Metabolic aspects of the early response of leaf rust-infected wheat

By

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"Conquering any difficulty always gives one a secret joy, for it means pushing back a boundary-line and adding to one's liberty."
- Henri Frederic Amiel, "The Private Journal of Henri Frederic Amiel"

That which you call your soul or spirit is your conciousness, and that which you call 'free will' is your mind's freedom to think or not, the only will you have, your only freedom, the choice that controls all the choices you make and determines your life and your character.
- Ayn Rand, "Atlas Shrugged"

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Abbreviations

A

ACC	1-aminocyclopropane-1-carboxylic acid
AP	Appressorium
ASSV	Aborted substomatal vesicle
ATP	Adenosine-5'-triphosphate
Avr	Avirulence

B

BBI	Bowman-Birk serine protease inhibitor
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
BTH	Benzothiadiazole

D

DCR	Dark control resistant
DCS	Dark control susceptible
dCTP	Deoxycytidine triphosphate
DDRT-PCR	Differential display reverse transcription PCR
DIR	Dark infected resistant
DIS	Dark infected susceptible
DTE	Dithioerythritol
DMSO	Dimethylsulfoxide
DMPC	Dimethyl pyrocarbonate
dNTP	Deoxynucleotide triphosphate

E

EDTA	Ethylenedinitrilotetraacetic acid
EST	Expressed sequence tag
ET	Ethylene
EtBr	Ethidium bromide

F

F_0	Ground state fluorescence
F_m	Maximum fluorescence of a dark adapted
$F_{m'}$	Maximum steady state fluorescence
F_s	Minimum steady state fluorescence
F_v/F_m	Maximum quantum yield of PSII
Φ_{PSII}	Quantum yield of PSII

G

G	Germtube
GST	Glutathione S-transferase

H

H_2O_2	Hydrogen peroxide
HMC	Haustorium mother cell
hpi	Hours post inoculation
HR	Hypersensitive response

I

IPTG	Isopropyl β-D-thiogalactopyranoside
INA	2,2-dichloroisonicotinic acid

J

JA	Jasmonic acid

L

LAR	Localized acquired resistance
LB	Luria-Bertani
Lr	Leaf rust resistance
LRR	Leucine-rich repeat

M

MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MeJA	Methyl jasmonate
MeSA	Methyl salicylate
MSB	Menadione sodium bisulphite

N

NBS	Nucleotide binding site
NCR	Control uninfected resistant
NCS	Control uninfected susceptible
NIR	Control infected resistant
NIS	Control infected susceptible
NO	Nitric oxide
NOS	Nitric oxide synthase
NPQ	Non-photochemical quenching

O

O_2^-	Superoxide radical
OH^-	Hydroxyl radical
ORF	Open reading frame

P

PAL	Phenylalanine ammonia-lyase
PCR	Polymerase chain reaction
PI	Protease inhibitor
PR	Pathogenesis-related
PS	Photosystem
PVP	Polyvinylpyrrolidone

Q

Q_A	Plastoquinone molecule
q_P	Proportion of open PSII

R

R	Resistance
RACE	Rapid Amplification of cDNA Ends
RbcL	RUBISCO large subunit
RbcS	RUBISCO small subunit
RLK	Receptor-like protein kinase
RPK	Receptor protein kinase
RT-PCR	Reverse transcription PCR
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
RuBisCO	Ribulose-1,5-bisphosphate carboxylase/oxygenase

S

SA	Salicylic acid
SAR	Systemic acquired resistance
SLG	*S*-locus glycoprotein
SDS	Sodium dodecyl sulfate
SRK	*S* receptor kinase
SSH	Suppression subtractive hybridization
SSV	Substomatal vesicle

T

Tris	Tris (hydroxymethyl)-aminomethane
Tween™ 20	Polyoxyethylene sorbitan monolaurate

