal anti-HSP70 antibody in Thatcher (IS) and Thatcher+*Lr34* (IR) wheat infected with leaf rust.

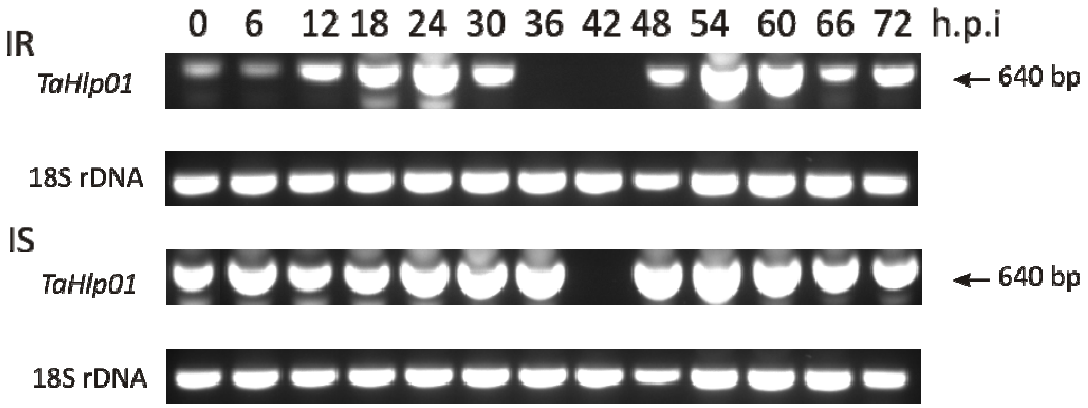

Figure 4.10: Expression analysis of *TaHlp01* in *P. striiformis* infected Avoset wheat (three leaf stage). IR indicates the expression in infected resistant and IS in infected susceptible wheat.

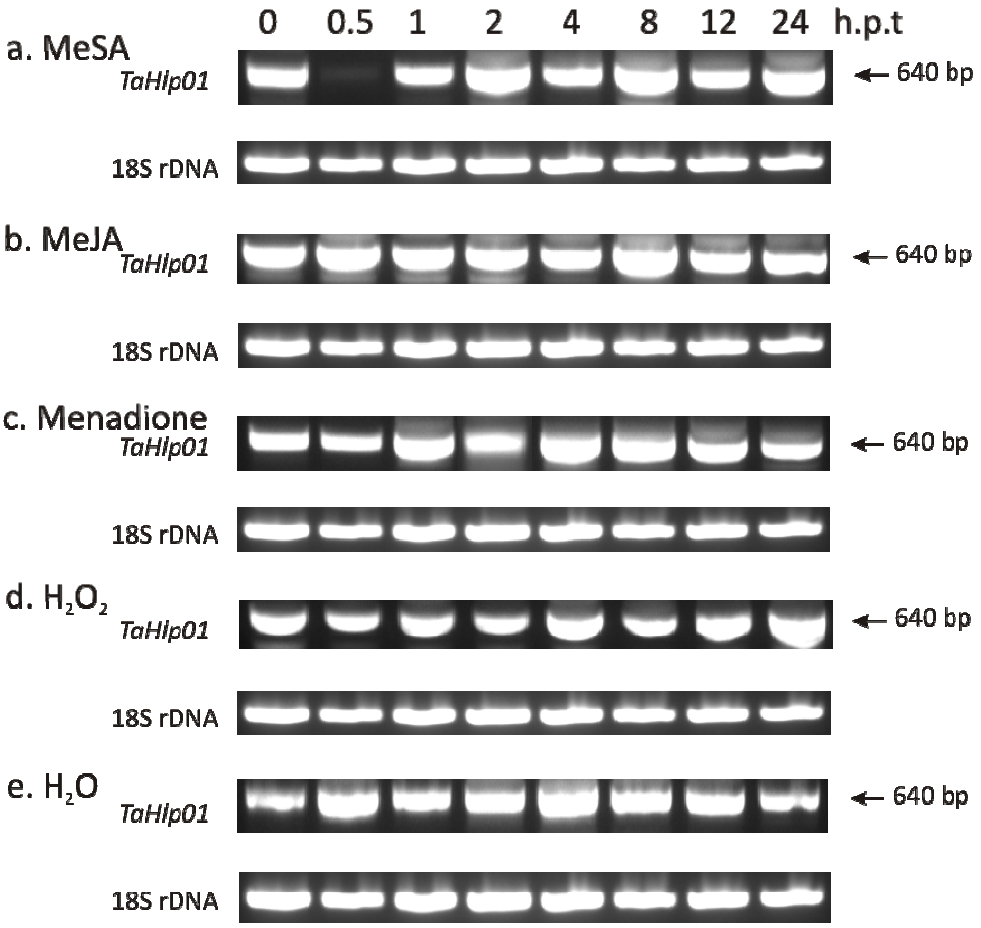

Figure 4.11: Expression analysis of *TaHlp01* during the treatment of Thatcher*+Lr34* seedlings with different chemicals.

Seedlings were used since *P. striiformis* infections on seedlings showed *TaHlp01* expression in seedlings. In the plants treated with MeSA (Fig 4.11a), *TaHlp01* expression was repressed at 0.5 hours post treatment (h.p.t.) where after it recovered with the highest levels of expression occurring at 2 - 8 h.p.t. During the MeJA treatment (Fig 4.11b), no substantial deviation in expression was found for the duration of the trial.

To determine whether *TaHlp01* was activated during oxidative stress, as was found for Hsps in animal systems, plants were treated with Menadione (Fig 4.11c) or H_2O_2 (Fig 4.11d). In the Menadione treated plants there was a slight increase in gene expression starting at 1 h.p.t while the H_2O_2 treated plants only showed a slight increase in gene expression at 4 h.p.t. The H_2O treated control plants (Fig 4.11e) showed no increases in gene expression, as was expected.

Since Hsps were originally identified in organisms undergoing heat stress, Thatcher and Thatcher*+Lr34* plants were subjected to heat stress at 42^{o}C (Fig 4.12). In the Thatcher*+Lr34* plants, there was a doubling of *TaHlp01* expression within 30 minutes after exposure reaching a maximum 60 minutes after exposure to elevated temperatures. In contrast, *TaHlp01* expression in the Thatcher plants did not show such high levels of expression and reached its maximum expression 2 h.p.t (Fig 4.12). In total contrast to *Thatcher+Lr34,* the expression in Thatcher was completely shut down at 0.5 h.p.t, where after expression returned to normal levels.

It is interesting to note that *TaHlp01* expression in the treated Thatcher+*Lr34* seedlings (Fig 4.11a-e) was much higher when compared to its expression in adult Thatcher+*Lr34* plants infected with leaf rust (Fig 4.8). In contrast, *TaHlp01* expression in seedlings (Fig 4.12) and adult Thatcher plants (Fig 4.8) was at the same high levels, irrespective of the age of the plant.

4.4 DISCUSSION

Hsps are a group of highly conserved proteins whose levels are increased in pro- and eukaryotes by elevated temperatures and a variety of other physical and chemical injuries

(Lindquist and Craig, 1988; Cronje and Bornman, 1999). The proteins are grouped into families according to their apparent molecular masses.

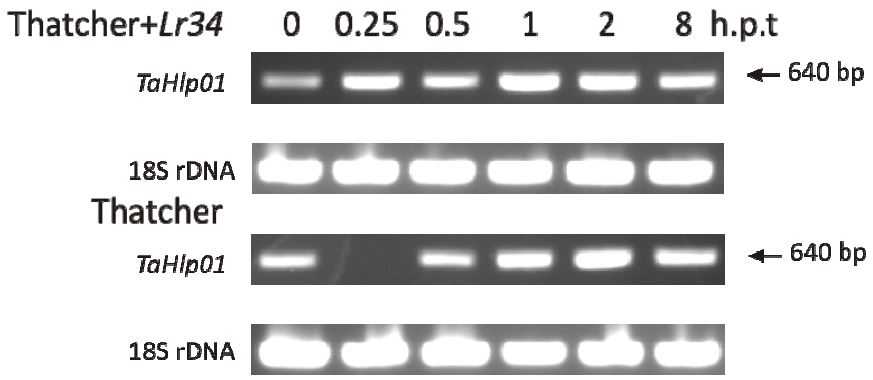

Figure 4.12: Expression analysis of *TaHlp01* during heat shock treatment of Thatcher and Thatcher*+Lr34* seedlings at 42^{o}C.

Some groups contain both constitutive and inducible members e.g. Hsc70 which is constitutively expressed and Hsp70 which is inducibly expressed (Cho and Hong, 2004; Cronje *et al.,* 2004; Tutar *et al.,* 2006).

Hsps play key roles in the maintenance of cellular homeostasis through chaperoning functions where they assist in the folding and assembly of immature or damaged polypeptides or proteins. They also help in protein transport and the degradation of denatured proteins (Bukau and Horwich, 1998; Forreiter and Nover, 1998; Sung *et al.,* 2001).

During this study, two expressed gene fragments were identified. *TaHsp70h* encoded a 70 kDa polypeptide that shared extensive homology with an *O. sativa* Hsp70 cognate gene. *TaHlp01* on the other hand encoded a 20.4 kDa polypeptide that shared homology to a *O. sativa* Hsp70 protein. When aligned to each other, the first part of each gene fragment that codes for a conserved Hsp70 domain, showed extensive homology, but the 3' sequences differed. This indicated that the two gene fragments may be coded for by different genes that are related or that they are the result of differential transcription of a single gene.

The theory of differential transcription was supported with a southern blot that showed only one cross hybridizing fragment on genomic level in the different cultivars. However, until the full genomic sequences of *TaHlp01* and/or *TaHsp70h* are available, it will be difficult to confirm this.

Upon comparison of the putative polypeptides, both contained a conserved Hsp70 motif at the amino end. This is followed by an ATPase domain and in the case of *TaHsp70h*, a carboxyl-terminal substrate-binding element. *TaHsp70h* contained two ATPase domains while *TaHlp01* had only one. Hsp70s interact with co-chaperones through the amino-terminal ATPase domain and with substrates by means of the carboxyl-terminal substrate binding domain (Nollen and Morimotto, 2002). Since *TaHlp01* lacks a substrate binding element, it might be possible that it forms part of a chaperone complex with other Hsps as was found by Kimura *et al.* (1995) where Hsp70 was necessary for Hsp90 function.

While the two highly conserved *TaHsp70h* and *TaHlp01* mRNAs were formed as proven with RT-PCR and a Northern blot, a single 70 kDa polypeptide was detected. It was concluded that this polypeptide represented *TaHsp70h*, based on the size of the polypeptide and the constitutive expression pattern as seen by RT-PCR. Since a polyclonal antibody was used for the Western blot, it might be possible that the antibody was generated against epitopes not available on *TaHlp01* due to its truncated size in comparison to *TaHsp70h*.

In response to pathogen infection, *TaHsp70h* expression remained constant in both resistant and susceptible wheat lines, indicating that it is not involved in the defence reaction against leaf rust. It might however be involved at a later stage than what was covered during this study. Since Dhankher *et al.* (1997) proved that Hsps are involved in normal developmental processes, it might explain the constitutive expression of *TaHsp70h* indicating that it could be involved in normal cellular activities rather than in the defence response.

In contrast, *TaHlp01* was inducibly expressed in the resistant cultivars as early as 6 h after infection with leaf rust and 12 h after infection with yellow rust. The expression of the gene was however constitutive in both susceptible cultivars. This clearly indicates a role for TaHlp01 during the defence response. The expression pattern of *TaHlp01* could also implicate one of two things: (a) The expression of *TaHlp01* is very important for the successful resistance response of the resistant plants but not for the susceptible plants or (b) the expression of *TaHlp01* is essential for a resistance response in both resistant and susceptible plants, but since it is already actively transcribed in the susceptible plants, it is not necessary to induce it's expression during infection. The resistant plants could thus conserve energy and resources by shutting *TaHlp01* expression down and only activate it when needed.

This repression and induction of *TaHlp01* expression in the resistant cultivars are most probably due to the Lr34 and Yr-1 resistance loci present, since the resistant and susceptible plants are identical except for the resistance locus. The regulation of *TaHlp01* expression is therefore most probably controlled by a gene located on the resistance locus that could also regulate other unidentified genes. The presence, nature and function of such a protein however still have to be proven.

During all the chemical treatment experiments of the Thatcher+*Lr34* seedlings, very high initial levels of *TaHlp01* were detected. The same high level of *TaHlp01* expression was also detected in the susceptible adult Thatcher plants. In contrast, in the adult Thatcher+*Lr34* plants used for the inoculation with leaf rust, the *TaHlp01* expression levels were much lower. It thus appears as if the expression of *TaHlp01* in Thatcher+*Lr34* is also developmentally regulated and is down regulated as a result of the activation of the adult leaf rust resistance locus in Thatcher*+Lr34*. This possible dual regulation of *TaHlp01* expression by age and genotype in the resistant plants will have to be further investigated.

Kanzaki *et al.* (2003) showed that members of the Hsp70 and 90 families were essential components of the plant defence signal transduction pathway, as well as for normal plant development. Plants in which the expression of these two genes was repressed, showed abnormal phenotypes while disease resistance was severely compromised which coincided with reduced expression of defence related genes. Repka (2006) and do Céu Silva *et al.* (2006) identified induced Hsp70 genes in the *Botrytis cinerea*/grapevine and the *Hemileia vastatrix*/coffee bean interactions respectively while Leckie *et al.* (1995) found increases in Hsp70 protein levels during a pea/*Erysiphe pisi* interaction. In the above mentioned studies the Hsp70 was postulated to be involved in the transport of newly synthesized polypeptides from the endoplasmic reticulum to the cytoplasm. These new polypeptides need to be kept denatured in order for them to be effectively transported through membranes into the cytoplasm where they are assembled by other proteins to become functional enzymes.

Upon the infection of wheat with leaf or yellow rust, the successful recognition of the pathogen by the resistant plants leads to the production of various proteins involved in the defence response. An example of such a protein is β-1,3-glucanase which has anti-fungal properties. This enzyme is active in the cytosol and was proven to be induced in wheat against leaf rust infection (Anguelova *et al.,* 1999; Kemp *et al.,* 1999; Appelgryn, results unpublished). Increased expression of *PR-2* encoding β-1,3-glucanase leads to increased β-1,3-glucanase polypeptides that needs to be transported to the cytoplasm or the apoplastic space. This in turn could lead to increased production of *TaHlp01* which could function to keep the polypeptides in a denatured state in order for them to be transported to their intended destinations.

When Thatcher+*Lr34* seedlings were treated with MeSA it was found that the expression of *TaHlp01* was repressed very early but at 1 h.p.t its expression level returned to normal and increased to a maximum at 2 h.p.t. A repression similar to the one observed during MeSA treatment was found in Thatcher+*Lr34* plants infected with leaf rust as well as in Avoset R and S plants infected with yellow rust. This indicates that an increase in SA levels associated with the normal defence response of wheat, could repress the expression of *TaHlp01*. This however needs to be confirmed by measuring the actual SA levels in the treated plants.

Cronje *et al.* (2004) showed increased Hsp70 gene expression mediated by SA in heat-stressed tomato plants. In this interaction, SA treatment induced Hsp70 production which in turn resulted in a decrease in apoptosis as a result of the temperature stress. In another study, Gális *et al.* (2004) found increased expression of SA-dependent genes, including Hsp70, providing increased resistance to white clover mosaic virus in *Phaseolus vulgaris* after treatment with SA. *TaHlp01* regulation by SA therefore does not fit into the normal expression pattern as seen with the other Hsp70 genes.

The induction of *TaHlp01* expression is independent of the jasmonic acid dependent signal transduction pathway, but it might be possible that the high expression of *TaHlp01* in the seedlings used for the study is overshadowing the effect that jasmonic acid might have.

Hsps have been shown to play an active role in the protection of animal cells against oxidative stress (Boston *et al.,* 1996; Bukau and Horwich, 1998) but its role in plants during oxidative stress is still not clear. Hamilton and Heckathorn (2001) suggested that Hsps might act as antioxidants. Upon treating Thatcher+*Lr34* with H_2O_2 and menadione, which are inducers of oxidative stress, an increase in *TaHlp01* gene expression was found after menadione, but not H_2O_2, treatment. It might thus be possible that the expression of *TaHlp01* is independent of H_2O_2 and that menadione produces a different kind of stress condition in which the increased expression of *TaHlp01* is one of the results. It will however be necessary to repeat above mentioned treatments on adult Thatcher*+Lr34* plants since the high levels of *TaHlp01* expression in the seedlings could conceal the real effect of the treatments.

During heat-induced stress conditions, there was a stronger, earlier expression of *TaHlp01* in the Thatcher*+Lr34* plants compared to the Thatcher plants where the expression was

initially switched off. This expression pattern is not typical of a heat shock protein, since the proteins are primarily involved in providing thermotolerance to plants, irrespective of the resistance or susceptible phenotype of a plant (Feder *et al*., 1996; Lee and Schöffl, 1996; Nollen *et al*., 1999). In this case, the higher expression patterns observed in the leaf rust-resistant plants might be due to an improved general defence response against any stress condition, whether it is biotic or abiotic.

To conclude, *TaHlp01* is a novel polypeptide that appears to play an important and unique role during the defence response of wheat after infection by rusts. The regulation of its expression is complex with both developmental cues and the resistance locus seemingly playing key roles. Based on its relationship with heat shock proteins, it is proposed to play a role in the protection and folding of native polypeptides, but this has to be confirmed in future research.

4.5 REFERENCES

Anguelova V.S., van der Westhuizen A.J. and Pretorius Z.A. 1999. **Intercellular proteins and β-1,3-glucanase activity associated with leaf rust resistance in wheat**. *Physiologia Plantarum* 106: 393-401

Banzet N., Richaud C., Deveaux Y., Kazmaier M., Gagnon J. and Triantaphylides C. 1998. **Accumulation of small heat shock proteins, including mitochondrial HSP22, induced by oxidative stress and adaptive response in tomato cells**. *The Plant Journal* 13: 519-527

Blake M.S., Johnston K.H., Russell-Jones G.J. and Gotschlich E.C. 1984. **A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots.** *Analytical Biochemistry* 136: 175-179

Borges A.A., Cools H.J. and Lucas J.A. 2003. **Menadione sodium bisulphite: a novel plant defence activator which enhances local and systemic resistance to infection by *Leptosphaeria maculans* in oilseed rape.** *Plant Pathology* 52: 429-436

Boston R.S., Viitanen P.V. and Vierling E. 1996. **Molecular chaperones and protein folding in plants.** *Plant Molecular Biology* 32: 191-222

Bradford M.M. 1976. **A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding**. *Analytical Biochemistry* 72: 248-253

Bukau B. and Horwich A.L. 1998. **The Hsp70 and Hsp60 chaperone machines.** *Cell* 92: 351-366

Burnett W.N. 1981. **Western blotting: electrophoretic transfer of proteins from SDS-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A**. *Analytical Biochemistry* 112: 195-203

Cho E.K. and Hong C.B. 2004. **Molecular cloning and expression pattern analyses of heat shock protein 70 genes from *Nicotiana tabacum.*** *Journal of Plant Biology* 47: 149-159

Chomczynski P. 1992. **One-hour downward alkaline capillary transfer for blotting of DNA and RNA**. *Analytical Biochemistry* 201: 134-139

Chomczynski P. and Mackey K. 1994. **One-hour downward capillary blotting of RNA at neutral pH.** *Analytical Biochemistry* 221: 303-305

Chomczynski P. and Sacchi N. 1987. **Single step method of RNA isolation by acid Guanidine thiocyanate – Phenol Chloroform extraction**. *Analytical Biochemistry* 162: 156-159

Cronje M.J. and Bornman L. 1999. **Salicylic acid influences Hsp70/Hsc70 expression in *Lycopersicon esculentum*: Dose- and time-dependent induction or potentiation**. *Biochemical and Biophysical Research Communications* 265: 422-427

Cronje M.J., Weir I.E. and Bornman L. 2004. **Salicylic acid-mediated potentiation of Hsp70 induction correlates with reduced apoptosis in tobacco protoplasts**. *Cytometry* 61: 76-87

Dhankher O.M., Drew J.E. and Gatehouse J.A. 1997. **Characterization of a pea hsp70 gene which is both developmentally and stress-regulated.** *Plant Molecular Biology* 34: 345-352

Dobson C.F. 2003. **Protein folding and misfolding**. *Nature* 426: 884-890

do Céu Silva M., Várzea V., Guerra-Guimarães L., Azinheira H.G., Fernandez D., Petitot A-S., Bertrand B., Lashermes P. and Nicole M. 2006. **Coffee resistance to the main diseases: leaf rust and coffee berry disease.** *Brazilian Journal of Plant Physiology* 18: 119-147

Falquet L., Pagni M., Bucher P., Hulo N., Sigrist C.J., Hofmann K. and Bairoch A. 2002. **The PROSITE database, its status in 2002.** *Nucleic Acids Research* 30: 235-238

Feder M.E., Cartano N.V., Milos L., Krebs R.A. and Lindquist S.L. 1996. **Effect of engineering Hsp70 copy number on Hsp70 expression and tolerance of ecologically relevant heat shock in larvae and pupae of *Drosophila melanogaster*.** *Journal of Experimental Biology* 199: 1837-1844

Fernandez D., Santos P., Agostini C., Bon M-C., Petitot A-S., Silva M.C., Guerra-Guimaraes L., Ribeiro A., Argout X. and Nicole M. 2004. **Coffee (*Coffea arabica* L.) genes early expressed**

during infection by the rust fungus (*Hemileia vastatrix*). *Molecular Plant Pathology* 5: 527-536

Forreiter C. and Nover L. 1998. **Heat stress-induced proteins and the concept of molecular chaperones.** *Journal of Biosciences 23: 287-302*

Gális I., Smith J.L. and Jameson P.E. 2004. **Salicylic acid-, but not cytokinin-induced, resistance to WCIMV is associated with increased expression of SA-dependent resistance genes in *Phaseolus vulgaris***. *Journal of Plant Physiology* 161: 459-466

Grene R. 2002. **Oxidative stress and acclimation mechanisms in plants.** *In* CR Somerville, EM Meyerowitz (eds). *The Arabidopsis Book*. American Society of Plant Biologists, Rockville, MD, doi: 10.1199/tab.0036.1, http://www.aspb.org/publications/arabidopsis/

Guy C.L. and Li Q.B. 1998. **The organization and evolution of the spinach stress 70 molecular chaperone gene family.** *The Plant Cell 10: 539-556*

Hamilton III E.W. and Heckathorn S.A. 2001. **Mitochondrial adaptations to NaCl. Complex I is protected by anti-oxidants and small heat shock proteins, whereas complex II is protected by proline and betaine**. *Plant Physiol*ogy 126: 1266-1274