U

U	Urediospore
UV	Ultraviolet

W

WIPK	Wound-induced protein kinase

X

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

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Chapter 1

Introduction

Pathogens utilize plants as a source of nutrients. This forces the plant to activate a range of survival mechanisms to overcome the disease. Included are both physical barriers (León *et al*., 2001) and biochemical defences (Pieterse *et al*., 2001). A typical resistance response of plants involves a rapid localized cell death at the site of infection that is mediated by elevated levels of reactive oxygen species characteristic of the hypersensitive response (Lam *et al*., 2001). In addition, various signalling molecules such as salicylic acid, jasmonic acid and ethylene are also involved. Infection also leads to the induced expression of defence-related genes (Hammond-Kosack and Jones, 1996) such as the pathogenesis-related genes (Van Loon *et al*., 2006). The production of these proteins has been observed upon infection or infestation of plants with viruses, fungi, bacteria or insects and is used as a marker for the induced defensive state (Bronner *et al*., 1991; Broderick *et al*., 1997; Van der Westhuizen *et al*., 1998; Velazhahan and Muthukrishnan, 2003; Cui *et al*., 2005; Bonfig *et al*., 2006; Chandra-Shekara *et al*., 2006).

Plant disease resistance in most cases, can be defined by a gene-for-gene interaction described by Flor (1956). The recognition of the pathogen by the plant is dependent on two genes, the disease resistance gene located within the plant and the pathogen-borne avirulence gene (Flor, 1971). Resistance gene products function as receptors that recognise and bind ligands or elicitors that could be either the avirulence gene product itself, or a molecule that was produced directly or indirectly by the avirulence protein (Baker *et al*., 1997). This recognition and binding of the ligand by the corresponding receptor protein then facilitates the activation of an appropriate defence signalling pathway (Haffani *et al*., 2004).

Receptor-like protein kinases frequently act as R-proteins as they are at the forefront of the recognition of these avirulence gene products due to their location on the plasma membrane and their ability to phosphorylate both serine and threonine amino acids (Walker, 1994). Receptors such as Lrk10 and Xa21 are both receptor-like protein kinases which confers resistance to *Puccinia triticina* and *Xanthomonas oryzae* in wheat and rice respectively (Song *et al*., 1995; Feuillet *et al*., 1997). In addition to the receptor-like protein kinases, other protein kinases such as mitogen

activated protein kinases also play key roles in the transfer of the signal from the plasma membrane to the nucleus (Jouannic *et al*., 2000).

Once the signal is efficiently relayed a complex defence response involving several different aspects is activated. Proteases are present in all plants, animals and microorganisms where it breaks down proteins by hydrolyzing peptide bonds (Fan and Wu, 2005). These proteases, when present in a high concentration, are detrimental for the plant. Their activity is thus regulated by protease inhibitors (Rawlings *et al*., 2004). Pathogens also secrete proteases during infection to digest plant proteins (De Leo and Gallerani, 2002). The plants react by inducing the expression of protease inhibitor genes. Protease inhibitors form a stable complex with target proteases thereby blocking, altering or preventing access to the active sites of the protease (Habib and Khalid, 2007). The involvement of protease inhibitors were shown during various plant-pathogen interactions (Eckelkamp *et al*., 1993; Lorito *et al*., 1994; Falco and Silva-Filho, 2003). Plant protease inhibitors also accumulate systemically after wounding (Eckelkamp *et al*., 1993) and show a similar induced expression pattern upon application of chemical elicitors (Zhao *et al*., 1996).

In addition to protease inhibitors, plants employ various other mechanisms as part of the defence response. This costs the plant a lot in terms of energy consumption. In addition, pathogens utilize plants for nutrients and while photosynthesis is the source of nutrients, the site of pathogen infection forms a sink for the nutrients (Thomson *et al*., 2003). The role of photosynthesis occurring in the chloroplasts is to use water, CO_2 from the atmosphere and energy generated by the sun to synthesise organic compounds (Mauseth, 1995). A reason for the suboptimal functioning of photosynthesis in susceptible plants is partly due to the breakdown of photosynthetic components such as the large subunit of RuBisCO by enzymes that are present in the infecting pathogen (Van der Westhuizen and Botha, 1993). Silencing of the oxygen-evolving complex of photosystem II increased susceptibility of *Nicotiana benthamiana* when infected with tobacco mosaic virus (Abbink *et al*., 2002).

Since photosynthesis relies on light energy, it is only logical that plant resistance might also be indirectly dependent on the presence of light. Light was shown to be required in the development of plant resistance during infection (Zeier *et al*., 2004; Chandra-Shekara *et al*., 2006). Evidence of this was shown when *Arabidopsis* plants were infected with Turnip Crinkle Virus (Chandra-Shekara *et al*., 2006). Salicylic acid glucoside levels were lower in plants placed in the dark compared to the normal plants suggesting that darkness also affects the SA biosynthetic pathway. To further confirm the importance of light certain wound related pathogenesis-related proteins have been shown to be regulated by phytochrome (Magliano and Casal, 1998). When plants are attacked by pathogens or placed under stress the photosynthetic capacity is compromised leading to the plant being less capable of fending off attacking pathogens (Lu and Zhang, 2000; Thomson *et al*., 2003).

Based on the above given background, the overall aim of this study was to identify and to study various aspects of the complex defence response shortly after wheat was infected by *Puccinia triticina*. SSH will initially be used to clone differentially expressed cDNA from *P. triticina* infected wheat. Once the identity of these genes are known, their involvement in various defence aspects will be studied in order to obtain a better idea of the complex response of infected wheat. The role of light during this specific interaction will also be confirmed.

Chapter 2

Literature review

2.1. Introduction

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

2.2. Plant defence

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

Exposure to various microorganisms or environmental stresses can lead to the activation of inducible defence mechanisms (Métraux *et al.*, 2002). The induced resistance response is dependent on the recognition of the pathogen by the plant. This recognition is extremely specific and can distinguish between different races of pathogens. The ability to recognise the pathogen speedily will determine the effectiveness of the resistance response. If not, disease will follow.

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

2.3. Gene-for-gene concept

As a whole, plants are naturally resistant to most pathogens (Dangl and Jones, 2001). Pathogens can however overcome these natural defences of the plant, making the plant susceptible to the particular organism (Bergelson *et al.*, 2001). Two genes are involved in this co-evolution of resistance and susceptibility, namely a plant-borne disease resistance (*R*) gene and a pathogenic avirulence (*avr*) gene.

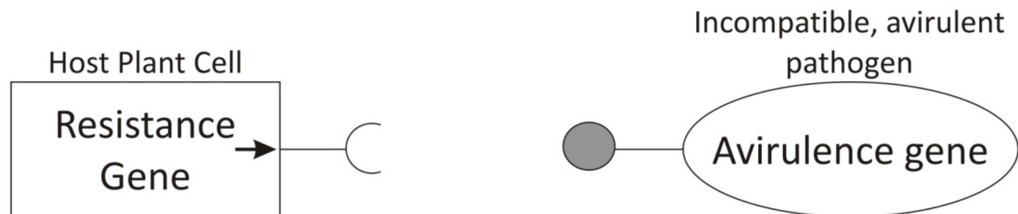
Resistance genes play an important role in the gene-for-gene interaction which confers resistance to pathogens carrying the corresponding avirulence genes (Flor, 1971). In 1956, Flor defined the gene-for-gene concept as being that, for every incompatible reaction, the resistant plant contains an *R* gene while the complementary *avr* gene resides in the invading pathogen. Specific pathogen recognition is dependent on the interaction between the encoded products of the *R* and *avr* genes, either directly or indirectly (Van der Biezen and Jones, 1998).