Hendrix R.W. 1979. **Purification and properties of GroE, a host protein involved in bacteriophage assembly.** *Journal of Molecular Biology* 129: 375-393

Henics T. 2003. **Extending the 'stressy' edge: molecular chaperones flirting with RNA**. *Cell Biology International* 27: 1-6

Hunt C. and Morimoto R.I. 1985. **Conserved features of eukaryotic *hsp70* genes revealed by comparison with the nucleotide sequence of human *hsp70***. *Proceedings of the National Academy of Sciences USA* 82: 6455-6459

Kanzaki H., Saitoh H., Ito A., Fujisawa S., Kamoun S., Katou S., Yoshioka H. and Terauchi R. 2003. **Cytosolic HSP90 and HSP70 are essential components of INF1-mediated hypersensitive response and non-host resistance to *Pseudomonas cichorii* in *Nicotiana benthamiana***. *Molecular Plant Pathology* 4: 383-391

Kemp G., Botha A-M., Kloppers F.J. and Pretorius Z.A. 1999. **Disease development and β-1,3-glucanase expression following leaf rust infection in resistant and susceptible near-isogenic wheat seedlings.** *Physiological and Molecular Plant Pathology* 55: 45-52

Kimura Y., Yahara I. and Lindquist S. 1995. **Role of the protein chaperone YDJ1 in establishing Hsp90-mediated signal transduction.** *Science* 268: 1362-1365

Klessig D.F., Durner J., Noad R., Navarre D.A., Wendehenne D., Kumar D., Zhou J.M., Shah J., Zhang S., Kachroo P., Trifa Y., Pontier D., Lam E. and Silva H. 2000. **Nitric oxide and salicylic acid defence in plant defence.** *Proceedings of the National Academy of Sciences USA* 97: 8849-8855

Laemmli U.K. 1970. **Cleavage of structural proteins during the assembly of the head of the bacteriophage T4**. *Nature* 227: 680-685

Larkindale J. and Knight M.R. 2002. **Protection against heat stress-induced oxidative damage in *Arabidopsis* involves calcium, abscisic acid, ethylene and salicylic acid**. *Plant Physiology 128: 682-695*

Leckie C.P., Callow J.A. and Green J.R. 1995. **Reorganization of the endoplasmic reticulum in pea leaf epidermal cells infected by the powdery mildew fungus *Erysiphe pisi***. *New Phytologist* 131: 211-221

Lee J.H. and Schöffl F. 1996. **An Hsp70 antisense gene affects the expression of HSP70/HSC70, the regulation of HSF and the acquisition of thermotolerance in transgenic *Arabidopsis thaliana*.** *Molecular Genetics and Genomics* 252**:** 11–19

Lee B.H., Won S.H., Lee H.S., Miyao M., Chung W.I., Kim I.J. and Jo J. 2000. **Expression of the chloroplast-localized small heat shock protein by oxidative stress in rice.** *Gene* 245: 283-290

Liang P. and Pardee A.B. 1992. **Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction.** *Science* 257: 967-971

Lindquist S. and Craig E.A. 1988. **The heat-shock proteins.** *Annual Review of Genetics* 22: 631-677

Martin G.B., Bogdanove A.J. and Sessa G. 2003. **Understanding the functions of plant disease resistance proteins.** *Annual Review of Plant Biology* 54: 23-61

Mayer M.P. and Bukau B. 2005. **Hsp70 chaperones: Cellular functions and molecular mechanism**. *Cellular and Molecular Life Sciences* 62: 670-684

Neill S.J., Desikan R., Clarke A., Hurst R.D. and Hancock J.T. 2002. **Hydrogen peroxide and nitric oxide as defence molecules in plants.** *Journal of Experimental Botany* 53: 1237-1247

Nollen E.A., Brunsting J.F., Roelofsen H., Weber L.A. and Kampinga H.H. 1999. ***In vivo*** **chaperone activity of heat shock protein 70 and thermotolerance.** *Molecular and Cellular Biology* 19: 2069–2079

Nollen E.A.A. and Morimoto R.I. 2002. **Chaperoning defence pathways: molecular chaperones as stress-sensing 'heat shock' proteins.** *Journal of Cell Science* 115: 2809-2816

Nürnberger T. and Lipka V. 2005. **Non-host resistance in plants: new insights into an old phenomenon.** *Molecular Plant Pathology* 6: 335-345

Raskin I. 1992. **Role of salicylic acid in plants.** *Annual Review of Plant Physiology and Plant Molecular Biology* 43: 439-463

Ren D., Yang K-Y., Li G-J., Liu Y. and Zhang S. 2006. **Activation of Ntf4, a tobacco mitogen-activated protein kinase, during plant defence response and its involvement in hypersensitive response-like cell death.** *Plant Physiology* 141: 1482-1493

Repka V. 2006. **Early defence responses induced by two distinct elicitors derived from a *Botrytis cinerea* in grapevine leaves and cell suspensions**. *Biologia Plantarum* 50: 94-106

Ryals J., Uknes S. and Ward E. 1994. **Systemic acquired resistance.** *Plant Physiology* 104: 1109-1112

Rybutt D.B. and Parish C.R. 1982. **Protein determination on an automatic spectrophotometer.** *Analytical Biochemistry* 121: 213-214

Sambrook J., Fritsch E.F. and Maniatis T. 1989. **Molecular cloning: a laboratory manual.** 2nd ed. USA: *Cold Spring Harbour press*

Schett G., Steiner C-W., Xu Q., Smolen J.S. and Steiner G. 2003. **TNFα mediates susceptibility to heat-induced apoptosis by protein phosphatase-mediated inhibition of the HSF1/hsp70 stress response.** *Cell Death and Differentiation* 10: 1126-1136

Shinnick T.M. 1991. **Heat shock proteins as antigens of bacterial and parasitic pathogens.** *Current Topics in Microbiology and Immunology* 167: 145-160

Staskawicz B.J., Ausubel F.M., Baker B.J., Ellis J.G. and Jones J.D.G. 1995. **Molecular genetics of plant disease resistance.** *Science* 268: 661-667

Sung D.Y., Vierling E. and Guy C.L. 2001. **Comprehensive expression profile analysis of the *Arabidopsis* Hsp70 gene family**. *Plant Physiology* 126: 789-800

Thatcher L.F., Anderson J.P. and Singh K.B. 2005. **Plant defence responses: what have we learned from *Arabidopsis*.** *Functional Plant Biology* 32: 1-19

Turner J.G., Ellis C. and Devoto A. 2002. **The jasmonate signal pathway**. *The Plant Cell* 14: S153-S164

Tutar Y., Song Y. and Masison D.C. 2006. **Primate chaperones Hsc70 (constitutive) and Hsp70 (induced) differ functionally in supporting growth and prion propagation in *Saccharomyces cerevisiae.*** *Genetics* 172: 851-861

Wang J.W. and Wu J.Y. 2005. **Nitric oxide is involved in methyl jasmonate-induced defence responses and secondary metabolism activities of taxus cells**. *Plant and Cell Physiology* 46: 923-930

Chapter 5

Evidence for volatile defence between leaf rust-infected and uninfected wheat

5.1 INTRODUCTION

Plants, as sedentary organisms, have to adjust to their surrounding environment during their life cycle. To compensate for their immobility, plants have evolved various mechanisms to interact with their environment including the release and detection of volatile organic compounds (VOC). Plant communication is a loaded term that has come to encompass a broad definition. Most would accept the definition with the requirement that information can be exchanged, regardless of intent or fitness consequence for either party (Baldwin *et al.,* 2002).

Systemic acquired resistance (SAR) is the process whereby distal parts of a plant receive a signal from an infected part (Scheel, 1998). This signal allows the distal parts to activate its own defence as a preventative strategy. In a similar way two neighbouring plants could communicate so that the uninfected plant could activate its defences based on an airborne signal coming from an infected neighbour. Since intra-plant communication and signal transduction have been described many times (Dicke *et al.,* 1990; Karban *et al.,* 2000; Arimura *et al.,* 2001; Tscharntke *et al.,* 2001; Huang *et al.,* 2003; Engelberth *et al.,* 2004; Ruther and Kleier, 2005), the possibility of communication and signal transfer between plants exists. If there are receptor proteins to facilitate intra-plant communication, there should also be ones to accept signals from other plants in the same area.

Plants respond to VOCs by changing the transcription patterns of defence-related genes (Bate and Rothstein, 1998; Arimura *et al*., 2000; Farag *et al*., 2005; Paschold *et al*., 2006). They may furthermore increase the production of defence-related plant hormones such as jasmonic acid (JA) and other VOCs (Engelberth *et al*., 2004; Ruther and Kleier, 2005), proteinase inhibitors (Tscharntke *et al*., 2001) and phenolic compounds (Baldwin and Schultz, 1983).

Engelberth *et al*. (2004) reported that maize plants are primed by volatiles that are released from damaged plants. Exposure to such volatiles caused undamaged maize plants to produce JA and terpenes more intensively and/or rapidly in response to caterpillar-induced damage than plants that were damaged without this pre-treatment. In contrast, no such

priming effects were observed in tobacco plants (Paschold *et al.,* 2006). Tobacco also did not respond to volatile methyl jasmonate at levels consistent with concentrations that occur under natural conditions (Preston *et al*., 2004).

Peanut plants infected with white mold, *Sclerotium rolfsii,* emitted a mixture of lipoxygenase products, terpenoids, indole and methyl salicylate (MeSA) which were both quantitatively and qualitatively different from volatiles collected from healthy plants (Huang *et al.,* 2003). Among these volatiles, (Z)-3-hexenyl acetate, linalool and MeSA significantly inhibited fungal growth on solid culture media.

While some studies found no evidence for the transfer of information between damaged and undamaged plants (Preston *et al.,* 2001), many others presented evidence supporting the hypothesis of information exchange between damaged and undamaged plants (Dicke *et al.,* 1990; Karban *et al.,* 2000; Arimura *et al.,* 2001).

The aim of the study was to confirm a putative interplant communication event between wheat infected with leaf rust and uninfected wheat. In a previous study it was discovered that uninfected wheat plants that was in the same general vicinity as infected wheat plants showed, on molecular level, increased defence responses while they showed no physical signs of infection. This led to the hypothesis that there must be some sort of signal transduction taking place between these infected and uninfected plants.

5.2 Materials and Methods

5.2.1 Biological Material

Triticum aestivum seed was germinated and grown in pots containing a sterilized 1:1 soil: peat mixture in a glasshouse at 25^{o}C with a 16 h day/8 h night cycle. For the leaf rust infection studies, the Thatcher and Thatcher*+Lr34* cultivars were grown to flag leaf stage before they were inoculated.

Puccinia triticina pathotype UVPrt9 was increased on susceptible Karee wheat seedlings from which fresh urediniospores were collected for inoculation purposes.

5.2.2 Leaf Rust Inoculation Of Wheat

Adult Thatcher and Thatcher+*Lr34* plants were sprayed with freshly collected *P. triticina* spores (approx 65000 spores/ml) that were suspended in distilled water containing a drop of Tween™ 20. Control plants were sprayed with distilled water containing Tween™ 20. The plants were left to dry and then placed in a dark dew-simulation cabinet at 18 to 20°C for 16 h to facilitate spore germination where after they were transferred to the glasshouse.

5.2.3 An Investigation Into Possible Plant Communication

To investigate possible interplant communication in wheat, infected resistant (IR) plants were placed in a closed chamber for 12 h where after uninfected resistant (UR) and uninfected susceptible (US) plants, were added. In another experiment, in another enclosed cabinet, infected susceptible (IS) plants were placed for 12 h where after UR and US plants were added. Additional uninfected control plants (CR and CS) were kept separately within another growth cabinet. Flag leaf material was collected from all plants initially at 12 h intervals up to 24 h and thereafter at 24 h intervals up to 72 h post inoculation (h.p.i). The first sample was taken directly after the uninfected plants were added to the infected plants. All samples were quick-frozen in liquid nitrogen and stored at -70°C. Tissue was ground to a fine powder in liquid nitrogen.

5.2.4 Western Blot Analysis

Total cellular protein was extracted from leaves in an extraction buffer (50 mM Tris-HCl pH 7.5, 2.0 mM EDTA, 2.0 mM PMSF, 10 mM β-mercaptoethanol). After centrifugation at 10 000 *g* for 10 min at 4°C, protein concentration of the cleared supernatant was determined according to Bradford (1976) on a microtiterplate reader (Bio-Rad Model 3550) according to the method described by Rybutt and Parish (1982).

Twenty microgram total cellular protein was separated on a 12% (w/v) SDS-Page gel according to Laemmli (1970) for 1 h at 150 V and constant current. The polypeptides were transferred to PVDF membranes (Amersham Biosciences) using a Mini Trans-Blot cell (Bio-Rad) for 1 h at 350 mA current according to the manufacturer's specifications. A Western blot was then performed according to the method described by Burnette (1981). The membranes were probed with a 1:9000 diluted rabbit-anti-β-1.3-glucanase antibody (Anguelova *et al.,* 1999). A 1:2000 diluted horseradish peroxidase conjugated goat-anti-

rabbit-IgG (Amersham Biosciences) was used as secondary antibody. Cross reacting polypeptides were detected with the ECL detection system (Amersham Biosciences) according to the manufacturers' specifications. To quantify induced expression, the band intensities were determined using Quantity One Analysis software (Biorad). Each time point was expressed relative to time 0.

5.2.5 β-1,3-GLUCANASE ACTIVITY DETERMINATION

β-1,3-glucanase activity was assayed by measuring the rate of reducing sugar production spectrophotometrically through the *p*-hydroxybenzoic acid hydrazide (PAHBAH) procedure (York *et al.,* 1985) with laminarin as substrate (Fink *et al.,* 1988) using total cellular protein. A standard curve relating absorbance at 410 nm to glucose concentration was used to calculate the specific β-1,3-glucanase activity which was expressed as mM glucose.µg protein^{-1}.h^{-1}.

5.2.6 RT-PCR ANALYSIS

Total RNA was extracted from wheat leaf tissue according to the method of Chomczynski and Sacchi (1987). The quantity and quality of the extracted RNA were determined as described by Sambrook *et al.* (1989).

RT-PCR assays were performed on 10 ng total RNA using the RobusT II RT-PCR kit (Finnzymes) in the presence of 2.5 µM sequence specific primers and 25 µM dNTP's. The amplification conditions were according to the RobusT kit specifications, with the temperature for the reverse transcription step being 48^{0}C while the PCR annealing temperature varied based on the particular primer combination. PCR products were analyzed on a 1% (w/v) agarose gel (Sambrook *et al.,* 1989). A control RT-PCR reaction was performed for the constitutively expressed 18S rDNA gene using specific primers (5'- CAA CTT TCG ATG GTA GGA TAG -3' and 5'- CTC GTT AAG GGA TTT AGA TTG -3'; 226 bp product). The expression levels of the individual amplified cDNA fragments at the different time intervals were determined by first expressing the obtained values relative to that of the amplified 18S rDNA and then to that of time 0.

The expression of the following genes was determined: *PR2* (5'- TAG GCG ATA CCT TGC CAA -3' and 5'- ACT TCA TAC TTA GAC TGT CG -3'; 800 bp product), *PR5* (5'- GCC GCG GGG GCT

CGT AGT TGC -3' and 5'- CA CTC CAG AAC TCC GCT TAA -3'; 500 bp product; Hu and Reddy, 1997), *TaHlp01* (5'- CAG CAG CTG CGC AAA GGC TC -3' and 5'- TCA CAC GGG TAG GGT AGT CC -3'; 600 bp product) and the wheat homologues of *Etr1* (5'- AGA TCC CAT GTC CAA AAC CA -3' and 5'- TTG ATT TTG CCA ATC GAA CA -3'; 950 bp product), *NPR1* (5'- GAT GGA TTC GCC GAT TCT TA -3' and 5'- TAT GGT TGA CAT CGG CAA GA -3'; 950 bp product) and *AtCor1* (5'- CCA ACG TTT TCC TCT GTG GT -3' and 5'- CCA ACT CAG GTG AAG GAT CAA -3'; 950 bp product; Benedetti *et al.,* 1998).

5.2.7 Fluorescence Microscopy

Thatcher*+Lr34* plants were inoculated with leaf rust and placed in a closed chamber for 12 h where after uninfected Thatcher and Thatcher*+Lr34* plants were added. A control set of Thatcher and Thatcher*+Lr34* plants were placed in a separate growth chamber. Both chambers were kept at the same conditions. After 72 h in the presence of the IR plants, the UR and US, as well as the CR and CS plants, were inoculated with leaf rust (5.2.2) and transferred to a glasshouse. Flag leaves were harvested, in triplicate sample bottles, 48 h.p.i and fixed in a mixture of ethanol/dichloromethane (3:1) containing 0.15% (v/v) TCA. The specimens were stained with 0.1% (w/v) Uvitex 2BT in 0.1 M Tris-HCl pH 5.8 according to the method of Rohringer *et al.* (1977).

Samples were stored in 50% (v/v) glycerol with a trace amount of lactophenol as preservative until they were examined using an Olympus AX70 microscope. Different infection structures were counted for each set of sample bottles and the length and width of each colony was measured under the microscope and used to determine its area. One week after inoculation, leaf material was collected and photographed to ascertain the success of the infection.

5.3 Results

It was previously found that uninfected wheat plants that were placed in the vicinity of leaf rust infected wheat, showed increased levels of defence related compounds (Appelgryn, unpublished results). These uninfected plants thus appeared to be primed on molecular level and ready to defend themselves against any potential infection. This led to the

hypothesis that an interplant communication event took place between the infected and uninfected wheat plants.

This possible communication was investigated by following the expression pattern of several genes. To confirm the activation of the defence response, Pathogenesis-related protein 2 (*PR2*) and Pathogenesis-related protein 5 (*PR5*) gene expression was determined. The expression of another gene that is induced during the wheat defence response, *TaHlp01* (Appelgryn, unpublished results), was also determined. In addition, β-1,3-glucanase activity was measured. Finally, the expression of three marker genes encoding proteins involved in the defence, perception and synthesis of different volatiles was also tested in order to postulate a possible candidate molecule(s) responsible for wheat volatile defence.

5.3.1 THATCHER INFECTION-RELATED COMMUNICATION

5.3.1.1 Thatcher Infected with leaf rust

To confirm the activation of the defence response in the infected susceptible and resistant plants, *PR2*, *PR5* and *TaHlp01* expression was investigated. In the IS plants, an up-regulation of both *PR2* (Fig 5.1a) and *PR5* expression (Fig 5.1b) occurred at 24 h.p.i. These elevated levels were maintained for the duration of the study. In addition, *TaHlp01* expression increased 24 h.p.i reaching a maximum at 84 h.p.i (Fig 5.1c). β-1,3-glucanase activity increased up to 36 h.p.i followed by a much stronger induction 84 h.p.i (Fig 5.2a). These increases in β-1,3-glucanase activity were accompanied by increased protein levels from 24 h.p.i onwards (Fig 5.2c).

In the CS plants *PR2*, *PR5* and *TaHlp01* gene expression was either repressed from 12 h.p.i onwards or the expression remained constant (Fig 5.1a-c). While there was a slight increase in β-1,3-glucanase activity 24 h.p.i (Fig 5.2a), the β-1,3-glucanase protein levels remained constant (Fig 5.2c). In the CR, *PR2* gene expression increased 12 h.p.i (Fig 5.1a) while *PR5* and *TaHlp01* expression remained either constant or decreased (Fig 5.1b and c). β-1,3-glucanase activity and protein levels also remained constant during the time trial (Fig 5.2b and c) with decreases in both at 72 h.p.i.

Overall these results indicate the successful activation of the defence response in the susceptible plants following infection. This defence response encompassed the induced expression of both *PR2* and *PR5*, as well as increased β-1,3-glucanase enzymatic activity.

5.3.1.2 Uninfected Thatcher and Thatcher*+Lr34*

In the US plants there was increased *PR2* (Fig 5.1a) and *PR5* expression 24 h.p.i (Fig 5.1b). Despite decreased expression at 48 h.p.i, the higher levels of expression returned at 72 h.p.i. *TaHlp01* also showed the same induced expression pattern (Fig 5.1c). For β-1,3-glucanase activity, there was an increase 48 h.p.i (Fig 5.2a) with increased β-1,3-glucanase protein levels from 48 h.p.i onwards (Fig 5.2c).

In the UR plants, there was no induced *PR2* gene expression (Fig 5.1a) while a repression of *PR5* gene expression occurred from 12 h.p.i onwards (Fig 5.1b). *TaHlp01* on the other hand showed a transient increase in expression 24 h.p.i (Fig 5.1c). β-1,3-glucanase activity was inhibited from 12 h.p.i (Fig 5.2b) with only a slight increase in activity back to the normal level occurring at 72 h.p.i. After an initial decrease in β-1,3-glucanase protein levels at 12 h.p.i, the levels increased again to that of time zero from 24 h.p.i onwards (Fig 5.2c).

To summarize, IS plants managed to activate an extensive defence response within uninfected Thatcher plants, both on expression level as well as protein activity. In the Thatcher+*Lr34* plants, the induced response was only partial with none of the acknowledged *PR* genes being involved.

5.3.1.3 Expression of volatile defence marker genes

Despite numerous attempts, *NPR1* gene expression was not detected in any of the plant tissues (results not shown). In contrast, both *Etr1* at 60 h.p.i (Fig 5.3a) and *Cor1* at 84 h.p.i (Fig 5.3b) was inducibly expressed in the IS plants. These two genes were either not expressed at all in the control plants, or showed increased expression at 12 h.p.i in the case of *Etr1* (Fig 5.3a).

In the US plants there was a slight increase in *Etr1* expression 24 h.p.i (Fig 5.3a) while *Cor1* showed a doubling in gene expression 72 h.p.i after an initial repression (Fig 5.3b).

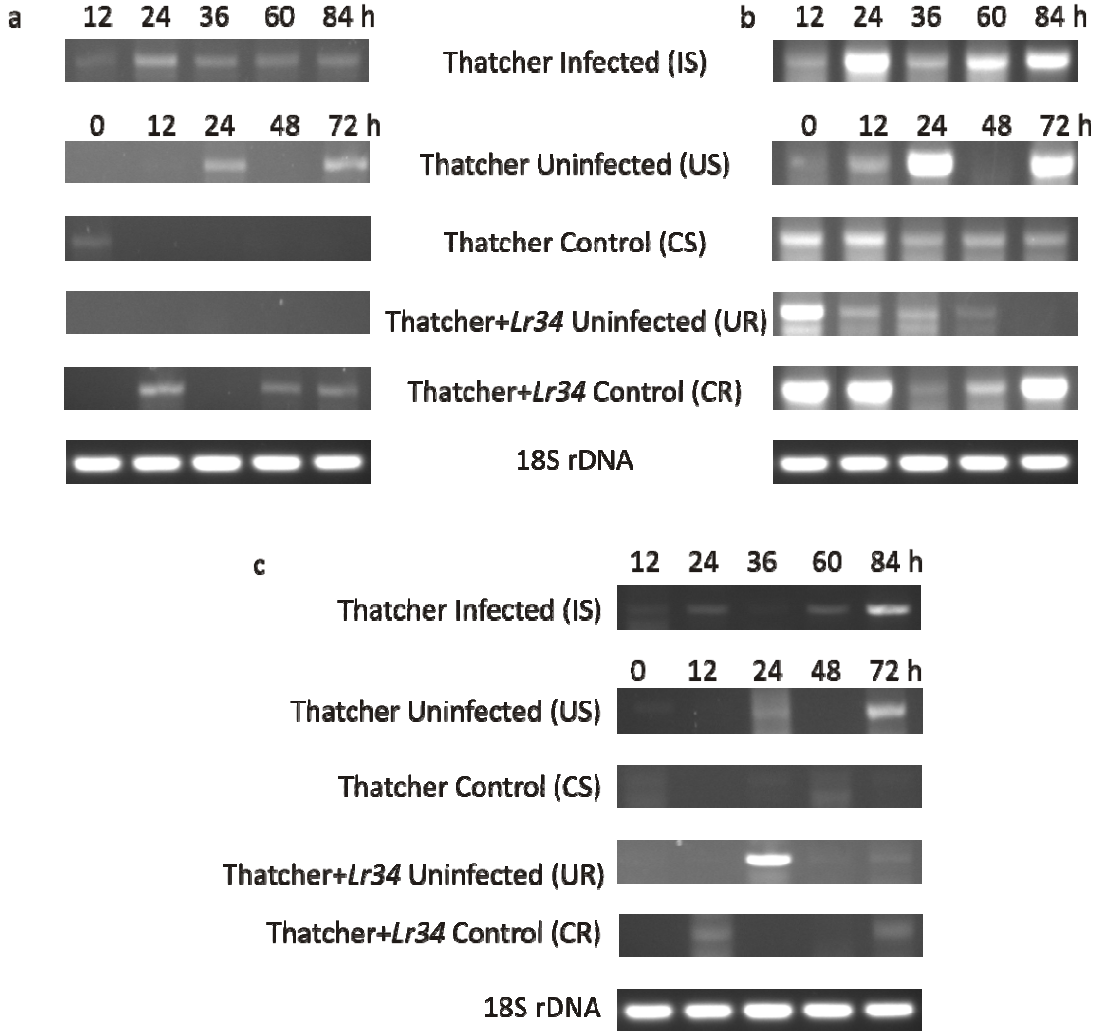

Figure 5.1: Selected defence related marker gene expression during the interaction between infected Thatcher (IS) and uninfected Thatcher (US) and *Thatcher+Lr34* (UR). In (a) *PR2* gene expression, in (b) *PR5* gene expression and in (c) *TaHlp01* gene expression is presented.

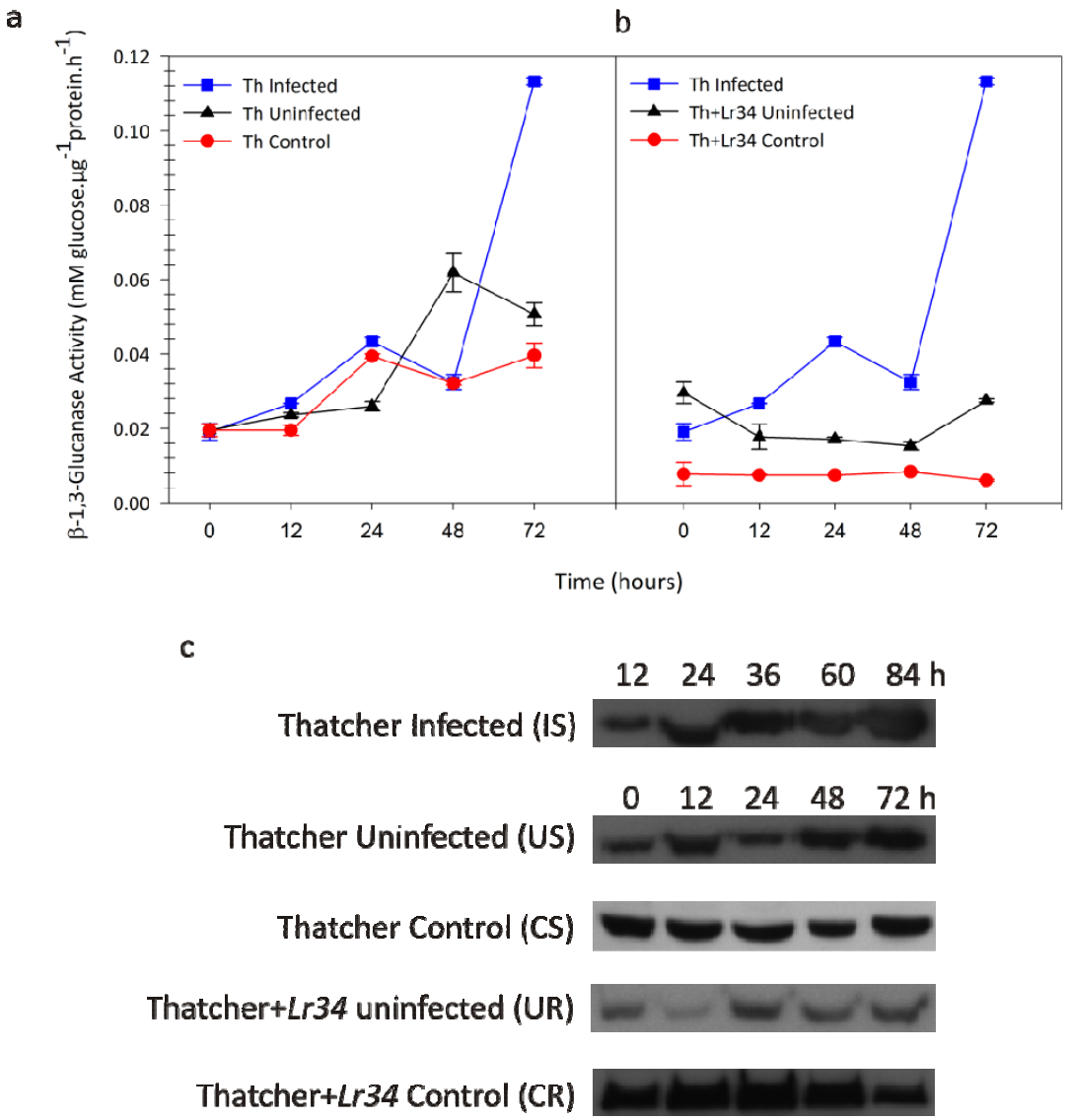

Figure 5.2: β-1,3-glucanase activity for infected Thatcher (IS) and its interaction with (a) Thatcher (US) and (b) Thatcher+*Lr34* (UR). In (c) β-1,3-glucanase protein levels were determined. Error bars indicate standard deviation and n=3.

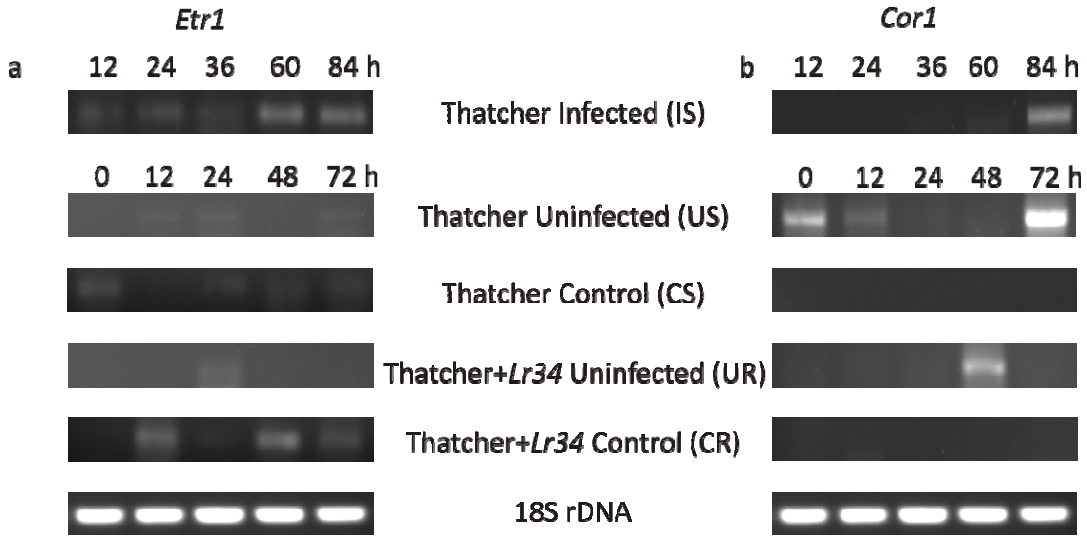

Figure 5.3: Expression of marker genes involved in different volatile defence pathways during the interaction between infected Thatcher (IS) and uninfected Thatcher (US) and uninfected Thatcher+*Lr34* (UR). In (a) *Etr1* and in (b) *Cor1* gene expression was determined.

In the UR plants there was a slight increase in *Etr1* expression 24 h.p.i (Fig 5.3a) while *Cor1* showed an increase in expression 48 h.p.i (Fig 5.3b).

Since the expression of both Etr1 and Cor1 was induced, it can be assumed that JA/ethylene defence play a key role in the defence response of the IS plants. Since Cor1 was inducibly expressed in US and UR plants, it can be concluded that these plants detected a JA related volatile signal emitted by the IS plants.

5.3.2 THATCHER+*LR34* INFECTION-RELATED COMMUNICATION

5.3.2.1 Thatcher*+Lr34* Infected with leaf rust

During the infection of *Thatcher+Lr34* plants, there was an increase in *PR2* gene expression, reaching a maximum expression level 36 h.p.i (Fig 5.4a). In contrast, *PR5* expression was repressed from 36 h.p.i onwards (Fig 5.4b) while the expression of *TaHlp01* was repressed and induced again at 36 h.p.i (Fig 5.4c).

β-1,3-glucanase activity increased in the IR plants, reaching a maximum from 24 to 48 h.p.i with a subsequent decrease at 72 h.p.i (Fig 5.5a). β-1,3-glucanase protein levels also increased from 24 h.p.i reaching a maximum level at 84 h.p.i (Fig 5.5c). This supported the induced expression pattern of *PR2*.

In the CS plants there was a repression of *PR2* (Fig 5.4a) and *PR5* gene expression (Fig 5.4b). *TaHlp01* expression remained constant (Fig 5.4c). For β-1,3-glucanase activity, the CS plants showed constant levels of activity (Fig 5.5a) as well as constant β-1,3-glucanase protein levels (Fig 5.5c).

In the CR plants, *PR2* gene expression increased 12 h.p.i (Fig 5.4a) while *PR5* and *TaHlp01* expression either stayed constant or decreased (Fig 5.4b and c). β-1,3-glucanase activity and protein levels also stayed constant during the time trial with a slight decrease in both at 72 h.p.i (Fig 5.5b and c).

Thus, even though the defence response of IR plants was activated as seen by *PR2* gene expression and β-1,3-glucanase activity, it is important to note that in contrast to IS plants, *PR5* and *TaHlp01* gene expression were not induced.

5.3.2.2 Uninfected Thatcher and Thatcher*+Lr34*

In the US plants, there was no increase in *PR2* or *TaHlp01* gene expression (Fig 5.4a and c) while a strong increase in *PR5* gene expression was evident from 12 h.p.i (Fig 5.4b). β-1,3-glucanase activity remained constant until 72 h.p.i when there was a slight increase in activity (Fig 5.5a). This increase coincided with an increase in β-1,3-glucanase protein levels 72 h.p.i (Fig 5.5c).

In the UR plants there was an increase in *PR2* gene expression 48 h.p.i (Fig 5.4a) as well as an increase in *PR5* gene expression 72 h.p.i after an initial repression (Fig 5.4b). The expression for *TaHlp01* increased from 24 h.p.i reaching the maximum level at 48 h.p.i (Fig 5.4c). β-1,3-glucanase activity showed an initial increase in activity 12 h.p.i, followed by another strong increase 72 h.p.i (Fig 5.5b). This increase in β-1,3-glucanase activity also coincided with an increase in β-1,3-glucanase protein levels 72 h.p.i (Fig 5.5c).

To conclude, US and UR plants reacted differently to volatile signals originating from IR plants. While the UR plants deployed an extensive defence response, only *PR5* gene expression was induced in the US plants. It seems however as if the *PR2* defence response is only later switched on in the US plants.

5.3.2.3 Expression of volatile defence marker genes

In the IR, US and UR plants, there was again no *NPR1* gene expression detected (results not shown). In the CS plants there was a constitutive expression for *Etr1* (Fig 5.6a), while there was no expression of *Cor1* detected (Fig 5.6b). In the CR plants, the expression of *Etr1* increased slightly at 12 h.p.i (Fig 5.6a) whereas no expression of *Cor1* was detected (Fig 5.6b).

In the US plants, there was an initial high expression of *Etr1* (Fig 5.6a) which was repressed from 12 h.p.i only to return to the initial levels 72 h.p.i. In the same set of plants there was only a slight increase in *Cor1* expression detectible at 12 h.p.i (Fig 5.6b). In the UR plants, there was a strong increase in *Etr1* expression 48 h.p.i (Fig 5.6a) with no visible expression of *Cor1* (Fig 5.6b). It is thus clear that in contrast to the IS plants, IR plants did not activate the JA/Ethylene pathway.

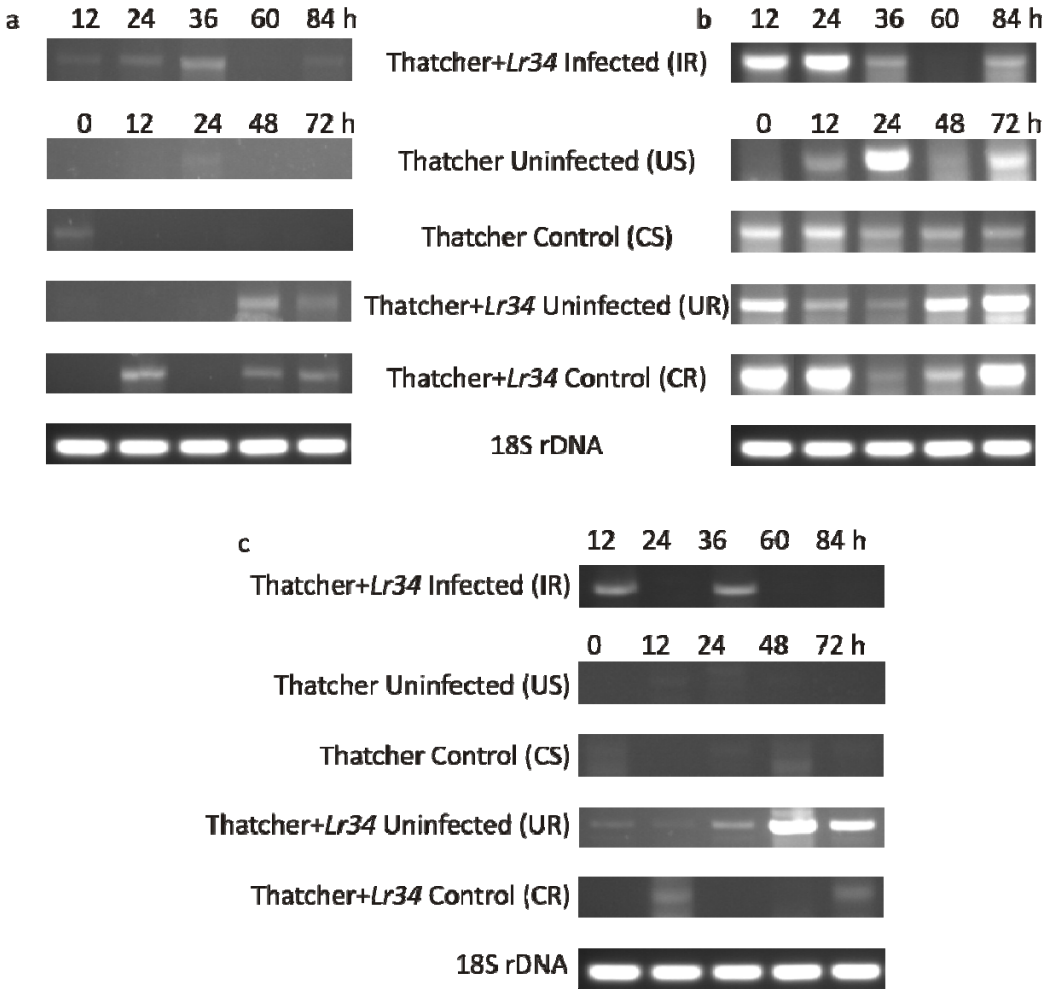

Figure 5.4: Selected defence related marker gene expression during the interaction between infected Thatcher*+Lr34* (IR) and uninfected Thatcher (US) and *Thatcher+Lr34* (UR). In (a) *PR2* gene expression, in (b) *PR5* gene expression and in (c) *TaHlp01* gene expression is presented.

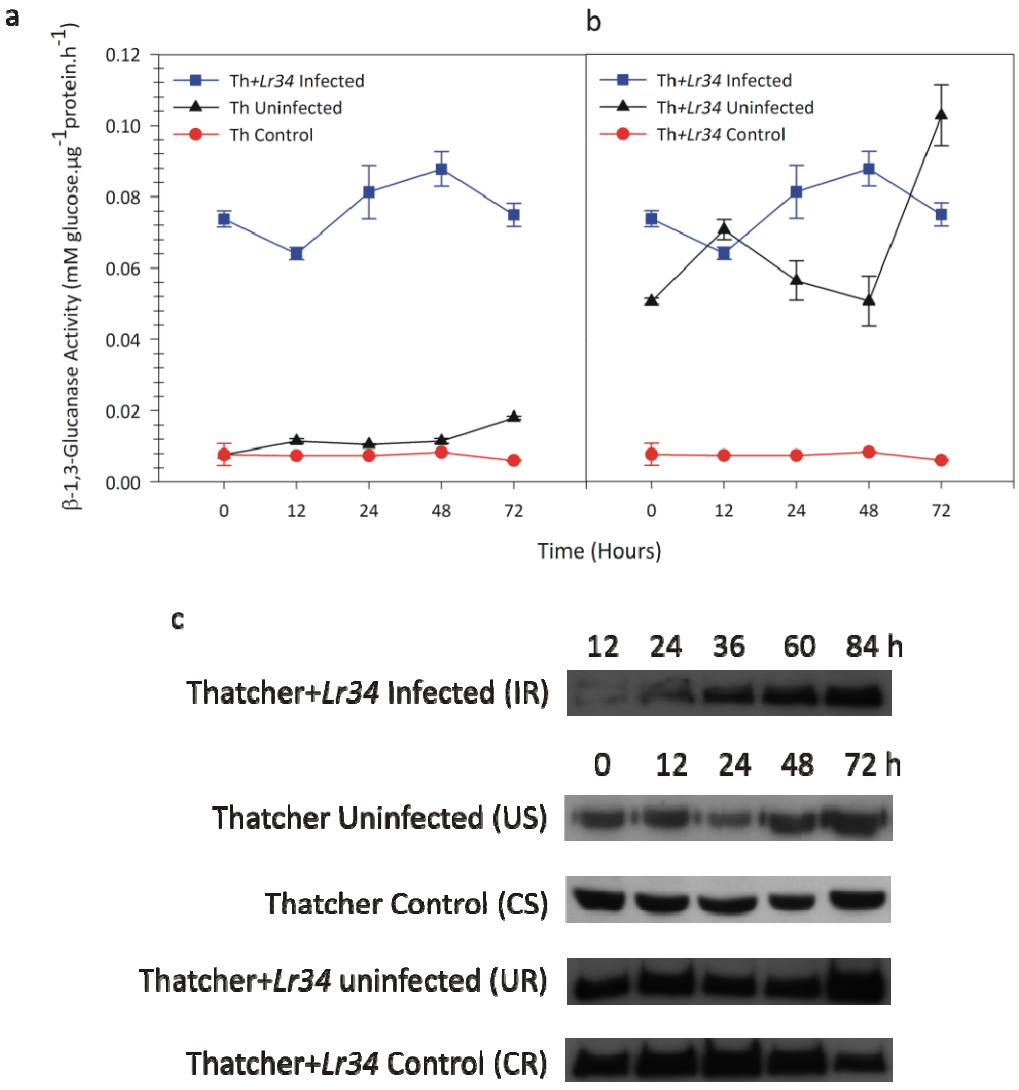

Figure 5.5: β-1,3-glucanase activity for infected Thatcher+*Lr34* (IR) and its interaction with (a) uninfected Thatcher (US) and (b) Thatcher+*Lr34* (UR). In (c) β-1,3-glucanase protein levels were determined. Error bars indicate standard deviation and n=3.

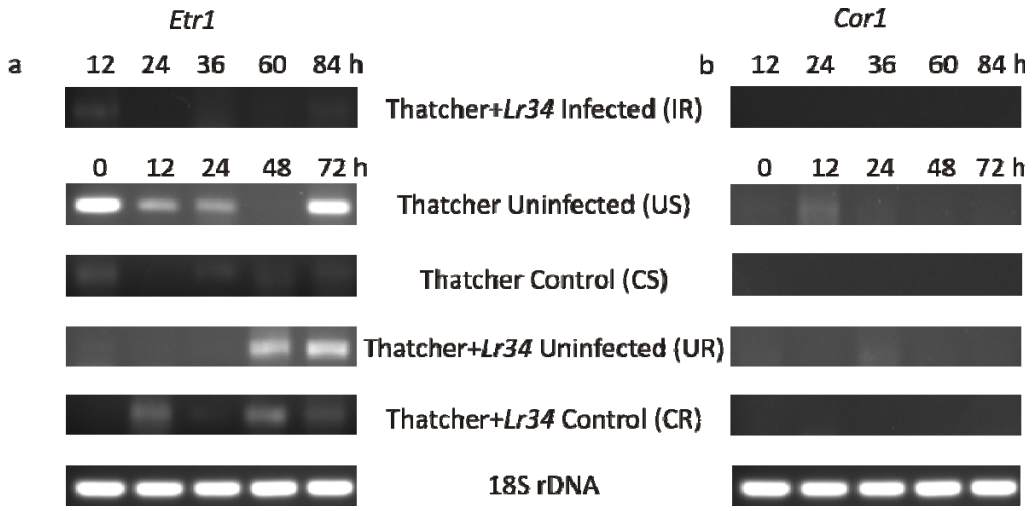

Figure 5.6: Expression of indicator genes involved in different volatile defence pathways during the interaction between infected Thatcher+*Lr34* (IR) and uninfected Thatcher (US) and uninfected Thatcher+*Lr34* (UR). In (a) *Etr1* and in (b) *Cor1* gene expression was determined.