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

2.4. Plants and disease

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

Resistance



Susceptibility

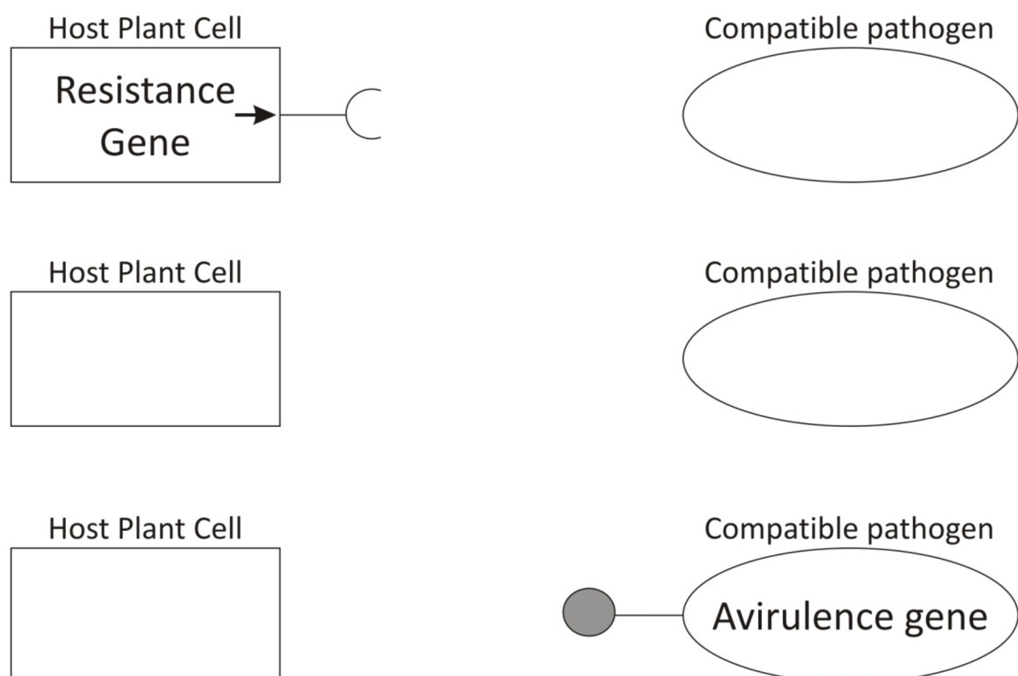


Figure 2.1. Molecular model of the gene-for gene interaction in plants (Staskawicz *et al.*, 1995).

Different aspects of plant defence have been revealed by genetic, genomic and biochemical analyses. There are several degrees of resistance, namely 1) non-host resistance, 2) race non-specific resistance, 3) race specific resistance and 4) basal defence (Hammond-Kosack and Parker, 2003).

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

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

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

2.5. Receptor proteins

Receptor proteins form part of the first line of recognition upon pathogen infection (Martin, 1999). After the plant recognises the pathogen, it can switch on the appropriate defence mechanism.

2.5.1. Resistance genes

All *R*-genes thus far described are receptor proteins involved in the binding of an appropriate elicitor (Hahn, 1996; Martin *et al.*, 2003). In several cases, the elicitor was proven to be the *avr* gene product (Greenberg, 1997; Boller and Keen, 1999; Keller *et al.*, 2000). R proteins can be placed into five different categories based on their structural characteristics: 1) intracellular protein kinases (e.g. Pto from tomato (Martin *et al.*, 1993)), 2) transmembrane receptor-like protein kinases (RLKs) with an extracellular leucine-rich repeat (LRR) domain and cytoplasmic protein kinase domain (e.g. Xa21 from rice (Song *et al.*, 1995)), 3) intracellular receptor-like proteins with LRR domains and nucleotide binding sites (NBS) (e.g. RPS2 and RPM1 from *Arabidopsis* (Bent *et al.*, 1994; Grant *et al.*, 1995) and *Prf* from tomato (Salmeron *et al.*, 1996)), 4) intracellular receptor-like proteins with LRR and NBS domains and a region of similarity to the *Drosophila* Toll and the mammalian interleukin-1 receptors (e.g. RPP5 from *Arabidopsis* (Parker *et al.*, 1997)) and 5) transmembrane receptor-like proteins with extracellular LRR domains (e.g. Cf-9 from tomato (Jones *et al.*, 1994)). The recognition by these receptors is the first step in the activation of the defence response (Skirpan *et al.*, 2001).

Xa21 and Xa26 confer resistance to *Xanthomonas oryzae* in *Oryza sativa* (Song *et al.*, 1995; Yang *et al.*, 2003). Xa21 and Xa26 both carry a LRR motif and a Ser/Thr kinase domain suggesting that it may play a role in cell surface recognition of a pathogenic ligand. *Xa26* has been observed to be constitutively expressed in the rice cultivar Minghui 63 which was inoculated with the *Xanthomonas oryzae* strain JL691 (Sun *et al.*, 2004).