Despite this, *Etr1* gene expression is induced in the UR plants and an effective defence was activated. It is thus conceivable that another volatile compound is released by the IR plants.

5.3.3 Phenotypic Analysis Of The Defence Response Of US And UR Plants

To determine whether the induced defence response visible in the UR and US plants was effective to protect the plants against leaf rust infection, US and UR plants that were placed in the presence of IR plants were inoculated with leaf rust. After inoculation, spores on the later infected susceptible (LIS) and later infected resistant (LIR) plants were allowed to germinate and develop for 48 h before the leaves were examined microscopically.

On control infected Thatcher plants, 62% (± 29%) of all counted structures were colonies, 20% (± 17%) were small colonies with less than 6 haustorium mother cells (HMCs), 13% (± 8%) were germinated spores that aborted growth and 6% (± 6%) were germinated spores that did not penetrate the leaf (Fig 5.7). This is characteristic of a susceptible plant infected with leaf rust. In contrast, on leaves of LIS plants, only 4% (± 4%) of spores developed into colonies, 6% (± 6%) developed into small colonies with less than 6 HMCs, while 85% (± 9%) showed aborted growth and 7% (± 4%) did not penetrate the leaf.

On control infected Thather*+Lr34* plants, 6% (± 6%) of all counted structures were colonies (Fig 5.7), 11% (± 8%) were small colonies with less than 6 HMCs, 80% (± 13%) represented aborted growth of germinated spores and 4% (± 2%) were germinated spores that did not penetrate the leaf. This again is characteristic of resistant plants upon infection with leaf rust. In contrast, LIR plants had an average of 2% (± 2%) colonies, 10% (± 5%) formed small colonies with less than 6 HMCs, 85% (± 10%) aborted growth and 4% (± 4%) did not penetrate.

On control Thatcher plants, colonies achieved an average size of 9578 μm^2 (± 4923 μm^2) while in the US plants the colonies were 2816 μm^2 (± 826 μm^2) in size on average. On control Thatcher*+Lr34* plants, colonies developed to an average size of 6513 μm^2 (± 2748 μm^2). In contrast, colonies on communication Thatcher*+Lr34* plants developed to only 5115 μm^2 (± 5 μm^2).

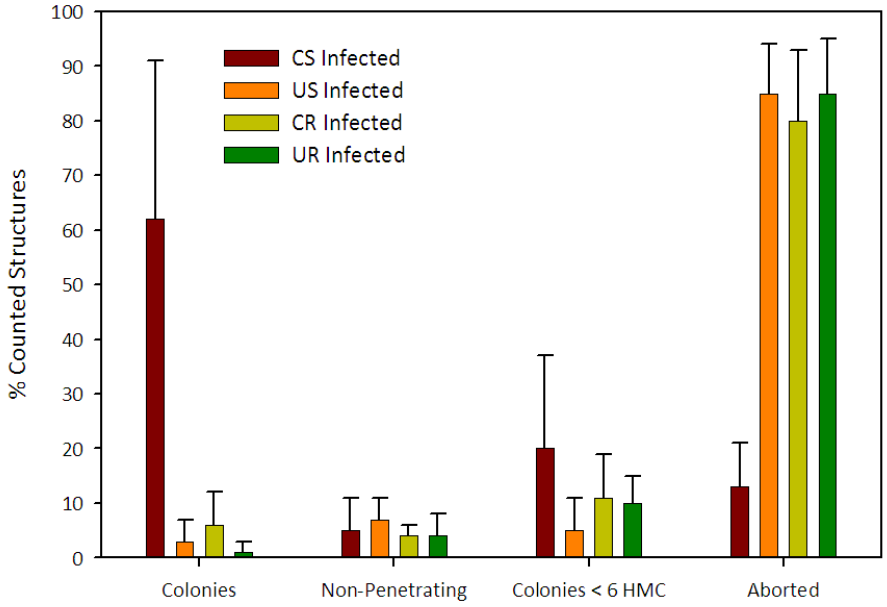

Figure 5.7: The effect of volatile emissions on the defence response of US and UR wheat. Error bars indicate the standard deviation and n=3.

Figure 5.8 indicates the flag leaves of the different plant sets after infection. White spots on the leaves are necrotic spots indicative of resistance while red-brown colonies are indications of susceptibility. The top leaf represents the original infected Thatcher*+Lr34* plants while the leaf second from the top represents the infected CR.

Both plants showed necrotic spots indicative of the HR leading to resistance. The third leaf from the top represents the LIR plants showing markedly more white necrotic lesions than the previous two. This indicates that a stronger defence response is functioning in the LIR plants leading to a strong resistance response. The fourth leaf from the top represents the infected CS plants while the fifth leaf represents the LIS plants. The former showed the characteristic red-brown pustules/colonies that are formed with very little necrotic lesions. The LIS plants on the other hand, showed many more necrotic lesions with fewer colonies, indicating a stronger defence response compared to the control.

5.4 DISCUSSION

Induced disease resistance in plants relies on the ability of the host to recognize the potential pathogen and trigger an appropriate response. Plants often employ distinct recognition mechanisms and defence pathways for different pathogenic elicitors. These pathways are not necessarily linear and the signals mediated by the major endogenous signal molecules SA, JA, ethylene and other VOC's appear to form a network of interactions (Li *et al.,* 2004).

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 (Oldroyed and Staskawicz, 1998) to name a few. This suggests that this enzyme plays a direct role in plant defence against pathogens. β-1,3-glucanase encoded by the *PR2* gene, is a pathogenesis related 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 as an increase in β-1,3-glucanase protein levels, would indicate the positive activation of a defence response.

Figure 5.8: The visible effect of volatile emissions on the defence responses of infected wheat. IR is infected resistant, CR is control resistant, LIR is later infected resistant, CS is control susceptible and LIS is later infected susceptible.

It was isolated from several plants after pathogen infection (Rebmann *et al.,* 1991; Reimmann and Dudler 1993; Xu and Reddy, 1997; Fagoaga *et al.,* 2001) and was found to have antifungal activity against several fungi.

Using these two marker genes, the activation of the defence response in both infected resistant and susceptible wheat was investigated. In both, *PR2* gene expression was induced within 24 h.p.i. which was accompanied by increased polypeptide levels. These elevated β-1,3-glucanase polypeptide levels in turn led to increased β-1,3-glucanase activity. In the IS plants, a six fold increase in enzyme activity was found at 72 h.p.i., while the IR plants achieved a maximum 1.5 fold increase in activity at 48 h.p.i. where after it decreased.

Anguelova *et al.* (1999) found an increase in β-1,3-glucanase activity within 48 h.p.i in adult *Thatcher+Lr35* wheat plants infected with leaf rust while Kemp *et al.* (1999) found an increase within 12 h.p.i in the Palmiet+*Lr29* and Palmiet+*Lr34* wheat cultivars infected with leaf rust. Dann *et al*. (1996) also found increases in β-1,3-glucanase activity 5 days after infection of green bean with *Colletotrichum lindemuthianum.*

When the expression of *PR5 is* considered*,* there was a clear difference between IS and IR plants. In the former, the gene was inducibly expressed but not in the IR plants where gene expression was repressed. It is therefore clear that in the IR plants, a limited activation of the defence response in the form of PR2 was sufficient to establish complete resistance against leaf rust infection. In contrast, IS plants activated an extensive defence response including both PR2 and PR5, yet a susceptible phenotype was the end result. One can therefore conclude that PR proteins alone are not sufficient to attain resistance against pathogen infection, but that other factors definitely also contribute to an effective resistance response.

In another study, *TaHlp01* was found to be inducibly expressed shortly after leaf rust infection of Thatcher and Thatcher+*Lr34* (Appelgryn, results unpublished). Even though the involvement of this gene in the defence response has not been proven, it was used as an additional marker to confirm the activation of a response in wheat following infection. Again there was a difference between IR and IS plants where the latter showed increased

expression, reaching a maximum at 72 h.p.i. In combination, these three marker genes confirm the activation a defence response in both cultivars following leaf rust infection.

These three genes were thus used to investigate the activation of a defence response in uninfected plants through a volatile defence event. When placed in the vicinity of IS plants, US plants showed increased expression of *PR2*, *PR5* and *TaHlp01.* This was accompanied by both increased β-1,3-glucanase protein levels and enzyme activity. These increases indicate the activation of the defence response even though the plants were not infected with the pathogen. These plants are therefore reacting to a putative volatile signal received from the IS plants.

In contrast, the UR plants exposed to IS plants showed no increase in *PR2* and *PR5* gene expression. This was also reflected in both protein levels and enzyme activity. There was however an increase in *TaHlp01* gene expression indicating that the UR plants did respond to a putative signal coming from the IS plants. This response however did not necessitate the activation of defence gene expression most probably since Thatcher+*Lr34* already have an effective defence mechanism that is capable to overcome leaf rust infection. The US plants thus reacted extensively on a volatile signal coming from the IS plants, while the response of the UR plants was only partial.

The exact opposite was found when US and UR plants were exposed to IR plants. In the US plants, there was increased *PR5* gene expression but no changes in *PR2* and *TaHlp01* gene expression. Both β-1,3-glucanase protein levels and activity was constant until 72 h.p.i. where slight increases in both were visible. In the UR plants increased expression was found for *PR2, PR5* and *TaHlp01* coinciding with a slightly later increase in β-1,3-glucanase activity and protein levels. During this interaction the UR plants responded with an extensive defence response compared to only a partial response in the US plants.

To investigate the effectiveness of this induced defence responses on intact plants, US and UR plants were infected with leaf rust after exposure to IR plants. It was evident that the LIS plants showed an increased resistance against leaf rust since there was a 6 fold drop in developed colonies and a 4 fold drop in colonies with less than 6 haustorium mother cells compared to the CS plants. In addition the abortion rate of germinated spores increased 8 fold. Infected leaves also showed more necrotic flecks and fewer colonies compared to the

control plants. This clearly supports the evidence that the defence response was activated within the LIS plants. Even though the Thatcher+*Lr34* line is resistant against leaf rust, the LIR plants exhibited the same tendencies than the LIS plants. It is thus evident that both the LIR and LIS plants were primed on molecular level to withstand leaf rust infection.

Arimura *et al.* (2001) showed that lima bean leaves infested with spider mites were able to activate *PR2* gene expression in uninfested leaves within one day after exposure to infested leaves. They however did not describe the influence that the induced defence response had on subsequent infesting spider mites. Karban *et al.* (2000) showed that tobacco plants exposed to damaged sagebrush showed increased resistance against herbivore attacks indicating the positive role that volatile emissions could play on neighbouring plants regarding defence.

To summarize, this work proves that wheat infected with leaf rust is able to prime neighbouring plants and thereby improving their resistance against a pathogen. It is furthermore evident that the most effective communication was between similar cultivars, but despite this, even partial defence activation in the US plants was still sufficient to improve the resistance of the susceptible cultivar.

Induced defence responses are regulated by a network of interconnecting signal transduction pathways in which the hormonal signals SA, JA and ethylene play a major role (Pieterse and Van Loon, 2004). Ethylene is produced upon wounding or infection by pathogens as well as by treatment with elicitors of defence responses (Boller, 1995). It is known to travel through the atmosphere to activate plant defensive genes (Farmer and Ryan, 1990) and was found to be involved in plant-plant interactions (Ruther and Kleier, 2005). The *Etr1* gene encodes a receptor of ethylene and its expression is induced upon exposure to ethylene (O'Malley *et al.,* 2005).

The expression of the *AtCor1* gene is induced upon treatment with methyl jasmonate (Li *et al.,* 2004) and is believed to belong to a family of related enzymes involved in the biosynthesis/hydrolysis of plant cell wall components (Benedetti *et al.,* 1998). Methyl jasmonate, an end product of the jasmonate pathway was proven to be involved in interplant communication in *Artemisia tridentate* (Farmer and Ryan, 1990), *Nicotiana attenuata* (Preston *et al.,* 2004) and in grapevine (Larronde *et al.,* 2003).

NPR1 is an essential regulator of plant systemic acquired resistance (SAR), which confers immunity to a broad-spectrum of pathogens (Pieterse and Van Loon, 2004). It accumulates in the nucleus and activates gene expression. Inhibition of *NPR1* prevents defence gene expression (Mou *et al.,* 2003).

During the investigation of volatile defence in wheat, the expression of these three genes was used as markers for the different signal transduction pathways mediated by ethylene, methyl jasmonate and methyl salicylate respectively.

As part of the resistance response of the IS plants, both *Etr1* and *Cor1* gene expression was induced. This indicates that both an ethylene and a JA based signal were produced during infection. In the IR plants, there was no detectable change in expression for either *Etr1* or *Cor1* at all. In both the infected cultivars, no expression of *NPR1* was found indicating that at this stage SA based defence is not functional. Several studies found evidence that JA defence negatively regulates the expression of SA-responsive genes in *Arabidopsis thaliana* (Kachroo *et al.,* 2001; Kloek *et al.,* 2001). This might explain the lack of *NPR1* expression in the IS plants since there was increased JA-related gene expression. This however does not explain the lack of *NPR1* gene expression in the IR plants since there were no detectable changes in JA-related gene expression in the IR plants. It is however possible that during later stages of infection where SAR might be functioning, that this aspect could come into play.

Karban *et al*. (2000) and Preston *et al.* (2001) found that sagebrush increased methyl jasmonate production by up to 16 times upon damage and can further 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). Xu *et al.* (1994) found that ethylene as well as methyl jasmonate were able to activate *PR5* gene expression when it was applied endogenously to tobacco seedlings.

While IS plants showed increased levels of both *Etr1* and *Cor1* gene expression, the US and UR plants only displayed increased *Cor1* gene expression. This clearly demonstrates that both UR and US plants only reacted on the JA based signal coming from the IS plants. When

exposed to IR plants on the other hand that did not show any induced expression for both *Etr1* and *Cor1*, only the UR plants showed increased expression of *Etr1*. This indicates that the US and UR plants reacted differently to volatile signals emitted from the IR plants. In the case of the UR plants they reacted strongly to an ethylene-based signal while the US plants did not.

To summarize, in reaction to a JA based signal coming from the IS plants, US plants activated an extensive defence response while the UR plants showed only a limited response. This was despite the fact that both ethylene and JA based volatiles were produced by the IS plants. In contrast, even though it seems as if no such volatiles were produced by the IR plants, the UR plants produced an extensive response that was seemingly based on an unknown volatile signal. It is tempting to speculate that since this study used near-isogenic wheat lines, either the production or the detection of the volatile signal could be linked to the resistance locus present in Thatcher+*Lr34.* The induced expression of *Etr1* in the UR plants could therefore be a byproduct of the extensive induced defence response of the UR plants that were kept in the presence of IR plants. The identity and nature of such a novel volatile that is produced by the IR plants will be investigated in the future using GC/MS analysis.

To conclude, the results strongly suggests that a communication event took place between infected and uninfected wheat and that this communication was in general the most effective between plants of the same cultivar. These communication events are not always noticeable in field conditions and the closed environment of a glasshouse probably enhanced the effect of volatiles emitted by infected plants (Arimura *et al.,* 2001). This phenomenon does however hold great potential to be exploited as a natural method to induce the defence responses of crops in field conditions.

5.5 REFERENCES

Anguelova V.S., van der Westhuizen A.J. and Pretorius Z.A. 1999. **Intercellular proteins and β-1,3-glucanase activity associated with leaf rust resistance in wheat**. *Physiologia Plantarum* 106: 393-401

Arimura G-I., Ozawa R., Shimoda T., Nishioka T., Boland W. and Takabayashi J. 2000. **Herbivory-induced volatiles elicit defence genes in Lima bean leaves**. *Nature* 406: 512-515

Arimura G-I., Ozawa R., Horiuchi J-I., Nishioka T. and Takabayashi J. 2001. **Plant-plant interactions mediated by volatiles emitted from plants infested by spider mites.** *Biochemical Systematics and Ecology* 29: 1049-1061

Baldwin I.T. and Schultz J.C. 1983. **Rapid changes in tree leaf chemistry induced by damage: evidence for communication between plants**. *Science* 221: 277-279

Baldwin I.T., Kessler A. and Halitschke R. 2002. **Volatile defence in plant-plant herbivore interactions: what is real?** *Current Opinion in Plant Biology* 5: 1-4

Bate N.J. and Rothstein S.J. 1998. **C_6-volatiles derived from the lipoxygenase pathway induce a subset of defence-related genes**. *The Plant Journal* 16: 561-569

Benedetti C.E., Costa C.L., Turcinelli S.L. and Arruda P. 1998. **Differential expression of a novel gene in response to coronatine, methyl jasmonate and wounding in the *Coi1* mutant of *Arabidopsis***. *Plant Physiology* 116: 1037-1042

Boller T. 1995. **Chemoperception of microbial signals in plant cells.** *Annual Review of Plant Physiology and Plant Molecular Biology* 46: 189-214

Bradford M.M. 1976. **A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.** *Analytical Biochemistry* 72: 248-253

Burnette W.N. 1981. **"Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic**

detection with antibody and radioiodinated protein A. *Analytical Biochemistry* 112: 195-203

Caruso C., Chilosi G., Caporale C., Leonardi L., Bertini L., Magro P. and Buonocore V. 1999. **Induction of pathogenesis-related proteins in germinating wheat seeds infected with *Fusarium culmorum.*** *Plant Science* 140: 107-120

Chomczynski P. and Sacchi N. 1987. **Single step method of RNA isolation by acid Guanidine thiocyanate – Phenol Chloroform extraction.** *Analytical Biochemistry* 162: 156-159

Dann E.K., Meuwly P., Métraux J-P. and Deverall B.J. 1996. **The effect of inoculation or chemical treatment on activities of chitinase and β-1,3-glucanase and accumulation of salicylic acid in leaves of green bean, *Phaseolus vulgaris* L.** *Physiological and Molecular Plant Pathology* 49: 307-319

Dicke M., Sabelis M.W., Takabayashi J., Bruin J. and Posthumus M.A. 1990. **Plant strategies of manipulating predator-prey interactions through allelochemicals: prospects for application in pest control.** *Journal of Chemical Ecology* 16: 3091-3118

Engelberth J., Alborn H.T., Schmelz E.A. and Tumlinson J.H. 2004. **Airborne signals prime plants against insect herbivore attack.** *Proceedings of the National Academy of Sciences USA* 101: 1781-1785

Fagoaga C., Rodrigo I., Conejero V., Hinarejos C., Tuset J.J., Arnau J., Pina J.A., Navarro L. and Pena L. 2001. **Increased tolerance to *Phytophthora citrophthora* in transgenic orange plants constitutively expressing a tomato pathogenesis-related protein PR-5.** *Molecular Breeding* 7: 175-185

Farag M.A., Fokar M., Zhang H.A., Allen R.D. and Pare P.W. 2005. **(Z)-3-hexenol induces defence genes and downstream metabolites in maize**. *Planta* 220: 900-909

Farmer E.E. and Ryan C.A. 1990. **Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves.** *Proceedings of the National Academy of Sciences USA* 87: 7713-7716

Fink W., Liefland M. and Mendgen K. 1988. **Chitinases and β-1,3-glucanases in the apoplastic compartment of oat leaves (*Avena sativa* L.).** *Plant Physiology* 88: 270-275

Huang J., Cardoza Y.J., Schmelz E.A., Riana R., Engelberth J. and Tumlinson J.H. 2003. **Differential volatile emissions and salicylic acid levels from tobacco plants in response to different strains of *Pseudomonas syringae***. *Planta* 217: 767-775

Hu X. and Reddy A.S.N. 1997. **Cloning and expression of a PR5-like protein from *Arabidopsis*: inhibition of fungal growth by bacterially expressed protein.** *Plant Molecular Biology* 34: 949-959

Jung J-L., Fritig B. and Hahne G. 1993. **Sunflower (*Helianthus annuus* L.) pathogenesis-related proteins.** *Plant Physiology* 101: 873-880

Kachroo P., Shanklin J., Shah J., Whittle E.J. and Klessig D.F. 2001. **A fatty acid desaturase modulates the activation of defence defence pathways in plants.** *Proceedings of the National Academy of Sciences USA* 98: 9448-9453

Karban R., Baldwin I.T., Baxter K.J., Laue G. and Felton G.W. 2000. **Communication between plants: induced resistance in wild tobacco plants following clipping of neighboring sagebrush.** *Oecologia* 125: 66-71

Kemp G., Botha A-M., Kloppers F.J. and Pretorius Z.A. 1999. **Disease development and β-1,3-glucanase expression following leaf rust infection in resistant and susceptible near-isogenic wheat seedlings.** *Physiological and Molecular Plant Pathology* 55: 45-52

Kloek A.P., Verbsky M.L., Sharma S.B., Schoelz J.E., Vogel J., Klessig D.F. and Kunkel B.N. 2001. **Resistance to *Pseudomonas syringae* conferred by an *Arabidopsis thaliana* coronatine-insenstive (coi1) mutation occurs through two distinct mechanisms.** *The Plant Journal* 26: 509-522

Laemmli U.K. 1970. **Cleavage of structural proteins during the assembly of the head of the bacteriophage T4.** *Nature* 227: 680-685

Larronde F., Gaudillère J.P., Krisa S., Decendit A., Deffieux G. and Mérillonn J.M. 2003. **Airborne methyl jasmonate induces stilbene accumulation in leaves and berries of grapevine plants.** *American Journal of Enology and Viticulture* 54: 63-66

Li J., Brader G. and Palva E.T. 2004. **The WRKY70 transcription factor: a node of convergence for Jasmonate-mediated and Salicylate-mediated signals in plant defence**. *The Plant Cell* 16: 319-331

Mou Z., Fan W. and Dong X. 2003. **Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes.** *Cell* 113: 935-944

Oldroyed G.E.D. and Staskawicz B.J. 1998. **Genetically engineered broad-spectrum disease resistance in tomato.** *Proceedings of the National Academy of Sciences USA* 95: 10300-10305

O'Malley R.C., Rodriguez F.I., Esch J.J., Binder B.M., O'Donnell P., Klee H.J. and Bleecker A.B. 2005. **Ethylene-binding activity, gene expression levels and receptor system output for ethylene receptor family members from *Arabidopsis* and tomato**. *The Plant Journal* 41: 651–659

Paschold A., Halitschke R. and Baldwin I.T. 2006. **Using "mute" plants to translate volatile signals.** *The Plant Journal* 45: 275-291

Pierpoint W.S., Jackson P.J. and Evans R.M. 1990. **The presence of a thaumatin-like protein, a chitinase and a glucanase among the pathogenesis-related proteins of potato (*Solanum tuberosum*).** *Physiological and Molecular Plant Pathology* 36: 325-338

Pieterse C.M.J. and Van Loon L.C. 2004. **NPR1: the spider in the web of induced resistance defence pathways.** *Current Opinion in Plant Biology* 7: 456-464

Preston C.A., Laue G. and Baldwin I.T. 2001. **Methyl jasmonate is blowing in the wind, but can it act as a plant-plant airborne signal?** *Biochemical Systematics and Ecology* 29: 1007-1023

Preston C.A., Laue G. and Baldwin I.T. 2004. **Plant–plant defence: application of *trans*- or *cis*-methyl jasmonate equivalent to sagebrush releases does not elicit direct defences in native tobacco**. *Journal of Chemical Ecology* 30: 2193-2214

Rebmann G., Mauch F. and Dudler R. 1991. **Sequence of a wheat cDNA encoding a pathogen-induced thaumatin-like protein.** *Plant Molecular Biology* 17: 283-285

Reimmann C. and Dudler R. 1993. **cDNA cloning and sequence analysis of a pathogen-induced thaumatin-like protein from rice (*Oryza sativa*).** *Plant Physiology* 101: 1113-1114

Rohringer R., Kim W.K., Samborski D.J. and Howes N.K. 1977. **Calcofluor: an optical brightener for fluorescence microscopy of fungal plant parasites in leaves.** *Phytopathology* 67: 808-810

Ruther J. and Kleier S. 2005. **Plant-plant defence: ethylene synergizes volatile emission in *Zea mays* induced by exposure to (Z)-3-Hexen-1-ol**. *Journal of Chemical Ecology* 31: 2217-2222

Rybutt D.B. and Parish C.R. 1982. **Protein determination on an automatic spectrophotometer.** *Analytical Biochemistry* 121: 213-214

Sambrook J., Fritsch E.F. and Maniatis T. 1989. **Molecular cloning: a laboratory manual.** 2nd ed. USA: *Cold Spring Harbour press*

Scheel D. 1998. **Resistance response physiology and signal transduction.** *Current Opinion in Plant Biology* 1: 305-310

Tscharntke T., Thiessen S., Dolch R. and Boland W. 2001. **Herbivory, induced resistance and interplant signal transfer in *Alnus glutinosa***. *Biochemical Systematics and Ecology* 29: 1025-1047

Xu Y., Chang P.F.L., Liu D., Narasimhan M.L., Raghothama K.G., Hasegawa P.M. and Bressan R.A. 1994. **Plant defence genes are synergistically induced by ethylene and methyl jasmonate**. *The Plant Cell* 6: 1077-1085

Xu H. and Reddy A.S.N. 1997. **Cloning and expression of a PR5-like protein from *Arabidopsis*: inhibition of fungal growth by bacterially expressed protein.** *Plant Molecular Biology* 34: 949-959

York W.S., Darvill A.G., McNeil M., Stevenson T.T. and Albersheim P. 1985. **Isolation and characterization of plant cell walls and cell wall components**. *Methods in Enzymology* 118: 3-40

Chapter 6

General Discussion

Plants are major targets for pathogenic microbes seeking a source of nutrition. A complex array of interactions has thus evolved between plants and microbes to reflect both the nutrient acquisition strategies of the microbes as well as the defence strategies of plants. To be a successful pathogen, a microorganism needs to interfere with one or more essential functions of the plant, thereby causing disease. Regardless of the type of pathogen, a prerequisite for pathogenicity is the ability to gain access to the plant's interior. This is achieved through different means. Some pathogens take advantage of natural openings such as stomata or lenticels or enter the plant through wounds, while others simply penetrate the leaf surfaces (Felle *et al.,* 2004; Gourgues *et al.,* 2004; Amiri and Bompeix, 2005; Franceschi *et al.,* 2005*;* Takano *et al.,* 2006). In most fungal diseases, the fungus not only penetrates the cuticle but the cell wall as well which is the next obstacle for pathogens after reaching the intercellular spaces or apoplast (Parniske, 2004; An *et al.,* 2006).

Due to the fortress-like cell structure of plants, their innate ability to recognize potential pathogens and their effective defences, plants are generally resistant to most pathogens (Heath, 2000; Nürnberger *et al.*, 2004). The ability to detect potential pathogens has been essential to the development of modern plants (Chisholm *et al.,* 2006). Perception of the pathogen is achieved through receptors, a complex surveillance system capable of recognizing both conserved molecular patterns and specific effector proteins. Once the pathogen is perceived, it leads to the activation of the corresponding defence responses in the plant (Montesano *et al.,* 2003). Thus, in order to be successful, a pathogen needs to evade the plant surveillance system or suppress the plant defences.

Plants defend themselves against invaders in two ways. The first is by means of structural barriers that inhibit the pathogen from gaining entrance and spreading throughout the plant. Secondly, plants employ biochemical pathways to produce toxic substances that inhibit the growth of the pathogen. Combinations of these two defence types vary between different plant-pathogen interactions (Glazebrook, 2005). If the plant fails to recognize the pathogen or an elicitor, the appropriate defences will not be activated and disease results. Alternatively, if the plant responds with a rapid and well-aimed activation of defences, the attempted infection is halted.

Basal defence, a constituent of both nonhost and host resistance, provides basal-level resistance (also called innate immunity or local induced resistance) that prevents infection by a wide range of microbes (Heath, 2000; Thordal-Christensen, 2003; Nürnberger *et al.,* 2004; Oh and Collmer, 2005). Some pathogens have acquired the ability to suppress basal defence responses and enhance their avirulence by delivering specific effector proteins to the plant cells that interfere with plant defence. Gene-for-gene or race-cultivar-specific resistance occurs when specific members of a plant species, but not the species as a whole, have acquired resistance to a particular pathogen. This type of resistance is usually restricted to a particular pathogen species and is effective against specific genotypes of that pathogen (Dangl and Jones, 2001; Bonas and Lahaye, 2002; Hammond-Kosack and Parker, 2003; Chisholm *et al.,* 2006).

Inducible plant defences are triggered by the perception of a pathogen or pathogen-derived molecules called elicitors. The elicitors can be either general or specific to certain pathogen strains. In addition, pathogens can release polysaccharide oligomers from the plant surface, which could activate the defence response (Montesano *et al.,* 2003; Nürnberger *et al.,* 2004; Chisholm *et al.,* 2006). Perception of these elicitors takes place either at the cell surface or inside the cell (Dardick and Ronald, 2006). Recognition of general and specific elicitors triggers overlapping defence responses in the plant (Espinosa and Alfano, 2004; Kim *et al.,* 2005). These defence pathways are not necessarily linear and the signals mediated by the major endogenous signal molecules SA, JA, ethylene and other VOC's appear to form a network of interactions (Li *et al.,* 2004).

Recognition of the elicitor induces several early responses including the phosphorylation and dephosphorylation of plasma membrane proteins, increase of cytosolic Ca^{2+} ion fluxes, alkalization of the apoplast, as well as the activation of transcription factors and the early expression of defence genes (Wojtaszek, 1997; Peck, 2003; Dardick and Ronald, 2006). The synthesis of various defence-related proteins (e.g. PR proteins), which have antimicrobial activity and thus serve to contain the infection, is also activated (Wojtaszek, 1997; Hammerschmidt, 1999; Van Loon and Van Strien, 1999). The signals originating from the local infection site can then evolve into a systemic defence response where distal, undamaged parts of the plant develop resistance to future pathogen infections.

The study of plant-pathogen interactions can provide tools for the development of more durable approaches. For example, knowledge of the types of molecular responses activated in the plant during the infection by different pathogens can have tremendous potential. Plant defence responses are a result of a complex network of defence events that involves the interplay of protein kinases, hormones and reactive oxygen species, leading to the reprogramming of the plant transcriptome (Navarro *et al.,* 2004; Devoto *et al.,* 2005; Brachmann, 2006). These responses aim at the production of defence compounds and, finally, resistance. Elucidation of the molecular components acting in these cascades provides useful tools for engineering more durable crops and resistance that is not easily broken down.

In this study, DDRT-PCR was used to identify genes whose expression was induced in wheat in response to infection with leaf rust. Several putative genes were cloned, but upon closer investigation, only four were inducibly expressed exclusively in the resistant Thatcher*+Lr34* cultivar. These genes coded for a putative monosaccharide transporter, a putative cell wall invertase, a putative molecular chaperone and a putative indole-3-glycerol phosphate lyase respectively. In several other studies, the involvement of similar genes has been found in the defence response against a pathogen (Mendgen and Nass, 1988; Frey *et al.* 2000; Hall and Williams, 2000; Büttner and Sauer, 2001; Roitsch *et al.,* 2003; Wang *et al.,* 2004). These encoded proteins will therefore play definite roles in the defence response of Thatcher+*Lr34* infected with leaf rust.

Wheat rust fungi are very specific obligate parasites that interact with wheat in a gene-for-gene relationship (Person, 1959; Flor, 1971). When a rust spore lands on a host plant, it germinates and grows to the nearest stomata (Schulze-Lefert and Panstruga, 2003). After entering the leaf, the pathogen has to redirect the host's metabolic flow to its own benefit without killing the host.

Cell wall invertases are proteins that catalyze the cleavage of sucrose released into the apoplast via sucrose transporters. The resulting monomers are transported into the sink cells by monosaccharide transporters (Hall and Williams, 2000; Roitsch *et al.,* 2003). The activation of cell wall invertase and monosaccharide transporter gene expression during this study could be the result of two scenarios. In the first, the plant's own invertases and

monosaccharide transporters are activated to replenish carbon sources lost as a result of the activated cellular defence response or to replenish nutrients withdrawn by the fungus (Biemelt and Sonnewald, 2006). Secondly, the fungus could release its own invertases and monosaccharide transporters into the apoplastic space to acquire nutrients (Voegele *et al.,* 2001). These two cloned genes are currently being investigated as part of another study to ascertain their respective origins and roles during this interaction.

Molecular chaperones are key components contributing to cellular homeostasis in cells under both optimal and abnormal growth conditions (Grene, 2002). They are responsible for the folding, assembly, translocation and degradation of proteins in a broad array of cellular processes, including stress conditions (Bukau and Horwich, 1998; Forreiter and Nover, 1998; Sung *et al.,* 2001). Many molecular chaperones are stress proteins and most were originally identified as heat-shock proteins (Hsp) (Lindquist, 1986; Lindquist and Craig, 1988). The putative molecular chaperone cloned during this study showed high similarity to Hsp70. Current models propose that Hsp70 binds to nascent polypeptides thereby preventing improper folding of the polypeptide and also appears to maintain these polypeptides in a form competent for transport (Boston *et al.,* 1996).

Thus, due to the high similarity of *TaHlp01* to Hsp70 and the fact that it is inducibly expressed upon pathogen infection, implicate that the encoded protein is involved in the defence response of wheat upon fungal infection. It is however possible that its expression is an indirect result of infection. As a result of the chaperone functions of Hsp70, *TaHlp01* is presumably involved in the transport and folding of defence related polypeptides that are synthesized as a direct result of the defence response. A second possible role is that *TaHlp01* could form part of a chaperone complex since the cloned *TaHlp01* lacked a carboxy-terminal substrate binding domain. It has been found that an Hsp70 was necessary for Hsp90 function during the activation of a protein kinase (Kimura *et al.,* 1995). By binding to another chaperone, *TaHlp01* could thus modulate the activity of the former. Another cue for the indirect involvement of *TaHlp01* during resistance is the inability of chemicals that normally activate the plant's defence response, to induce the expression of *TaHlp01*. In fact, its expression is repressed by SA, a key component of SAR. It is important to note that the *TaHlp01* gene is present and expressed in both resistant and susceptible wheat cultivars, but is only inducibly expressed in the resistant cultivars after infection with rust. This

indicates that its regulation with regard to defence is dependent on the resistance loci in these wheat lines. Future studies will however have to investigate its role during infection with other pathogens such as bacteria or viruses.

Upon appropriate stimulation, resistance can also be induced systemically in the noninfected tissues of the plant. Pathogens, soilborne microorganisms, various chemicals and several forms of stress can enhance the tolerance of a plant to future pathogen attacks. Induced resistance is often associated with an enhanced capacity to mobilize cellular defence responses. Plants expressing these genes are thus "primed" for potentiated induction of defence responses when they encounter a pathogen attack (Conrath *et al.,* 2002; Van Hulten *et al.,* 2006). The classic example of induced resistance is SAR that is controlled by a defence pathway that depends on endogenous accumulation of SA (Malamy *et al.,* 1990; Métraux *et al.,* 1990; Uknes *et al.,* 1992; Durrant and Dong, 2004). SAR is associated with the accumulation of defence compounds such as PR proteins in the uninfected parts of the plant, and it is mainly effective against biotrophic pathogens (Glazebrook, 2005).

Plants have evolved to respond with sophisticated mechanisms to attack by herbivores and certain pathogens that rapidly destroy plant tissues. Wounding induces the expression of defensive foliar compounds that have toxic effects on the invader. In addition, plants under attack can also emit volatile substances that act indirectly by attracting predators of the herbivore (Schilmiller and Howe, 2005; Wasternack *et al.,* 2006). Importantly, volatile signals originating from the initial wound site can induce systemic resistance in healthy plants located considerable distances away that will protect these plants against a broad spectrum of future attackers (Howe, 2004).

When uninfected resistant and susceptible plants were thus exposed to infected resistant and susceptible plants respectively, increases in defence related gene expression, including *TaHlp01* and *PR2*, were found. This indicated that the infected plants were able to induce the expression of defence related genes in uninfected plants via a volatile signal that was transmitted. It was also evident that the activation of the defence responses was more effective between plants of the same cultivar than between plants of different cultivars. This could indicate that resistant plants may communicate more effectively with each other,

possibly through an unique defence ability due to the presence of the resistance locus. Even though a specific volatile signal has not yet been identified, it is evident from the marker gene expression, that methyl jasmonate, amongst others, is a prime candidate (Farmer and Ryan, 1990; Kessler *et al.,* 2006). Hamilton and Coleman (2001) found that methyl jasmonate was able to induce the accumulation of Hsp70 in distant untreated leaves of *Nicotinia attenuata*. In contrast to this, no accumulation of *TaHlp01* in uninfected plants exposed to infected plants was noted. This again strengthens the assumption that *TaHlp01* expression is an indirect result of the defence response.

The effectiveness of the interplant communication was confirmed when it was found that the induction of plant defences was not limited to induced gene expression, but that it actually improved the phenotypical resistance as well. When uninfected resistant and susceptible plants were infected after exposure to infected resistant plants, a 4 fold drop in colony formation was observed in both. The susceptible plants however showed a bigger increase in resistance compared to the resistant plants. This clearly indicates that the exposure to infected plants improved the resistance in these plants and warrants further investigation of its application in field trials. This phenomenon could possibly be exploited as a cheap, natural alternative for combating disease. However, it might be possible that the results obtained are enhanced by the closed space of the glasshouse (Arimura *et al.,* 2001) and that during field conditions, the effect of the volatile emissions might be muted as a result of this.

To summarize, this study contributed to the better understanding of plant defence in several ways. In the first instance, a novel truncated heat shock like protein was identified whose expression seems to be regulated by the resistance loci present in resistant wheat cultivars. The role of the encoded protein is still unclear, but it can be foreseen that the protein should play a key role during the controlled chaos following infection. Secondly, a possible volatile defence event between infected and uninfected plants was investigated. This particular field of research is very controversial due to several reasons (Karban *et al.*, 2000; Dicke and Bruin, 2001), but this study has proven the existence of such an event within the accepted limitations and borders of published work. Even though the effectiveness of this defence is not clearly evident in field conditions, controlled laboratory conditions have proven its existence. Future studies on both the abovementioned aspects

would aim to further establish the roles and importance of *TaHlp01* and volatile defence in the interaction between wheat and rusts.

References

References

Abeles F.B., Bosshart R.P., Forrence L.E. and Habig W.H. 1971. **Preparation and purification of glucanase and chitinase from bean leaves.** *Plant Physiology* 47: 129-134

Alborn H.T., Turlings T.C., Jones T.H., Stenhagen G., Loughrin J.H. and Tumlinson J.H. 1997. **An elicitor of plant volatiles from beet armyworm oral secretion.** *Science* 276: 945-949

Amiri A. and Bompeix G. 2005. **Diversity and population dynamics of *Penicillium* spp. on apples in pre- and postharvest environments: consequences for decay development.** *Plant Pathology* 54: 74-81

An Q., Hückelhoven R., Kogel K-H. and van Bel A.J.E. 2006. **Multivesicular bodies participate in a cell wall-associated defence response in barley leaves attacked by the pathogenic powdery mildew fungus.** *Cellular Microbiology* 8: 1009–1019

Anderson P.A., Lawrence G.J., Morrish B.C., Ayliffe M.A., Finnegan E.J. and Ellis J.G. 1997. **Inactivation of the flax rust resistance gene *M* associated with the loss of a repeated unit within the leucine-rich repeat coding region.** *The Plant Cell* 9: 641-651

Anguelova V.S., van der Westhuizen A.J. and Pretorius Z.A. 1999. **Intercellular proteins and β-1,3-glucanase activity associated with leaf rust resistance in wheat**. *Physiologia Plantarum* 106: 393-401

Arimura G-I., Ozawa R., Shimoda T., Nishioka T., Boland W. and Takabayashi J. 2000. **Herbivory-induced volatiles elicit defence genes in Lima bean leaves**. *Nature* 406: 512-515

Arimura G-I., Ozawa R., Horiuchi J-I., Nishioka T. and Takabayashi J. 2001. **Plant-plant interactions mediated by volatiles emitted from plants infested by spider mites.** *Biochemical Systematics and Ecology* 29: 1049-1061

Arrigo A.P. 2002. **Small stress proteins: chaperones that act as regulators of intracellular redox state and programmed cell death.** *Journal of Biological Chemistry* 379: 19-26

Axtell M.J. and Staskawicz B.J. 2003. **Initiation of *RPS2*-specified disease resistance in *Arabidopsis* is coupled to the AvrRpt2-directed elimination of RIN4**. *Cell* 112: 369-377

References

Bai J., Pennill L.A., Ning J., Lee S.W., Ramalingam J., Webb C.A., Zhao B., Sun Q., Nelson J.C., Leach J.E. and Hulbert S.H. 2002**. Diversity in nucleotide binding site-leucine-rich repeat genes in cereals.** *Genome Research* 12: 1871-1884

Baker B., Zambryski P., Staskawicz B. and Dinesh-Kumar S.P. 1997. **Signalling in plant-microbe interactions.** *Science* 276: 726-733

Baldwin I.T. and Schultz J.C. 1983. **Rapid changes in tree leaf chemistry induced by damage: evidence for communication between plants**. *Science* 221: 277-279

Baldwin I.T., Kessler A. and Halitschke R. 2002. **Volatile defence in plant-plant herbivore interactions: what is real?** *Current Opinion in Plant Biology* 5: 1-4

Banzet N., Richaud C., Deveaux Y., Kazmaier M., Gagnon J. and Triantaphylides C. 1998. **Accumulation of small heat shock proteins, including mitochondrial HSP22, induced by oxidative stress and adaptive response in tomato cells**. *The Plant Journal* 13: 519-527

Bate N.J. and Rothstein S.J. 1998. **C6-volatiles derived from the lipoxygenase pathway induce a subset of defence-related genes**. *The Plant Journal* 16: 561–569

Bendahmane A., Farnham G., Moffett P. and Baulcombe D.C. 2002. **Constitutive gain-of-function mutants in a nucleotide binding site-leucine rich repeat protein encoded at the *Rx* locus of potato.** *The Plant Journal* 32: 195-204

Bell E. and Mullet J.E. 1991. **Lipoxygenase gene expression is modulated in plants by water deficit, wounding and methyl jasmonate**. *Molecular Genetics and Genomics* 230: 456-462

Benedetti C.E., Costa C.L., Turcinelli S.L. and Arruda P. 1998. **Differential expression of a novel gene in response to coronatine, methyl jasmonate and wounding in the *Coi1* mutant of *Arabidopsis***. *Plant Physiology* 116: 1037-1042

Bent A.F., Kunkel B.N., Dahlbeck D., Brown K.L., Schmidt R., Giraudat J., Leung J. and Staskawicz B.J. 1994. ***RPS2* of *Arabidopsis thaliana:* a leucine-rich repeat class of plant disease resistance genes**. *Science* 265: 1856–1860

References

Bestwick C.S., Brown I.R. and Mansfield J.W. 1998. **Localized changes in peroxidase activity accompany hydrogen peroxide generation during the development of a nonhost hypersensitive reaction in lettuce.** *Plant Physiology* 118: 1067-1078

Blake M.S., Johnston K.H., Russell-Jones G.J. and Gotschlich E.C. 1984. **A rapid, sensitive method for detection of alkaline phosphatase-conjugated anti-antibody on Western blots.** *Analytical Biochemistry* 136: 175-179

Biemelt S. and Sonnewald U. 2006. **Plant-microbe interactions to probe regulation of plant carbon metabolism.** *Journal of Plant Physiology* 163: 307-318

Blumwald E., Aharon G.S. and Lam B.C-H. 1998. **Early signal transduction pathways in plant-pathogen interactions**. *Trends in Plant Science* 3: 342-346

Boller T. 1995. **Chemoperception of microbial signals in plant cells.** *Annual Review of Plant Physiology and Plant Molecular Biology* 46: 189-214

Bonas U. and Lahaye T. 2002. **Plant disease resistance triggered by pathogen-derived molecules: refined models of specific recognition.** *Current Opinion in Microbiology* 5: 44-50

Borges A.A., Cools H.J. and Lucas J.A. 2003. **Menadione sodium bisulphite: a novel plant defence activator which enhances local and systemic resistance to infection by *Leptosphaeria maculans* in oilseed rape.** *Plant Pathology* 52: 429-436

Boston R.S., Viitanen P.V. and Vierling E. 1996. **Molecular chaperones and protein folding in plants.** *Plant Molecular Biology* 32: 191-222

Boudart G., Dechamp-Guillaume G., Lafitte C., Ricart G., Barthe J.P., Mazau D. and Esquerre-Tugaye M.T. 1995. **Elicitors and suppressors of hydroxyproline-rich glycoprotein accumulation are solubilized from plant cell walls by endopolygalacturonase.** *European Journal of Biochemistry* 232: 449-457