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

Lrk10 that was isolated from wheat, forms part of the *wlrk* family of plant RLKs that was mapped to the *Lr10* disease resistance locus (Feuillet *et al.*, 1997). *Lr10* confers resistance to leaf rust. LRK10 bears an extracellular domain to which no other protein showed similarity to. More than 50 leaf rust resistance (*Lr*) genes (Table 2.1) have been identified in wheat (Feuillet *et al.*, 2003).

2.5.2. Receptor-like protein kinases

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

The first plant RLK gene was cloned from maize and was identified by Walker and Zhang (1990). Plant RLKs are similar to RPKs except that autophosphorylation is Ser/Thr specific (Walker, 1994). Only one plant RLK was found to be a tyrosine specific protein kinase, namely PRK1 (Mu *et al.*, 1994). Table 2.2 is a summary of the RLKs identified in plants (Stein *et al.*, 1991; Chang *et al.*, 1992; Kohorn *et al.*, 1992; Walker, 1993). A database has been set up to regulate the information on RLKs.

Table 2.1. Leaf rust resistance genes in wheat with their functions (http://www.cdl.umn.edu/res_gene/wlr.html).

Lr gene	Origin	Linkage	Tester source	Remarks	Gene references
1	Common Wheat		*Centenario*		
2a	Common Wheat		*Webster*		Dyck P.L and Samborski D.J. (1968a)
2b	Common Wheat		*Carina*		Dyck P.L. and Samborski D.J. (1974)
2c	Common Wheat		*Brevit*		Dyck P.L. and Samborski D.J. (1974)
3a	Common Wheat	*Sr*11	*Democrat*		Dyck P.L and Samborski D.J. (1968a)
3bg	Common Wheat	*Sr*11	*Bage*		Haggag M.E. and Dyck P.L. (1973)
3ka	Common Wheat	*Sr*11	*Klein Aniversario*		Haggag M.E. and Dyck P.L. (1973)
9	*Aegilops umbellulata*		*Transfer*		Sears E.R. (1956)
10	Common Wheat		*Exchange*		Dyck P.L. and Kerber E.R. (1971)
11	Common Wheat		*Hussar*		Dyck P.L and Johnson R. (1983)
12	Common Wheat		*Exchange*	adult plant resistance	Dyck P.L. *et al*. (1966)
13	Common Wheat	*Ne*2m, *Lr*23	*Frontana*	adult plant resistance	"
14a	*Yaroslav emmer*		*Selkirk*		Dyck P.L. and Samborski D.J. (1970)
14b	Common Wheat		*Maria Escobar*		"
15	Common Wheat	*Lr*2, *Sr*6	*W1483*		Luig N.H. and McIntosh R.A. (1968)
16	Common Wheat	*Sr*23	*Exchange*		Dyck P.L. and Samborski D.J. (1968b)
17a	Common Wheat	*Lr*37, *Sr*38, *Yr*17	*Klein Lucero*		"
17b	Common Wheat	*Lr*37, *Sr*38, *Yr*17	*Harrier*		
18	*T. timopheevi*		*Africa 43*		"
19	*Thinopyrum ponticum*	*Sr*25	*Thinopyrum ponticum*		Sharma D. and Knott D.R. (1966)
20	Common Wheat	*Pm*1, *S*15, *Sr*22	*Timmo*		Browder L.E. (1972)
21	*T. tauschii*		*T. tauschii*		Rowland G.G. and Kerber E.R. (1974)
22a	*T. tauschii*	Tg, *W*2	*T. tauschii*	adult plant resistance	"
22b	Common Wheat	Tg, *W*2	*Thatcher*	adult plant resistance	Dyck P.L. (1979)
23	Durum Wheat	*Lr*13, *Sr*9	*Gabo*	test at 25°C	McIntosh R.A. and Dyck P.L. (1975)
24	*Thinopyrum ponticum*	*Sr*24	*Agent*		Browder L.E. (1973)
25	*Secale cereale*	*Pm*7	*Transec*		Driscoll C.J. and Anderson L.M. (1967)
26	*Secale cereale*	*Sr*31, *Yr*9	St-1-25		Zeller F.J. (1973)
27	Common Wheat	*Sr*2	*Gatcher*	Functional only with *Lr*31	Singh R.P. and McIntosh R.A. (1984)
28	*A. speltoides*		C-77-1		McIntosh R.A. *et al*. (1982)
29	*Thinopyrum ponticum*		CS7D-Ag#11		Sears E.R. (1973)
30	Common Wheat		*Terenzio*		Dyck P.L. and Kerber E.R. (1981)