Brachmann A. 2006. **Plant-fungal symbiosis *en gros* and *en détail***. *New Phytologist* 171: 242-246

References

Bradford M.M. 1976. **A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.** *Analytical Biochemistry* 72: 248-253

Bradley D.J., Kjellbom P. and Lamb C.J. 1992. **Elicitor- and wound- induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defence response.** *Cell* 70: 21-30

Buchmeier N.A. and Heffron F. 1990. **Induction of *Salmonella* stress proteins upon infection of macrophages.** *Science* 248: 730-732

Bukau B. and Horwich A.L. 1998. **The Hsp70 and Hsp60 chaperone machines.** *Cell* 92: 351-366

Burnette W.N. 1981. **"Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A.** *Analytical Biochemistry* 112: 195-203

Büttner M. and Sauer N. 2001. **Monosaccharide transporters in plants: structure, function and physiology.** *Biochimica et Biophysica Acta* 1465: 263-274

Cano-Delgado A., Penfield S., Smith C., Catley M. and Bevan M. 2003. **Reduced cellulose synthesis invokes lignification and defence responses in *Arabidopsis thaliana*.** *The Plant Journal* 34: 351-362

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

Caruso C., Chilosi G., Caporale C., Leonardi L., Bertini L., Magro P. and Buonocore V. 1999. **Induction of pathogenesis-related proteins in germinating wheat seeds infected with *Fusarium culmorum.*** *Plant Science* 140: 107-120

Century K.S., Shapiro A.D., Repetti P.P., Dahlbeck D., Holub E. and Staskawicz B.J. 1997. ***NDR1*, a pathogen-induced component required for *Arabidopsis* disease resistance.** *Science* 278: 1963-1965

References

Cervone F., Castoria R., Leckie F. and De Lorenzo G. 1997. **Perception of fungal elicitors and signal transduction.** *In Signal transduction in plants.* Birkhäuser Verlag. Basel Switzerland. 153-177

Chen W. and Singh K.B. 1999. **The auxin, hydrogen peroxide and salicylic acid induced expression of the *Arabidopsis* GST6 promoter is mediated in part by an *ocs* element**. *The Plant Journal* 19: 667-677

Chisholm S., Coaker G., Day B. and Staskawicz B. 2006. **Host-microbe interactions: shaping the evolution of the plant immune response.** *Cell* 124: 803-814

Cho E.K. and Hong C.B. 2004. **Molecular cloning and expression pattern analyses of heat shock protein 70 genes from *Nicotiana tabacum.*** *Journal of Plant Biology* 47: 149-159

Chomczynski P. and Sacchi N. 1987. **Single step method of RNA isolation by acid Guanidine thiocyanate – Phenol Chloroform extraction.** *Analytical Biochemistry* 162: 156-159

Chomczynski P. 1992. **One-hour downward alkaline capillary transfer for blotting of DNA and RNA.** *Analytical Biochemistry* 201: 134-139

Chomczynski P. and Mackey K. 1994. **One-Hour Downward Capillary Blotting of RNA at Neutral pH.** *Analytical Biochemistry* 221: 303-305

Conrath U., Pieterse C.M.J. and Mauch-Mani B**.** 2002. **Priming in plant–pathogen interactions.** *Trends in Plant Science* 7: 210-216

Creelman R.A., Tierney M.L. and Mullet J.E. 1992. **Jasmonic acid/methyl jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression.** *Proceedings of the National Academy of Sciences USA* 89: 4938-4941

Creelman R.A. and Mullet J.E. 1997. **Biosynthesis and action of jasmonates in plants.** *Annual Review of Plant Physiology and Plant Molecular Biology* 48: 355-381

Croft K.P.C., Juttner F. and Slusarenko A.J. 1993. **Volatile products of the lipoxygenase pathway evolved from *Phaseolus vulgaris (L.)* leaves inoculated with *Pseudomonas syringae pv. phaseolicola.*** *Plant Physiology* 101: 13-24

References

Cronje M.J. and Bornman L. 1999. **Salicylic acid influences Hsp70/Hsc70 expression in *Lycopersicon esculentum*: Dose- and time-dependent induction or potentiation.** *Biochemical and Biophysical Research Communications* 265: 422-427

Cronje M.J., Weir I.E. and Bornman L. 2004. **Salicylic acid-mediated potentiation of Hsp70 induction correlates with reduced apoptosis in Tobacco protoplasts.** *Cytometry* 61: 76-87

Dann E.K., Meuwly P., Métraux J-P. and Deverall B.J. 1996. **The effect of inoculation or chemical treatment on activities of chitinase and β-1,3-glucanase and accumulation of salicylic acid in leaves of green bean, *Phaseolus vulgaris* L.** *Physiological and Molecular Plant Pathology* 49: 307-319

Delaney T.P., Friedrich L. and Ryals J.A. 1995. ***Arabidopsis* signal transduction mutant defective in chemically and biologically induced disease resistance.** *Proceedings of the National Academy of Sciences USA* 92: 6602-6606

Dangl J.L. and Jones J.D. 2001. **Plant pathogens and integrated defence responses to infection.** *Nature* 411: 826-833

Dangl J.L. and McDowell J.M. 2006. **Two modes of pathogen recognition by plants**. *Proceedings of the National Academy of Sciences USA* 103: 8575-8576

Dardick C. and Ronald P**.** 2006. **Plant and animal pathogen recognition receptors signal through non-RD kinases**. PLoS Pathogens 2: 14-28

Delledonne M., Zeier J., Marocco A. and Lamb C. 2001. **Signaling interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response.** *Proceedings of the National Academy of Sciences USA* 98: 13454-13459

del Rio L.A., Pastori G.M., Palma J.M., Sandalio L.M., Sevilla F., Corpas F.J., Jimenez A., Lopez-Huertas E. and Hernandez J.A. 1998. **The activated oxygen role of peroxisomes in senescence.** *Plant Physiology* 116: 1195-1200

Dempsey D.A., Shah J. and Klessig D.F. 1999. **Salicylic acid and disease resistance in plants.** *Critical Review of Plant Science* 18: 547-575

References

Desikan R., Mackerness S., Hancock J.T. and Neill S.J. 2001. **Regulation of the *Arabidopsis* transcriptome by oxidative stress.** *Plant Physiology* 127: 159-172

Després C., De Long C., Glaze S., Liu E. and Fobert P.R. 2000. **The *Arabidopsis* NPR1/NIM1 protein enhances the DNA binding activity of a subgroup of the TGA family of bZIP transcription factors.** *The Plant Cell* 12: 279-290

Després C. and Fobert P.R. 2006. ***In vivo* biochemical characterization of transcription factors regulating plant defence response to disease.** *Canadian Journal of Plant Pathology* 28: 3-15

Devoto A., Ellis C., Magusin A., Chang H-S., Chilcott C., Zhu T. and Turner J.G. 2005. **Expression profiling reveals *COI1* to be a key regulator of genes involved in wound- and methyl jasmonate-induced secondary metabolism, defence and hormone interactions.** *Plant Molecular Biology* 58: 497-513

Dicke M., Sabelis M.W., Takabayashi J., Bruin J. and Posthumus M.A. 1990. **Plant strategies of manipulating predator-prey interactions through allelochemicals: prospects for application in pest control.** *Journal of Chemical Ecology* 16: 3091-3118

Dicke M. and Bruin J. 2001. **Chemical information transfer between plants: back to the future.** *Biochemical Systematics and Ecology* 29: 981-994

Di Gaspero G. and Cipriani G. 2003. **Nucleotide binding site/leucine-rich repeats, Pto-like and receptor-like kinases related to disease resistance in grapevine.** *Molecular Genetics and Genomics* 269: 612–623

Dilks D.W., Ring R.H., Khawaja X.Z., Novak T.J. and Aston C. 2003. **High-throughput confirmation of differential display PCR results using reverse Northern blotting.** *Journal of Neuroscience Methods* 123: 47-54

Ding X.Z., Tsokos G.C. and Kiang J.G. 1998**. Overexpression of HSP-70 inhibits the phosphorylation of HSF1 by activating protein phosphatase and inhibiting protein kinase C activity.** *FASEB Journal* 12: 451–459

References

Dixon R.A., Achnine L., Kota P., Liu C-J., Reddy M.S.S. and Wang L. 2002. **The phenylpropanoid pathway and plant defence - a genomics perspective.** *Molecular Plant Pathology* 3: 371-390

Dobson C.F. 2003. **Protein folding and misfolding**. *Nature* 426: 884-890

do Céu Silva M., Várzea V., Guerra-Guimarães L., Azinheira H.G., Fernandez D., Petitot A-S., Bertrand B., Lashermes P. and Nicole M. 2006. **Coffee resistance to the main diseases: leaf rust and coffee berry disease.** *Brazilian Journal of Plant Physiology* 18: 119-147

Dodds P.N., Lawrence G.J., Catanzariti A-M., Teh T., Wang C-I.A., Ayliffe M.A., Kobe B. and Ellis J.G. 2006. **Direct protein interaction underlies gene-for-gene specificity and coevolution of the flax resistance genes and flax rust avirulence genes.** *Proceedings of the National Academy of Sciences USA* 103: 8888-8893

Dong X. 2001. **Genetic dissection of systemic acquired resistance**. Current Opinion in Plant Biology 4: 309-314

Dudareva N., Pichersky E. and Gershenzon J. 2004. **Biochemistry of plant volatiles.** *Plant Physiology* 135: 1893-1902

Durner J. and Klessig D.F. 1995. **Inhibition of ascorbate peroxidases by salicylic acid and 2,6-dichloroisonicotinic acid, two inducers of plant defence response.** *Proceedings of the National Academy of Sciences USA* 92: 11312-11316

Durrant W.E. and Dong X. 2004. **Systemic acquired resistance.** *Annual Review of Phytopathology* 42: 185-209

Engelberth J., Alborn H.T., Schmelz E.A. and Tumlinson J.H. 2004. **Airborne signals prime plants against insect herbivore attack.** *Proceedings of the National Academy of Sciences USA* 101: 1781-1785

Epple P., Apel K. and Bohlmann H. 1995. **An *Arabidopsis thaliana* thionin gene is inducible via a signal transduction pathway different from that for pathogenesis related proteins**. *Plant Physiology* 109: 813-820

References

Espinosa A. and Alfano J.R. 2004. **Disabling surveillance: bacterial type III secretion system effectors that suppress innate immunity.** *Cellular Microbiology* 6**:** 1027-1040

Eulgem T., Rushton P.J., Robatzek S. and Somssich I.E. 2000. **The WRKY superfamily of plant transcription factors.** *Trends in Plant Science* 5: 199-206

Fagoaga C., Rodrigo I., Conejero V., Hinarejos C., Tuset J.J., Arnau J., Pina J.A., Navarro L. and Pena L. 2001. **Increased tolerance to *Phytophthora citrophthora* in transgenic orange plants constitutively expressing a tomato pathogenesis-related protein PR-5.** *Molecular Breeding* 7: 175-185

Fall R. Karl T., Hansel A., Jordan A. and Lindinger W. 1999. **Volatile organic compounds emitted after leaf wounding: on-line analysis by proton-transfer-reaction mass spectrometry.** *Journal of Geophysical Research Atmospheres* 104: 15963-15974

Falquet L., Pagni M., Bucher P., Hulo N., Sigrist C.J., Hofmann K. and Bairoch A. 2002. **The PROSITE database, its status in 2002.** *Nucleic Acids Research* 30: 235-238

Fan C.Y., Lee S. and Cyr D.M. 2003. **Mechanisms for regulation of Hsp70 function by Hsp40.** *Cell Stress and Chaperones* 8: 309-316

Farag M.A., Fokar M., Zhang H.A., Allen R.D. and Pare P.W. 2005. **(Z)-3-hexenol induces defence genes and downstream metabolites in maize**. *Planta* 220: 900-909

Farmer E.E. and Ryan C.A. 1990. **Interplant communication: Airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves**. *Proceedings of the National Academy of Sciences USA* 87: 7713-7716

Feder M.E., Cartano N.V., Milos L., Krebs R.A. and Lindquist S.L. 1996. **Effect of engineering hsp70 copy number on hsp70 expression and tolerance of ecologically relevant heat shock in larvae and pupae of *Drosophila melanogaster*.** *Journal of Experimental Biology* 199: 1837–1844

Felle H.H., Herrmann A., Hanstein S., Hückelhoven R. and Kogel K-H. 2004. **Apoplastic pH defence in barley leaves attacked by the powdery mildew fungus *Blumeria graminis* f. sp. *hordei*.** *Molecular Plant-Microbe Interactions* 17: 118-123

References

Fernandez D., Santos P., Agostini C., Bon M-C., Petitot A-S., Silva M.C., Guerra-Guimaraes L., Ribeiro A., Argout X. and Nicole M. 2004. **Coffee (*Coffea arabica* L.) genes early expressed during infection by the rust fungus (*Hemileia vastatrix*).** Molecular Plant Pathology 5: 527-536

Feuillet C., Shachermayr G. and Keller B. 1997. **Molecular cloning of a new receptor-like kinase gene encoded at the Lr10 disease resistance locus of wheat.** *The Plant Journal* 11: 45-52

Feys B.J. and Parker J.E. 2000. **Interplay of defence pathways in plant disease resistance**. *Trends in Genetics* 16: 449-455.

Fink W., Liefland M. and Mendgen K. 1988. **Chitinases and β-1,3-glucanases in the apoplastic compartment of oat leaves (*Avena sativa* L.).** *Plant Physiology* 88: 270-275

Flor H.H. 1947. **Inheritance of reaction to rust in flax.** *Journal of Agricultural Research* 74: 241-262

Flor H.H. 1956. **The complementary genetic systems in flax and flax rust.** *Advances in Genetics* 8: 29-54

Flor H.H. 1971. **Current status of the gene-for-gene concept.** *Annual Review of Phytopathology* 9: 275-296

Flors C. and Nonell S. 2006. **Light and singlet oxygen in plant defence against pathogens: phototoxic phenalenone phytoalexins.** *Accounts of Chemical Research* 39: 293-300

Forreiter C. and Nover L. 1998. **Heat stress-induced proteins and the concept of molecular chaperones.** *Journal of Biosciences* 23: 287-302

Fotopoulos V., Gilbert M.J., Pittman J.K., Marvier A.C., Buchanan A.J., Sauers S., Hall J.L. and Williams L. 2003. **The monosaccharide transporter gene, *AtSTP4*, and the cell-wall invertase, *At_fruct1*, are induced in *Arabidopsis* during infection with the fungal biotroph *Erysiphe cichoracearum*.** *Plant Physiology* 132: 821-829

References

Franceschi V.R., Krokene P., Christiansen E. and Krekling T. 2005. **Anatomical and chemical defences of conifer bark against bark beetles and other pests.** *New Phytologist* 167: 353-376

Frey M., Stettner C., Pare P.W., Schmelz E.A., Tumlinson J.H. and Gierl A. 2000. **An herbivore elicitor activates the gene for indole emission in maize.** *Proceedings of the National Academy of Sciences USA* 97: 14801–14806

Fritig B., Heinz M. and Legrand M. 1998. **Antimicrobial proteins in induced plant defence.** *Current Opinion in Immunology* 10: 16-22

Fujimoto S.Y., Ohta M., Usui A., Shinshi H. and Ohme-Takagi M. 2000. ***Arabidopsis* ethylene-responsive element binding factors act as transcriptional activators or repressors of GCC box–mediated gene expression.** *The Plant Cell* 12: 393-404

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

Gális I., Smith J.L. and Jameson P.E. 2004. **Salicylic acid-, but not cytokinin-induced, resistance to WClMV is associated with increased expression of SA-dependent resistance genes in Phaseolus vulgaris.** *Journal of Plant Physiology* 161: 459-466

Gang D.R., Wang J.H., Dudareva N., Nam K.H., Simon J.E., Lewinsohn E. and Pichersky E. 2001. **An investigation of the storage and biosynthesis of phenylpropenes in sweet basil.** *Plant Physiology* 125: 539-555

Garrido C., Bruey J.M., Fromentin A., Hammann A., Arrigo A.P. and Solary E. 1999. **HSP27 inhibits cytochrome c-dependent activation of procaspase-9.** *FASEB Journal* 13: 2061-2070

Ghannam A., Jacques A., De Ruffray P., Baillieul F. and Kauffmann S. 2005. **Identification of tobacco ESTs with a hypersensitive response (HR)-specific pattern of expression and likely involved in the induction of the HR and/or localized acquired resistance (LAR).** *Plant Physiology and Biochemistry* 43: 249-459

References

Gierl A. and Frey M. 2001. **Evolution of benzoxazinone biosynthesis and indole production in maize.** *Planta* 213: 493-498

Glazebrook J. 1999. **Genes controlling expression of defence responses in *Arabidopsis*.** *Current Opinion in Plant Biology* 2: 280-286

Glazebrook J. 2005. **Contrasting mechanisms of defence against biotrophic and necrotrophic pathogens.** *Annual Review of Phytopathology* 43: 205-227

Gómez S. and Stuefer J.F. 2006. **Members only: induced systemic resistance to herbivory in a clonal plant network**. *Oecologia* 147: 461-468

Gourgues M., Brunet-Simon A., Lebrun M-H. and Levis C. 2004. **The tetraspanin BcPls1 is required for appressorium mediated penetration of *Botrytis cinerea* into host plant leaves.** *Molecular Microbiology* 51: 619-629

Gozzo F. 2003. **Systemic acquired resistance in crop protection: from nature to a chemical approach.** *Journal of Agricultural and Food Chemistry* 51: 4487-4503

Grant J.J. and Loake G.J. 2002. **Role of reactive oxygen intermediates and cognate redox defence in disease resistance.** *Plant Physiology* 124: 21-29

Grant M.R., Godiard L., Straube E., Ashfield T., Lewald J., Sattler A., Innes R.W. and Dangl J.L. 1995. **Structure of the *Arabidopsis RPM1* gene enabling dual specificity disease resistance**. *Science* 269: 843-846

Grene R. 2002. **Oxidative stress and acclimation mechanisms in plants.** *In* CR Somerville, EM Meyerowitz (eds) *The Arabidopsis Book*. American Society of Plant Biologists, Rockville, MD, doi: 10.1199/tab.0036.1, http://www.aspb.org/publications/arabidopsis/

Gundlach H., Muller M.J., Kutchan T.M. and Zenk M.H. 1992. **Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures.** *Proceedings of the National Academy of Sciences USA* 89: 2389-2393

Guy C.L. and Li Q.B. 1998. **The organization and evolution of the spinach stress 70 molecular chaperone gene family.** *The Plant Cell* 10: 539-556

References

Hall J.L. and Williams L.E. 2000. **Assimilate transport and partitioning in fungal biotrophic interactions**. *Australian Journal of Plant Physiology* 27**:** 549–560

Hamberg M. and Gardner H.W. 1992. **Oxylipin pathway to jasmonates-biochemistry and biological significance**. *Biochimica et Biophysica Acta* 1165: 1–18

Hamilton E.W. III and Coleman J.S. 2001. **Heat shock proteins are induced in unstressed leaves of *Nicotiana attenuata* (Solanaceae) when distant leaves are stressed.** *American Journal of Botany* 88: 950-955

Hamilton III E.W. and Heckathorn S.A. 2001. **Mitochondrial adaptations to NaCl. Complex I is protected by anti-oxidants and small heat shock proteins, whereas complex II is protected by proline and betaine.** *Plant Physiol*ogy 126: 1266-1274

Hammerschmidt R. 1999. **Phytoalexins: what have we learned after 60 years?** *Annual Review of Phytopathology* 34: 387-412

Hammond-Kosack K.E. and Parker J.E. 2003. **Deciphering plant–pathogen communication: fresh perspectives for molecular resistance breeding.** *Current Opinion in Biotechnology* 14: 177-193

Hanks S.K., Quinn A.M. and Hunter T. 1988. **The protein kinase family: conserved features and deduced phylogeny of the catalytic domains.** *Science* 241: 42-52

Hartl F.U. and Hayer-Hartl M. 2002. **Molecular chaperones in the cytosol: from nascent chain to folded protein.** *Science* 295: 1852-1858

Haslbeck M. 2002. **sHsps and their role in the chaperone network.** *Cellular and Molecular Life Sciences* 59: 1649-1657

Heath M. 1997. **Signalling between pathogenic rust fungi and resistant or susceptible host plants.** *Annals of Botany* 80: 713-720

Heath M.C. 2000. **Hypersensitive response-related death.** *Plant Molecular Biology* 44: 321-334

References

Heath M.C. 2001. **Non-host resistance to plant pathogens: nonspecific defence or the result of specific recognition events?** *Physiological and Molecular Plant Pathology* 58: 53-54

Hendrix R.W. 1979. **Purification and properties of GroE, a host protein involved in bacteriophage assembly.** *Journal of Molecular Biology* 129: 375-393

Henics T. 2003. **Extending the 'stressy' edge: molecular chaperones flirting with RNA**. *Cell Biology International* 27: 1-6

Herrmann K.M. and Weaver L.M. 1999. **The shikimate pathway.** *Annual Review of Plant Physiology and Plant Molecular Biology* 50: 473-503

Hiebert C., Thomas J. and McCallum B. 2005. **Locating the broad-spectrum wheat leaf rust resistance gene *Lr52 (LrW*) to chromosome 5B by a new cytogenetic method.** *Theoretical and Applied Genetics* 110: 1453-1457

Holmes F.O. 1938. **Inheritance of resistance to tobacco-mosaic disease in tobacco.** *Phytopathology* 28: 553-561

Holopainen J.K. 2004. **Multiple functions of inducible plant volatiles.** *Trends in Plant Science* 9: 529-533

Howe G. 2004. **Jasmonates as signals in the wound response.** *Journal of Plant Growth Regulation* 23: 223-237

Hu X. and Reddy A.S.N. 1997. **Cloning and expression of a PR5-like protein from *Arabidopsis*: inhibition of fungal growth by bacterially expressed protein.** *Plant Molecular Biology* 34: 949-959

Huang J., Cardoza Y.J., Schmelz E.A., Riana R., Engelberth J. and Tumlinson J.H. 2003. **Differential volatile emissions and salicylic acid levels from tobacco plants in response to different strains of *Pseudomonas syringae***. *Planta* 217: 767-775

Huang J., Schmelz E.A., Alborn H., Engelberth J. and Tumlinson J.H. 2005. **Phytohormones mediate volatile emissions during the interaction of compatible and incompatible**

pathogens: the role of ethylene in *Pseudomonas syringae* infected tobacco. *Journal of Chemical Ecology* 31: 439-459