31	Common Wheat		*Gatcher*	Functional only with *Lr*27	Singh R.P. and McIntosh R.A. (1984)
32	*T. tauschii*		*T. tauschii*		Kerber, E.R. (1987)
33	Common Wheat	*Lr*26	PI58458		Dyck P.L. *et al*. (1987)
34	Common Wheat	*Yr*18, *Bdv*1	PI58548	test at 10°C	Dyck P.L. (1987)
35	*A. speltoides*	*Sr*32?	*A. speltoides*	adult plant resistance, linked to stem rust resistance	Kerber E.R. and Dyck P.L. (1990)
36	*A. speltoides*		*A. speltoides*		Dvorak J. and Knott D.R. (1990)
37	*A. ventricosa*	*Sr*38, *Yr*17	VPM	test at 18°C	Bariana H.S. and McIntosh R.A. (1993)
38	*Thinopyrum intermedium*		*Thinopyrum intermedium*		Friebe B. *et al*. (1992)
39*	*T. tauschii*		*T. tauschii*		
40*	*T. tauschii*		*T. tauschii*		
41	*T. tauschii*		*T. tauschii*		Cox T.S. *et al*. (1993)
42	*T. tauschii*		*T. tauschii*		"
43*	*T. tauschii*		*T. tauschii*		
44	*T. spelta*	*Lr*33	*T. aestivum spelta* 7831		Dyck P.L. and Sykes E.E. (1994)
45	*Secale cereale*		*Secalis cereale*		McIntosh R.A. *et al*. (1995)
46	Common Wheat	*Yr*29	*Pavon* 76		Singh R.P. *et al*. (1998)
47	*A. speltoides*		*Pavon*		Dubcovsky J. *et al*. (1998)
48	Common Wheat		CSP 44	adult plant resistance with *Lr*34	Saini R.G. *et al*. (2002)
49	Common Wheat		VL 404	adult plant resistance with *Lr*34	"
50	*T. timopheevi*		*T. timopheevii* subsp. *armeniacum*		Brown-Guedira G.L. *et al*. (2003)
51	*A. speltoides*		*A. speltoides*	translocation of segment of 1S	Helguera M. *et al*. (2005)
52	Common Wheat				
53	*T. dicoccoides*		*T. dicoccoides*		
54	*A. kotschyi*		*A. kotschyi*		
55	*E. trachycaulis*		*E. trachycaulis*		

*** *Lr*39 = *Lr*41; *Lr*40 = *Lr*21; *Lr*43 is not a unique gene, germplasm line had *Lr*21 and *Lr*39.**

Table 2.2. Plant RLKs with their functions (modified and updated from Torii and Clark, 2000; Becraft, 2002). Highlighted fields are RLKs involved in plant defence.