Hunt C. and Morimoto R.I. 1985. **Conserved features of eukaryotic hsp70 genes revealed by comparison with the nucleotide sequence of human hsp70**. *Proceedings of the National Academy of Science USA* 82: 6455-6459

Ivanov S., Miteva L., Alexieva V., Karjin H. and Karanov E. 2005. **Alterations in some oxidative parameters in susceptible and resistant wheat plants infected with *Puccinia recondita* f.sp. *tritici*.** *Journal of Plant Physiology* 162: 275-279

Jabs T. 1999. **Reactive oxygen intermediates as mediators of programmed cell death in plants and animals.** *Biochemical Pharmacology* 57: 231-245

Jakoby M., Weisshaar B., Droge-Laser W., Vicente-Carbajosa J., Tiedemann J., Kroj T. and Parcy F. 2002. **bZIP transcription factors in *Arabidopsis*.** *Trends in Plant Science* 7: 106-111

Johal G.S., Gray J., Gruis D. and Briggs S.P. 1995. **Convergent insights into mechanisms determining disease and resistance response in plant-fungal interactions.** *Canadian Journal of Botany* 73: S468-S474

Jones D.A., Thomas C.M., Hammond-Kosack K.E., Balint-Kurti P.J. and Jones D.G.J. 1994. **Isolation of the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* by transposon tagging.** *Science* 266: 789-793

Jung J.-L., Fritig B. and Hahne G. 1993. **Sunflower (*Helianthus annuus* L.) pathogenesis-related proteins.** *Plant Physiology* 101: 873-880

Kachroo P., Shanklin J., Shah J., Whittle E.J. and Klessig D.F. 2001. **A fatty acid desatuase modulates the activation of defence defence pathways in plants.** *Proceedings of the National Academy of Sciences USA* 98: 9448-9453

Kanzaki H., Saitoh H., Ito A., Fujisawa S., Kamoun S., Katou S., Yoshioka H. and Terauchi R. 2003. **Cytosolic HSP90 and HSP70 are essential components of INF1-mediated hypersensitive response and non-host resistance to *Pseudomonas cichorii* in *Nicotiana benthamiana*.** *Molecular Plant Pathology* 4: 383-391

References

Karban R., Agrawal A.A., Thaler J.S. and Adler L.S. 1999. **Induced plant responses and information content about risk of herbivory.** *Trends in Ecology and Evolution* 14: 443-447

Karban R., Baldwin I.T., Baxter K.J., Laue G. and Felton G.W. 2000. **Communication between plants: induced resistance in wild tobacco plants following clipping of neighboring sagebrush.** *Oecologia* 125: 66-71

Karban R. 2001. **Communication between sagebrush and wild tobacco in the field.** *Biochemical Systematics and Ecology* 29: 995-1005

Karpinski S., Reynolds H., Karpinska B., Wingsle G., Creissen G. and Mullineaux P. 1999. **Systemic defence and acclimation in response to excess excitation energy in *Arabidopsis*.** *Science* 284: 654-657

Kelley W.L. 1998. **The J-domain family and the recruitment of chaperone power.** *Trends in Biochemical Sciences* 23: 222-227

Kemp G., Botha A-M., Kloppers F.J. and Pretorius Z.A. 1999. **Disease development and β-1,3-glucanase expression following leaf rust infection in resistant and susceptible near-isogenic wheat seedlings.** *Physiological and Molecular Plant Pathology* 55: 45-52

Kende H. 1993. **Ethylene biosynthesis.** *Annual Review of Plant Physiology and Plant Molecular Biology* 44: 283-307

Kessler A., Halitschke R., Diezel C. and Baldwin I.T. 2006. **Priming of plant defence responses in nature by airborne defence between *Artemisia tridentata* and *Nicotiana attenuate*.** *Oecologia* 148: 280-292

Kessmann H., Staub T., Hofmann C., Maetzke T., Herzog J., Ward E., Uknes S. and Ryals J. 1994. **Induction of systemic acquired disease resistance in plants by chemicals.** *Annual Review of Phytopathology* 32: 439-459

Kim M.G., da Cunha L., McFall A.J., Belkhadir Y., DebRoy S., Dangl J.L. and Mackey D. 2005. **Two *Pseudomonas syringae* type III effectors inhibit RIN4-regulated basal defence in *Arabidopsis*.** *Cell* 121: 749-759

References

Kimura Y., Yahara I. and Lindquist S. 1995. **Role of the protein chaperone YDJ1 in establishing Hsp90-mediated signal transduction.** *Science* 268: 1362-1365

Klessig D.F., Durner J., Noad R., Navarre D.A., Wendehenne D., Kumar D., Zhou J.M., Shah J., Zhang S., Kachroo P., Trifa Y., Pontier D., Lam E. and Silva H. 2000. **Nitric oxide and salicylic acid defence in plant defence.** *Proceedings of the National Academy of Sciences USA* 97: 8849-8855

Kloek A.P., Verbsky M.L., Sharma S.B., Schoelz J.E., Vogel J., Klessig D.F. and Kunkel B.N. 2001. **Resistance to *Pseudomonas syringae* conferred by an *Arabidopsis thaliana* coronatine-insenstive (coi1) mutation occurs through two distinct mechanisms.** *The Plant Journal* 26: 509-522

Koda Y. 1992. **The role of jasmonic acid and related compounds in the regulation of plant development**. *International Review of Cytology* 135: 155-199

Kogel K.H., Beckhove U., Dreschers J., Munch S. and Romme Y. 1994. **Acquired resistance in barley - the resistance mechanism induced by 2,6-dichloroisonicotinic acid is a phenocopy of a genetically based mechanism governing race-specific powdery mildew resistance.** *Plant Physiology* 106: 1269-1277

Koiwa H., Bressan R.A. and Hasegawa P.M. 1997. **Regulation of protease inhibitors and plant defence**. *Trends in Plant Science* 2: 379-384

Kolmer J.A. 1996. **Genetics of resistance to wheat leaf rust.** *Annual Review of Phytopathology* 34: 435-455

Kolmer J.A. 1999. **Virulence dynamics, phenotypic diversity and virulence complexity in two populations of *Puccinia triticina* in Canada from 1987–1997.** *Canadian Journal of Botany* 77: 333-338

Kolmer J.A. 2005. **Tracking wheat rust on a continental scale.** *Current Opinion in Plant Biology* 8: 441-449

Kumar D. and Klessig D.F. 2003. **High-affinity salicylic acid binding protein 2 is required for plant innate immunity and has salicylic acid-stimulated lipase activity.** *Proceedings of the National Academy of Sciences USA* 100: 16101-16106

References

Kunkel B.N. and Brooks D.M. 2002. **Cross talk between defence pathways in pathogen defence**. *Current Opinion in Plant Biology* 5: 325-331

Ladyzhenskaya E.P. and Protsenko M.A. 2002. **Plasma membrane-bound mechanisms of signal transduction in the control of plant dormancy and resistance.** *Biochemistry Moscow* 67: 151-160

Laemmli U.K. 1970. **Cleavage of structural proteins during the assembly of the head of the bacteriophage T4.** *Nature* 227: 680-685

Larkindale J. and Knight M.R. 2002. **Protection against heat stress-induced oxidative damage in Arabidopsis involves calciom, abscisic acid, ethylene and salicylic acid**. *Plant Physiology* 128: 682-695

Larronde F., Gaudillère J.P., Krisa S., Decendit A., Deffieux G. and Mérillonn J.M. 2003. **Airborne methyl jasmonate induces stilbene accumulation in leaves and berries of grapevine plants.** *American Journal of Enology and Viticulture* 54: 63-66

Lebel E., Heifets P., Thorne L., Uknes S., Ryals J. and Ward E. 1998. **Functional analysis of regulatory sequences controlling *PR1* gene expression in *Arabidopsis*.** *The Plant Journal* 16: 223-233

Lee J.H. and Schöffl F. 1996. **An Hsp70 antisense gene affects the expression of HSP70/HSC70, the regulation of HSF and the acquisition of thermotolerance in transgenic *Arabidopsis thaliana*.** *Molecular Genetics and Genomics* 252**:** 11–19

Leckie C.P., Callow J.A. and Green J.R. 1995. **Reorganization of the endoplasmic reticulum in pea leaf epidermal cells infected by the powdery mildew fungus *Erysiphe pisi***. *New Phytologist* 131: 211-221

Li J., Brader G. and Palva E.T. 2004. **The WRKY70 transcription factor: a node of convergence for Jasmonate-mediated and Salicylate-mediated signals in plant defence**. *The Plant Cell* 16: 319-331

Lee B.H., Won S.H., Lee H.S., Miyao M., Chung W.I., Kim I.J. and Jo J. 2000. **Expression of the chloroplast-localized small heat shock protein by oxidative stress in rice.** *Gene* 245: 283-290

References

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

Ligterink W., Kroj T., zur Nieden U., Hirt H. and Scheel D. 1997. **Receptor-mediated activation of a MAP Kinase in pathogen defence of plants.** *Science* 276: 2054-2057

Lindquist S. 1986. **The heat-shock response.** *Annual Review of Biochemistry* 55: 1151-1191

Lindquist S. and Craig E.A. 1988. **The heat-shock proteins.** *Annual Review of Genetics* 22: 631-677

Liu Y., Schiff M., Marathe R. and Dinesh-Kumar S.P. 2002. **Tobacco Rar1, EDS1 and NPR1/NIM1 like genes are required for N-mediated resistance to tobacco mosaic virus.** *The Plant Journal* 30: 415-429

Luan S. 1998. **Protein phosphatases and signalling cascades in higher plants.** *Trends in Plant Science* 3: 271-275

Malamy J., Carr J.P., Klessig D.F. and Raskin I. 1990. **Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection.** *Science* 250: 1002-1004

Marois E., Van den Ackerveken G. and Bonas U. 2002. **The *Xanthomonas* type III effector protein *AvrBs3* modulates plant gene expression and induces cell hypertrophy in the susceptible host**. *Molecular Plant-Microbe Interactions* 15: 637-646

Marsalis M.A. and Goldberg N.P. 2006. **Leaf, stem and stripe rust diseases of wheat.** Guide A-415 - New Mexico State University

Martin G.B., Bogdanove A.J. and Sessa G. 2003. **Understanding the functions of Plant Disease Resistance Proteins.** *Annual Review of Plant Biology* 54: 23-61

Mason H., De Wald D., Creelman R. and Mullet J. 1992. **Coregulation of soybean vegetative storage protein gene expression by methyl jasmonate and soluble sugars.** *Plant Physiology* 98: 859-867

Matern U., Grimmig B. and Kneusel R.E. 1995. **Plant cell wall reinforcement in the disease resistance response – molecular composition and regulation.** *Canadian Journal of Botany* 73: S511-S517

References

Mauch F., Hadwiger L.A. and Boller T. 1984. **Ethylene: symptom, not signal for the induction of chitinase and β-1,3-glucanase in pea pods by pathogens and elicitors.** *Plant Physiology* 76: 607-611

May M.J., Vernoux T., Leaver M., van Montagu M. and Inze D. 1998. **Glutathione homeostasis in plants: implications for environmental sensing and plant development.** *Journal of Experimental Botany* 49: 649-667

Mayer M.P., Brehmer D., Gassler C.S. and Bukau B. 2001. **Hsp70 chaperone machines.** *Advances in Protein Chemistry* 59: 1-44

Mayer M.P. and Bukau B. 2005. **Hsp70 chaperones: Cellular functions and molecular mechanism**. *Cellular and Molecular Life Sciences* 62: 670-684

McDowell J.M. and Dangl J.L. 2000. **Signal transduction in the plant immune response.** *Trends in Biochemical Science* 25: 79-82

McLusky S.R., Bennett M.H., Beale M.H., Lewis M.J., Gaskin P. and Mansfield J.W. 1999. **Cell wall alterations and localized accumulation of feruloyl-3'-methoxytyramine in onion epidermis at sites of attempted penetration by *Botrytis allii* are associated with actin polarization, peroxidase activity and suppression of flavonoid biosynthesis.** *The Plant Journal* 17: 523-534

Mehlen P., Schulze-Osthoff K. and Arrigo A-P. 1996. **Small stress proteins as novel regulators of apoptosis.** *The Journal of Biological Chemistry* 271: 16510-16514

Mendgen K. and Hahn M. 2002. **Plant infection and the establishment of fungal biotrophy**. *Trends in Plant Science* 7: 352-356

Mendgen K. and Nass P. 1988. **The activity of powdery-mildew haustoria after feeding the host cells with different sugars, as measured by potentiometric cyanine dye**. *Planta* 174**:** 283–288

Métraux J.P., Signer H., Ryals J., Ward E., Wyss-Benz M., Gaudin J., Raschdorf K., Schmid E., Blum W. and Inverardi B. 1990. **Increase in salicylic acid at the onset of systemic acquired resistance in cucumber.** *Science* 250: 1004-1006

References

Mitchell A. and Walters D. 1995. **Systemic protection in barley against powdery mildew infection using methyl jasmonate.** *Aspects of Applied Biology* 42: 323-326

Mittler R., Vanderauwera S., Gollery M. and van Breusegem F. 2004. **Reactive oxygen gene network of plants**. *Trends in Plant Science* 9: 490-498

Montesano M., Brader G. and Palva E.T. 2003. **Pathogen derived elicitors: searching for receptors in plants.** *Molecular Plant Pathology* 4: 73-79

Morris E.R. and Walker J.C. 2003. **Receptor-like protein kinases: the keys to response.** *Current Opinion in Plant Biology* 6: 1-4

Morrissey J.P. and Osbourn A.E. 1999. **Fungal resistance to plant antibiotics as a mechanism of pathogenesis.** *Microbiology and Molecular Biology Reviews* 63: 708-724

Mou Z., Fan W. and Dong X. 2003. **Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes.** *Cell* 113: 935-944

Nathan C. and Shiloh M.U. 2000. **Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens.** *Proceedings of the National Academy of Sciences USA* 97: 8841-8848

Navarro L., Zipfel C., Rowland O., Keller I., Robatzek S., Boller T. and Jones J.D.G. 2004. **The transcriptional innate immune response to flg22. Interplay and overlap with Avr gene-dependent defence responses and bacterial pathogenesis.** *Plant Physiology* 135: 1113-1128

Nawrath C. and Métraux J-P. 1999. **Salicylic acid induction–deficient mutants of *Arabidopsis* express *PR-2* and *PR-5* and accumulate high levels of Camalexin after pathogen inoculation**. *The Plant Cell* 11: 1393-1404

Neill S., Desikan R. and Hancock J. 2002a. **Hydrogen peroxide defence.** *Current Opinion in Plant Biology* 5: 388-395

Neill S., Desikan R., Clarke A., Hurst R.D. and Hancock J.T. 2002b. **Hydrogen peroxide and nitric oxide defence molecules in plants.** *Journal of Experimental Botany* 53: 1237-1247

References

Niggeweg R., Thurow C., Weigel R., Pfitzner U. and Gatz C. 2000. **Tobacco TGA factors differ with respect to interaction with NPR1, activation potential and DNA-binding properties.** *Plant Molecular Biology* 42: 775-788

Nicholson R.L. and Hammerschmidt R. 1992. **Phenolic compounds and their role in disease resistance.** *Annual Review of Phytopathology* 30: 369-389

Noctor G., Arisi A-C., Joanin L., Kunert K., Rennenberg H. and Foyer C. 1998. **Glutathione: biosynthesis, metabolism and relationship to stress tolerance exploited in transformed plants.** *Journal of Experimental Botany* 49: 623-647

Nollen E.A., Brunsting J.F., Roelofsen H., Weber L.A. and Kampinga H.H. 1999. **In vivo chaperone activity of heat shock protein 70 and thermotolerance.** *Molecular and Cellular Biology* 19: 2069–2079

Nollen E.A.A. and Morimoto R.I. 2002. **Chaperoning defence pathways: molecular chaperones as stress-sensing 'heat-shock' proteins.** *Journal of Cell Science* 115: 2809-2816

Nürnberger T., Brunner F., Kemmerling B. and Piater L. 2004. **Innate immunity in plants and animals: striking similarities and obvious differences.** *Immunological Reviews* 198: 249-266

Nürnberger T. and Lipka V. 2005**. Non-host resistance in plants: new insights into an old phenomenon. *Molecular Plant Pathology* 6: 335-345**

O'Brien I.E.W., Baguley B.C., Murray B.G., Morris B.A.M. and Ferguson I.B. 1998. **Early stages of the apoptotic pathway in plant cells are reversible.** *The Plant Journal* 13: 803-814

Odjakova M. and Hadjiivanova C. 2001. **The complexity of pathogen defence in plants.** *Bulgarian Journal of Plant Physiology* 27: 101-109

Oh H.S. and Collmer A. 2005. **Basal resistance against bacteria in *Nicotiana benthamiana* leaves is accompanied by reduced vascular staining and suppressed by multiple *Pseudomonas syringae* type III secretion system effector proteins.** *The Plant Journal* 44: 348-359

References

Oldroyed G.E.D. and Staskawicz B.J. 1998. **Genetically engineered broad-spectrum disease resistance in tomato.** *Proceedings of the National Academy of Sciences USA* 95: 10300-10305

O'Malley R.C., Rodriguez F.I., Esch J.J., Binder B.M., O'Donnell P., Klee H.J. and Bleecker A.B. 2005. **Ethylene-binding activity, gene expression levels and receptor system output for ethylene receptor family members from *Arabidopsis* and tomato**. *The Plant Journal* 41: 651–659

Oñate-Sánchez L. and Singh K.B. 2002. **Identification of *Arabidopsis* ethylene responsive element binding factors with distinct induction kinetics after pathogen infection.** *Plant Physiology* 128: 1313-1322

Ori N., Eshed Y., Paran I., Presting G., Aviv D., Tanksley S., Zamir D. and Fluhr R. 1997. **The *I2C* family from the wilt disease resistance locus *I2* belongs to the nucleotide-binding, leucine-rich repeat superfamily of plant disease resistance genes.** *The Plant Cell* 9: 521-532

Orozco-Cárdenas M.L., Narváez-Vásquez J. and Ryan C.A. 2001. **Hydrogen peroxide acts as a second messenger for the induction of defence genes in tomato plants in response to wounding, systemin and methyl jasmonate.** *The Plant Cell* 13: 179-191

Palma K. and Kermode A.R. 2003. **Metabolism of hydrogen peroxide during reserve mobilization and programmed cell death of barley (*Hordeum vulgare* L.) aleurone layer cells.** *Free Radical Biology and Medicine* 35: 1261-1270

Panchuk I.I., Volkov R.A. and Schöffl F. 2002. **Heat stress- and heat shock transcription factor dependent expression and activity of ascorbate peroxidase in *Arabidopsis.*** *Plant Physiology* 129: 838-853

Panstruga R. 2003. **Establishing compatibility between plants and obligate biotrophic pathogens.** *Current Opinion in Plant Biology* 6: 320-326

Parker J.E., Holub E.B., Frost L.N., Falk A., Gunn N.D. and Daniels M.J. 1996. **Characterization of *eds1*, a mutation in *Arabidopsis* suppressing resistance to *Peronospora parasitica* specified by several different *RPP* genes.** *The Plant Cell* 8: 2033-2046

Parker J.E., Coleman M.J., Szabo V., Frost L.N., Schmidt R., Van der Biezen E., Moores T., Dean C., Daniels M.J. and Jones J.D.G. 1997. **The *Arabidopsis* downy mildew resistance gene *RPP5* shares similarity to the Toll and interleukin-1 receptors with *N* and *L6*.** *The Plant Cell* 9: 879-894

Parniske M. 2004. **Molecular genetics of the arbuscular mycorrhizal symbiosis.** *Current Opinion in Plant Biology* 7: 414-421

Paschold A., Halitschke R. and Baldwin I.T. 2006. **Using "mute" plants to translate volatile signals.** *The Plant Journal* 45: 275-291

Pastori G.M. and Foyer C.H. 2002. **Common components, networks and pathways of cross-tolerance to stress. The central role of "Redox" and Abscisic acid-mediated controls.** *Plant Physiology* 129: 460-468

Peck S.C. 2003. **Early phosphorylation events in biotic stress**. *Current Opinion in Plant Biology* 6: 334-338

Person C. 1959. **Gene-for-gene relationships in host: parasite systems.** *Canadian Journal of Botany* 37: 1101-1130

Pickett J.A. and Poppy G.M. 2001. **Switching on plant genes by external chemical signals**. *Trends in Plant Science* 6: 137-139

Pierpoint W.S., Jackson P.J. and Evans R.M. 1990. **The presence of a thaumatin-like protein, a chitinase and a glucanase among the pathogenesis-related proteins of potato (*Solanum tuberosum*).** *Physiological and Molecular Plant Pathology* 36: 325-338

Pieterse C.M.J., van Wees S.C.M., van Pelt J.A., Knoester M., Laan R., Gerrits H., Weisbeek P.J. and van Loon L.C. 1998. **A novel defence pathway controlling induced systemic resistance in *Arabidopsis***. *The Plant Cell* 10: 1571-1580

Pieterse C.M.J. and Van Loon L.C. 2004. **NPR1: the spider in the web of induced resistance defence pathways.** *Current Opinion in Plant Biology* 7: 456-464

References

Preston C.A., Laue G. and Baldwin I.T. 2001. **Methyl jasmonate is blowing in the wind, but can it act as a plant-plant airborne signal?** *Biochemical Systematics and Ecology* 29: 1007-1023

Preston C.A., Laue G. and Baldwin I.T. 2004. **Plant–plant defence: application of *trans*- or *cis*-methyl jasmonate equivalent to sagebrush releases does not elicit direct defences in native tobacco**. *Journal of Chemical Ecology* 30: 2193-2214

Raskin I. 1992**. Role of salicylic acid in plants.** *Annual Review of Plant Physiology and Plant Molecular Biology* 43: 439-463

Rebmann G., Mauch F. and Dudler R. 1991. **Sequence of a wheat cDNA encoding a pathogen-induced thaumatin-like protein.** *Plant Molecular Biology* 17: 283-285

Reichmann J.L., Heard J., Martin G., Reuber L., Jiang C., Keddie J., Adam L., Pineda O., Ratcliffe O.J., Samaha R.R., Creelman R., Pilgrim M., Broun P., Zhang J. Z., Ghandehari D., Sherman B. K. and Yu G-L. 2000. ***Arabidopsis* transcription factors: genome-wide comparative analysis among eukaryotes.** *Science* 290: 2105-2110

Reimmann C. and Dudler R. 1993. **cDNA cloning and sequence analysis of a pathogen-induced thaumatin-like protein from rice (*Oryza sativa*).** *Plant Physiology* 101: 1113-1114