RLK/Class	Plant species	Biological function (if not known, expression pattern)	Reference
S-domain class			
SRK	*Brassica oleracea*	Self-incompatibility recognition	Stein *et al.,* 1991
SFR2	*Brassica oleracea*	Defence response signalling	Pastuglia *et al.,* 1997
ARK1	*Arabidopsis thaliana*	(Leaf cell expansion)	Tobias *et al.,* 1992
ARK2		(Cotyledon, leaf, sepal)	Dwyer *et al.,* 1994
ARK3		(Flower pedicles)	Dwyer *et al.,* 1994
RLK1		(Rosettes)	Walker, 1993
RLK4		(Root-hypocotyl boundary, base of lateral root, base of the petiole)	Coello *et al.,* 1999
ZmPK1	*Zea mays*	(Seedling roots, shoots and silks)	Walker and Zhang, 1990
KIK1		(Husks, etiolated shoots)	Braun *et al.,* 1997
OsPK10	*Oryza sativa*	(Upregulated by light)	Zhao *et al.,* 1994
LRR class			
BRI1	*Arabidopsis thaliana*	BR perception	Li and Chory, 1997
CLAVATA1		Meristem and flower development	Clark *et al.,*1997
ERECTA		Organ elongation	Torii *et al.,* 1996
PRK1	*Petunia inflate*	Pollen development	Lee *et al.,* 1996
SERK	*Daucus carota*	Correlation with embryogenic potential	Schmidt *et al.,* 1997
Xa21	*Oryza sativa*	Resistance to *Xanthomonas oryzae*	Song *et al.,* 1995
Xa26	*Oryza sativa*	Resistance to *Xanthomonas oryzae*	Sun *et al.*, 2004
LePRK1, 2	*Lycopersicon esculentum*	(pollen-pistil interaction)	Muschietti *et al., 1998*
RKF1	*Arabidopsis thaliana*	(anther specific)	Takahashi *et al.,* 1998
RPK1		(osmotic-stress induced)	Hong *et al.,* 1997
LRRPK		(Light-repressed)	Deeken and Kaldenhoff, 1997
TMK1		(Abscisic acid-, dehydration-, high salt- and cold-induced)	Chang *et al.,* 1992; Hong *et al.,* 1997
RLK5/HAESA		Floral abscission	Jinn *et al.,* 2000
LTK1, 2, 3	*Zea mays*	(endosperm specific)	Li and Wurtzel, 1998
OsTMK1	*Oryza sativa*	Gibberellin-induced cell division and elongation	van der Knaap *et al.,* 1999
EILP	*Nicotiana tabacum*	Non-host disease resistance	Takemoto *et al.*, 2000
OsLRK1	*Oryza sativa*	Floral meristem activity	Kim *et al.,* 2000
SARK	*Phaseolus vulgaris*	Senescence induced	Hajouj *et al.,* 2000
SbRLK1	*Sorghum bicolor*	(mesophyll cells)	Annen and Stockhaus, 1999
LRPKm1	*Malus x domestica*	Disease resistance	Komjanc *et al.*, 1999
TNFR class			
CRINKLY 4 (CR4)	*Zea mays*	Epidermal cell specification	Becraft *et al.,* 1996
EGF class			
WAK1, 2, 3, 4	*Arabidopsis thaliana*	Cell expansion and disease response	He *et al.,* 1996; Wagner and Kohorn, 2001
PR5 class			
PR5K	*Arabidopsis thaliana*	Disease/stress response	Wang *et al.,* 1996
Lectin class			
LecRK1	*Arabidopsis thaliana*	Development and adaptation	Riou *et al.,* 2002
Other class			
CrRLK1	*Catharanthus roseus*		Schulze-Muth *et al., 1996*
RKF2, 3	*Arabidopsis thaliana*	(Ubiquitous)	Takahashi *et al.,* 1998
Lrk10	*Triticum aestivum*	Leaf rust resistance	Feuillet *et al.,* 1997
At-RLK3	*Arabidopsis thaliana*	(induced by oxidative stress, pathogen attack)	Czernic *et al.,* 1999
PvRK20-1	*Phaseolus vulgaris*	(plant-microbe interaction)	Lange *et al.,* 1999

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

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

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

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

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

2.6. Mitogen-activated protein kinases

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

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

Activation of the downstream end of the cytoplasmic MAPK module often induces the translocation of the MAPK into the nucleus where the kinase activates the expression of certain sets of genes through the phosphorylation of specific transcriptional factors (Hirt, 2000). Figure 2.2 shows an example of the MAPK cascade activated during hypo-osmotic stress or a mechanical stimulus (Taylor *et al.*, 2001). Activation of the MAPKK is stimulated by a hypo-osmotic or mechanical stimulus which then in turn activates a MAPK. The MAPK activates the internal Ca^{2+} store by opening the anion channel regulating the Ca^{2+} concentration in the cell. The change in Ca^{2+} concentration activates another MAPKK and MAPK which will then activate NADPH oxidase to convert O_2 to superoxide to form hydrogen peroxide (H_2O_2).