Ren D., Yang K-Y., Li G-J., Liu Y. and Zhang S. 2006. **Activation of Ntf4, a tobacco mitogen-activated protein kinase, during plant defence response and its involvement in hypersensitive response-like cell death.** *Plant Physiology* 141: 1482-1493

Repka V. 2006. **Early defence responses induced by two distinct elicitors derived from a Botrytis cinerea in grapevine leaves and cell suspensions.** *Biologia Plantarum* 50: 94-106

Rhoades D. 1983. **Responses of alder and willow to attack by tent caterpillars and webworms: Evidence for pheromonal sensitivity of willows**. *American Chemical Society Symposium Series* 208: 55-68

Rock F.L., Hardiman G., Timans J.C., Kastelein R.A. and Bazan J.F. 1998. **A family of human receptors structurally related to *Drosophila* Toll**. *Proceedings of the National Academy of Sciences USA* 95: 588-593

References

Roelfs A.P. 1989. **Epidemiology of the cereal rusts in North America**. *Canadian Journal of Plant Pathology* 11: 86-90

Rohringer R., Kim W.K., Samborski D.J. and Howes N.K. 1977. **Calcofluor: an optical brightener for fluorescence microscopy of fungal plant parasites in leaves.** *Phytopathology* 67: 808-810

Roitsch T., Balibrea M.E., Hofmann M., Proels R. and Sinha A.K. 2003. **Extracellular invertase: key metabolic enzyme and PR protein.** *Journal of Experimental Botany* 54: 513-524

Romeis T. 2001. **Protein kinases in the plant defence response.** *Current Opinion in Plant Biology* 4: 407-414

Rushton P.J. and Somssich I.E. 1998. **Transcriptional control of plant genes responsive to pathogens.** *Current Opinion in Plant Biology* 1: 311-315

Ruther J. and Kleier S. 2005. **Plant-plant defence: ethylene synergizes volatile emission in *Zea mays* induced by exposure to (Z)-3-Hexen-1-ol**. *Journal of Chemical Ecology* 31: 2217-2222

Ryals J., Uknes S. and Ward E. 1994. **Systemic acquired resistance.** *Plant Physiology* 104: 1109-1112

Rybutt D.B. and Parish C.R. 1982. **Protein determination on an automatic spectrophotometer.** *Analytical Biochemistry* 121: 213-214

Salmeron J.M., Oldroyd G.E.D., Rommens C.M.T., Scofield S.R., Kim H.S., Lavelle D.T., Dahlbeck D. and Staskawicz B.J. 1996. **Tomato *Prf* is a member of the leucine-rich repeat class of plant disease resistance genes and lies embedded within the *Pto* kinase gene cluster.** *Cell* 86: 123-133

Sambrook J., Fritsch E.F. and Maniatis T. 1989. **Molecular cloning: a laboratory manual.** 2nd ed. USA: *Cold Spring Harbour press*

Scheel D. 1998. **Resistance response physiology and signal transduction.** *Current Opinion in Plant Biology* 1: 305-310

References

Schett G., Steiner C-W., Xu Q., Smolen J.s. and Steiner G. 2003. **TNFα mediates susceptibility to heat-induced apoptosis by protein phosphatase-mediated inhibition of the HSF1/hsp70 stress response**. *Cell Death and Differentiation* 10: 1126-1136

Schilmiller A.L. and Howe G.A. 2005. **Systemic defence in the wound response.** *Current Opinion in Plant Biology* 8: 369-377

Schulze-Lefert P. and Panstruga R. 2003. **Establishment of biotrophy by parasitic fungi and reprogramming of host cells for disease resistance.** *Annual Review of Phytopathology* 41: 641-667

Schweizer P., Gees R.E.M. and Mösinger E. 1993. **Effect of jasmonic acid on the interaction of barley (*Hordeum vulgare* L.) with the powdery mildew *Erysiphe graminis* f. sp. *hordei***. *Plant Physiology* 102: 503-511

Sembdner G. and Parthier B. 1993. **The biochemistry and physiological and molecular actions of jasmonates.** *Annual Review of Plant Physiology and Plant Molecular Biology* 44: 569-589

Sessa G. and Martin G.B. 2000. **Protein kinases in the plant defence response.** *In Advances in Botanical Research Vol. 32,* Academic Press, 379-404

Shah J. 2003. **The salicylic acid loop in plant defence.** *Current Opinion in Plant Biology* 6: 365-371

Shen B., Zheng Z. and Dooner H.K. 2000. **A maize sesquiterpene cyclase gene induced by insect herbivory and volicitin: characterization of wild-type and mutant alleles.** *Proceedings of the National Academy of Sciences USA* 97: 14807-14812

Shinnick T.M. 1991. **Heat shock proteins as antigens of bacterial and parasitic pathogens.** *Current Topics in Microbiology and Immunology* 167: 145-160

Shulaev V., Silverman P. and Raskin I. 1997. **Airborne defence by methyl salicylate in plant pathogen resistance.** *Nature* 385: 718-721

Singh K.B., Foley R.C. and Oñate-Sánchez L. 2002. **Transcription factors in plant defence and stress responses.** *Current Opinion in Plant Biology* 5: 430-436

References

Slaymaker D.H., Navarre D.A., Clark D., Del Pozo O., Martin G.B. and Klessig D.F. 2002. **The tobacco salicylic acid-binding protein 3 (SABP3) is the chloroplast carbonic anhydrase, which exhibits antioxidant activity and plays a role in the hypersensitive defence response.** *Proceedings of the National Academy of Sciences USA* 99**:** 11640-11645

Snyder B.A. and Nicholson R.L. 1990. **Synthesis of phytoalexins in sorghum as a site-specific response to fungal ingress.** *Science* 248: 1637-1639

Somssich I. and Hahlbrock K. 1998. **Pathogen defence in plants – a paradigm of biological complexity.** *Trends in Plant Science* 3: 86-90

Song W-Y., Wang G-L. and Chen L-L. 1995. **A receptor kinase-like protein encoded by the rice disease resistance gene, *Xa-21*.** *Science* 270: 1804-1806

Staskawicz B.J., Ausubel F.M., Baker B.J., Ellis J.G. and Jones J.D.G. 1995. **Molecular genetics of plant disease resistance.** *Science* 268: 661-667

Staswick P.E., Su W.P. and Howell S.H. 1992. **Methyl jasmonate inhibition of root growth and induction of a leaf protein are decreased in an *Arabidopsis thaliana* mutant**. *Proceedings of the National Academy of Sciences USA* 89: 6837-6840

Stintzi A., Heitz T. and Prasad V. 1993. **Plant 'pathogenesis-related' proteins and their role in defence against pathogens.** *Biochimie* 75: 687-706

Sung D.Y., Vierling E. and Guy C.L. 2001. **Comprehensive expression profile analysis of the *Arabidopsis* Hsp70 gene family.** *Plant Physiology* 126: 789-800

Takabayashi J. and Dicke M. 1996. **Plant-carnivore mutualism through herbivore-induced carnivore attractants.** *Trends in Plant Science* 1: 109-113

Takahashi H., Miller J., Nozaki Y., Takeda M., Shah J., Hase S., Ikegami M., Ehara Y. and Dinesh-Kumar S.P. 2002. **RCY1, an *Arabidopsis thaliana* RPP8/HRT family resistance gene, conferring resistance to cucumber mosaic virus requires salicylic acid, ethylene and a novel signal transduction mechanism.** *The Plant Journal* 32: 655-667

References

Takano Y., Takayanagi N., Hori H., Ikeuchi Y., Suzuki T., Kimura A. and Okuno T. 2006. **A gene involved in modifying transfer RNA is required for fungal pathogenicity and stress tolerance of *Colletotrichum lagenarium.*** *Molecular Microbiology* 60: 81-92

Tang X., Xie M., Kim Y.J., Zhou J., Klessig D.F. and Martin G.B. 1999. **Overexpression of *Pto* activates defence responses and confers broad resistance.** *The Plant Cell* 11: 15-29

Thaler J.S., Fidantsef A.L. and Bostock R.M. 2002. **Antagonism between jasmonate- and salicylate- mediated induced plant resistance: effects of concentration and timing of elicitation on defence-related proteins, herbivore and pathogen performance in tomato.** *Journal of Chemical Ecology* 28: 1131-1159

Thatcher L.F., Anderson J.P. and Singh K.B. 2005. **Plant defence responses: what have we learned from *Arabidopsis*.** *Functional Plant Biology* 32: 1-19

Thomas C.M., Jones D.A., Parniske M., Harrison K., Balint-Kurti P.J., Hatzixanthis K. and Jones J.D.G. 1997. **Characterization of the tomato *Cf-4* gene for resistance to *Cladosporium fulvum* identifies sequences that determine recognitional specificity in Cf-4 and Cf-9.** *Plant Cell* 9: 2209-2224

Thordal-Christensen H., Zhang Z., Wei Y. and Collinge D.B. 1997. **Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction.** *Plant Journal* 11: 1187-1194

Thordal-Christensen H. 2003. **Fresh insights into processes of nonhost resistance**. *Current Opinion in Plant Biology* 6: 351-357

Torres M.A., Dangl J.L. and Jones J.D.G. 2002. ***Arabidopsis* gp91^{phox} homologues *AtrbohD* and *AtrbohF* are required for accumulation of reactive oxygen intermediates in the plant defence resonse.** *Proceedings of the National Academy of Sciences USA* 99: 517-522

Tscharntke T., Thiessen S., Dolch R. and Boland W. 2001. **Herbivory, induced resistance, and interplant signal transfer in *Alnus glutinosa***. *Biochemical Systematics and Ecology* 29: 1025-1047

Turlings T.C.J., Tumlinson J.H. and Lewis W.J. 1990. **Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps.** *Science* 250: 1251-1253

References

Turlings T.C., Tumlinson J.H., Heath J.H., Proveau A.T. and Doolittle R.E. 1991. **Isolation and identification of allelochemicals that attract the larval parasitoid *Cortesia marginiventris* (Cresson) to the microhabitat of one of its hosts.** *Journal of Chemical Ecology* 17: 2235-2251

Turner J.G., Ellis C. and Devoto A. 2002. **The jasmonate signal pathway**. The Plant Cell 14: S153-S164

Tutar Y., Song Y. and Masison D.C. 2006. **Primate chaperones Hsc70 (constitutive) and Hsp70 (induced) differ functionally in supporting growth and prion propagation in *Saccharomyces cerevisiae*.** *Genetics* 172: 851-861

Uknes S., Mauch-Mani B., Moyer M., Potter S., Williams S., Dincher S., Chandler D., Slusarenko A., Ward E. and Ryals J. 1992. **Acquired resistance in *Arabidopsis***. *The Plant Cell* 4: 645-656

Van den Boom C.E.M., van Beek T.A., Posthumus M.A., de Groot A. and Dicke M. 2004. **Qualitative and quantitative variation among volatile profiles induced by *Tetranychus urticae* feeding on plants from various families.** *Journal of Chemical Ecology* 30: 69-89

Van Hulten M., Pelser M., Van Loon L.C., Pieterse C.M.J. and Ton J. 2006. **Costs and benefits of priming for defence in *Arabidopsis*.** *Proceedings of the National Academy of Sciences USA* 103: 5602-5607

Van Loon L.C. and Antoniw J.F. 1982. **Comparison of the effects of salicylic acid and ethephon with virus-induced hypersensitivity and acquired resistance in tobacco.** *Netherlands Journal of Plant Pathology* 88: 237-256

Van Loon L.C. 1997. **Induced resistance in plants and the role of pathogenesis-related proteins.** *European Journal of Plant Pathology* 103: 753-765

Van Loon L.C. and Van Strien E.A. 1999. **The families of pathogenesis-related proteins, their activities and comparative analysis of PR1 type proteins.** *Physiological and Molecular Plant Pathology* 55: 85-97

References

Van Wees S.C.M. and Glazebrook J. 2003. **Loss of non-host resistance of *Arabidopsis NahG* to *Pseudomonas syringae pv phaseolicola* is due to degradation products of salicylic acid.** *The Plant Journal* 33: 733-742

Veljovic-Jovanovic S.D., Pignocchi C., Noctor G. and Foyer C.H. 2001. **Low ascorbic acid in the *vtc-1* mutant of *Arabidopsis* is associated with decreased growth and intracellular redistribution of the antioxidant system.** *Plant Physiology* 127: 426-435

Veronese P., Ruiz M.T., Coca M.A., Hernandez-Lopez A., Lee H., Ibeas J.I., Damsz B., Pardo J.M., Hasegawa P.M., Bressan R.A. and Narasimhan M.L. 2003. **In defence against pathogens. Both plant sentinels and foot soldiers need to know the enemy.** *Plant Physiology* 131: 1580-1590

Vranová E., Inzé D. and Van Breusegem F. 2002. **Signal transduction during oxidative stress.** *Journal of Experimental Botany* 53: 1227-1236

Voegele R.T., Struck C., Hahn M. and Mendgen K. 2001. **The role of haustoria in sugar supply during infection of broad bean by the rust fungus *Uromyces fabae*.** *Proceedings of the National Academy of Sciences USA* 98: 8133-8138

Wang J.W. and Wu J.Y. 2005. **Nitric oxide is involved in methyl jasmonate-induced defence responses and secondary metabolism activities of taxus cells**. *Plant Cell Physiology* 46: 923-930

Wang W., Vinocur B., Shoseyov O. and Altman A. 2004. **Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response.** *Trends in Plant Science* 9: 244-252

Ward E.R., Uknes S.J., Williams S.C., Dincher S.S., Wiederhold D.L., Alexander D.C., Ahl-Goy P., Métraux J-P. and Ryals J.A. 1991. **Coordinate gene activity in response to agents that induce systemic acquired resistance.** *The Plant Cell* 3: 1085-1094

Wasternack C. and Parthier B. 1997. **Jasmonate-signalled plant gene expression.** *Trends in Plant Science* 2: 302–307

References

Wasternack C., Stenzel I., Hause B., Hause G., Kutter C., Maucher H., Neumerkel J., Feussner I. and Miersch O. 2006. **The wound response in tomato - role of jasmonic acid.** *Journal of Plant Physiology* 163: 297-306

Weiler E.W., Albrecht T., Groth B., Xia Z-Q., Luxem M., Liss H., Andert L. and Spengler P. 1993. **Evidence for the involvement of jasmonates and their octadecanoid precursors in the tendril coiling response of *Bryonia dioica***. *Phytochemistry* 32: 591-600

Wojtaszek P. 1997. **Oxidative burst: an early plant response to pathogen infection.** *Biochemical Journal* 322: 681-692

Xing T., Quellet Th-R-S.E. and Miki B.L. 2002. **Towards genomic and proteomic studies of protein phosphorylation in plant-pathogen interactions.** *Trends in Plant Science* 7:224-230

Xu Y., Chang P.F.L., Liu D., Narasimhan M.L., Raghothama K.G., Hasegawa P.M. and Bressan R.A. 1994. **Plant defence genes are synergistically induced by ethylene and methyl jasmonate**. *Plant Cell* 6: 1077-1085

Xu H. and Reddy A.S.N. 1997. **Cloning and expression of a PR5-like protein from *Arabidopsis*: inhibition of fungal growth by bacterially expressed protein.** *Plant Molecular Biology* 34: 949-959

Yamamoto S., Nakano T., Suzuki K. and Shinshi H. 2004. **Elicitor-induced activation of transcription via W box-related *cis*-acting elements from a basic chitinase gene by WRKY transcription factors in tobacco.** *Biochimica et Biophysica Acta* 1679: 279-287

York W.S., Darvill A.G., McNeil M., Stevenson T.T. and Albersheim P. 1985. **Isolation and characterization of plant cell walls and cell wall components**. *Methods in Enzymology* 118: 3-40

Yoshimura S., Yamanouchi U., Katayose Y., Toki S., Wang Z-X., Kono I., Kurata N., Yano M., Iwata N. and Sasaki T. 1998. **Expression of *Xa1*, a bacterial blight-resistance gene in rice, is induced by bacterial inoculation.** *Proceedings of the National Academy of Sciences USA* 95: 1663-1668

Young P.A. 1926. **Penetration phenomena and facultative parasitism to *Alternaria, Diplodia* and other fungi.** *Botanical Gazette* 81: 258-279

References

Zeringue Jr. H.J., Brown R.L., Neucere J.N. and Cleveland T.E. 1996. **Relationships between C_6-C_{12} alkanal and alkenal volatile contents and resistance of maize genotypes to *Aspergillus flavus* and aflatoxin production**. *Journal of Agricultural and Food Chemistry* 44: 403-407

Zhang Y., Fan W., Kinkema M., Li X. and Dong X. 1999. **Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the *PR1* gene.** *Proceedings of the National Academy of Sciences USA* 96: 6523-6528

Zügel U. and Kaufmann S.H.E. 1999. **Heat shock proteins in protection from and pathogenesis of infectious diseases.** *Clinical Microbiology Reviews* 12: 19-39

Summary

SUMMARY

The aim of this study was to investigate the early events following the infection of wheat with leaf rust. An attempt was made to identify and characterize genes putatively involved in these early events. More specifically, the aim was to obtain genes whose role in infected plants could be linked to the resistance locus within the resistant plant.

DDRT-PCR was used to isolate differentially expressed genes from the resistant Thatcher+*Lr34* plants during the first 15 h after infection. Four cDNA fragments were cloned and sequenced. The first clone coded for a monosaccharide transporter, while the second clone encoded a cell wall invertase. Both these clones formed part of a different study to postulate a role for these proteins during infection.

The third cDNA clone, coded for a putative heat shock protein. Heat shock proteins are molecular chaperones and are normally involved in ensuring cellular homeostasis by preventing the aggregation of denatured proteins and assisting in the folding and transport of new and denatured proteins. The fourth clone encoded an indole-3-glycerol phosphate lyase.

The expression of the putative heat shock protein increased 86 fold within 9 h.p.i in infected resistant wheat and was chosen for further analysis. The gene shared very high sequence similarity to an *O. sativa* HSP70 gene and was called *TaHlp01* (*Triticum aestivum* Heat shock Like Protein 01). *TaHlp01* was inducibly expressed upon infection of resistant wheat with leaf rust as well as yellow rust but its expression remained constant in the infected susceptible cultivars. This indicated that the regulation of expression is dependent on the presence of the resistance locus within the resistant cultivars. A transient repression of *TaHlp01* expression was found during the later stages of infection in both the IR and IS plants that were similar to a transient repression of *TaHlp01* expression after Thatcher+*Lr34* plants were treated with salicylic acid. *TaHlp01* expression was also found to be induced by heat stress, indicating a possible role during heat stress.

A possible interplant communication event was also examined. It was found that infected plants were able to induce the defence response of uninfected plants. This communication

between infected and uninfected plants was more effective between plants of the same cultivars than between different cultivars. It appears as if resistant plants were able to induce a more controlled defence response than susceptible plants. When uninfected resistant and susceptible plants exposed to infected plants, was infected themselves, they exhibited a more resistant phenotype compared to plants that was not exposed. The communication event during this interaction most likely involves jasmonic acid.

Keywords: *Triticum aestivum*, *Puccinia triticina*, plant defence, interplant communication, *TaHlp01*

Opsomming

OPSOMMING

Die doel van hierdie studie was om die vroeë gebeure na die infeksie van koring met blaarroes te bestudeer. ‘n Poging was aangewend om gene wat moontlik by hierdie vroeë interaksie betrokke is, te identifiseer en te karakteriseer. Klem is egter gelê op die klonering van gene waarvan die rol in geinfekteerde plante direk met die weerstandslokus gekoppel kan word.

DDRT-PCR was gebruik om gene wat binne die eerste 15 h na infeksie van weerstandbiedende Thatcher+*Lr34* plante met blaarroes, differensieël tot uiting kom te isoleer. Vier cDNA fragmente is gekloneer en hul DNA volgorde bepaal. Die eerste kloon kodeer vir ‘n monosakkaried draer proteïen en die tweede vir ‘n selwand invertase proteïen. Beide hierdie gene vorm deel van ‘n ander studie wat die rol van hierdie twee proteïene gedurende infeksie bepaal.

Die derde geen kodeer vir ‘n potensiële hitteskok protein. Hitteskok proteïene is molekulêre begeleiers wat normaalweg die sellulêre ewewig handhaaf deur te verhoed dat ongevoude en gedenatureerde proteïene aggregeer. Verder speel hulle ook ‘n rol in die vouing en vervoer van nuwe en gedenatureerde proteïene. Die vierde kloon het vir ‘n indool-3-gliserolfosfaat proteïen gekodeer.

Die uiting van die potensiële hitteskok proteïen het is binne 9 uur na infeksie 86 keer verhoog. Die geen is dus gekies vir verdere analise. Die geen toon baie hoë homologie getoon met ‘n *O. sativa* Hsp70 geen en is *TaHlp01* genoem (*Triticum aestivum* Heat Shock Like Protein 01). *TaHlp01* se uiting was aangeskakel tydens die infeksie van weerstandbiedende koring met blaarroes sowel as geelroes, maar het konstant gebly in vatbare plante. Dit het gelei tot die gevolgtrekking dat die uiting van *TaHlp01* in weerstandbiedende koring ofhanklik is van die weerstandslokus. ‘n Tydelike onderdrukking van *TaHlp01* uiting het voorgekom in beide vatbaar sowel as weerstandbiedend geïnfekteerde plante terwyl ‘n soortgelyke onderdrukking in plante wat met salisielsuur behandel was, voorgekom het. Dit dui op die moontlike regulering van *TaHlp01* deur

salisielsuur. *TaHlp01* se uitdrukking was ook geïnduseer tydens hitteskok wat dui op ‘n moontlike rol van die proteïen tydens hitteskok.

‘n Verdere aspek wat bestudeer was, was die oordraging van ‘n vlugtige sein tussen verskillende koring plante. Daar is gevind dat geinfekteerde koring plante in staat was om die verdedigingsmeganismes van ongeinfekteerde plante aan te skakel. Hierdie kommunikasie was ook meer effektief tussen plante van dieselfde kultivar as tussen verskillende kultivars. Dit wil ook voorkom asof geinfekteerde weerstandbiedende plante in staat is om ‘n meer gekontroleerde verdedigingsrespons in die ongeinfekteerde plante aan te skakel as vatbare plante. Tydens die infeksie van beide vatbare en weerstandbiedende plante wat voorheen in kontak was met weerstandbiedend geïnfekteerde plante, het hulle ‘n beter verdedigingsresponse getoon as plante wat nie in kontak was nie. Dit wil voorkom asof die sein wat betrokke is tydens hierdie kommunikasie, bes moontlik jasmoniensuur is.

Kernwoorde: *Triticum aestivum, Puccinia triticina,* plant verdediging, interplant kommunikasie, *TaHlp01*