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

Various MAP kinases have also been found to play a role during the plant defence response. MPK6 in *Arabidopsis* (Menke *et al.*, 2004) plays a role in the basal resistance of the plant against a virulent bacterial pathogen. When MPK6 was silenced, plants showed increased susceptibility. p48 SIP kinase in tobacco belongs to

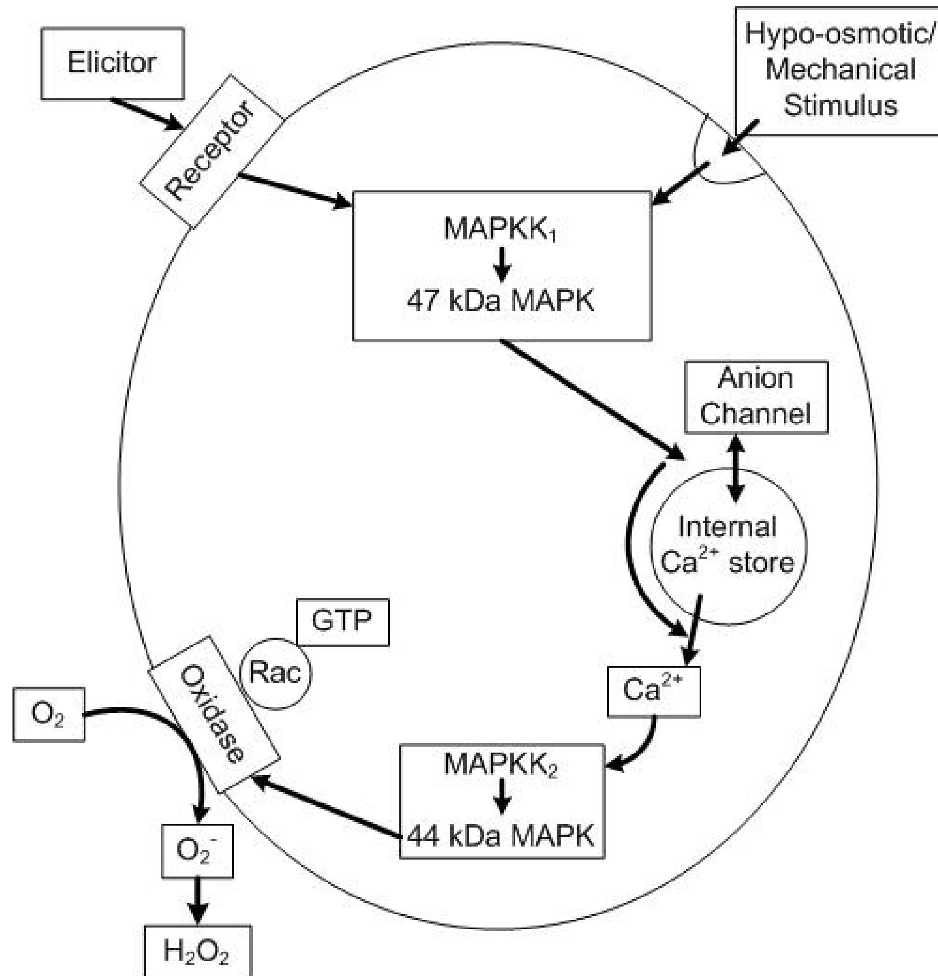


Figure 2.2. Schematic diagram of a MAPK signal-transduction pathway leading to the oxidative burst (Taylor *et al.*, 2001).

the MAP kinase family and is inducibly transcribed by SA treatment (Zhang and Klessig, 1997). Since p48 SIP kinase is activated by SA which plays an important role in signalling the defence response, it was suggested that p48 SIP kinase is also involved in the activation of defence responses.

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

OsBIMK1 shows similarity to previously reported MAPK genes (Song and Goodman, 2002). The expression of *OsBIMK1* is activated upon treatment of plants with benzothiadiazole (BTH) as well as INA, probenazole, JA, MeJA, *Pseudomonas syringae* pv. *syringae* or wounding. This suggests that *OsBIMK1* plays an important role in rice disease resistance.

Further studies on the role of MAP kinase signalling pathways will enable a better understanding of the molecular mechanisms controlling plant development and plant responses to various stresses. In addition to MAPK, RLKs and a variety of other protein kinases partake in the defence response of plants against pathogens. This clearly indicates the importance of phosphorylation and dephosphorylation in the activation of plant defence.

2.7. Biochemical defences

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

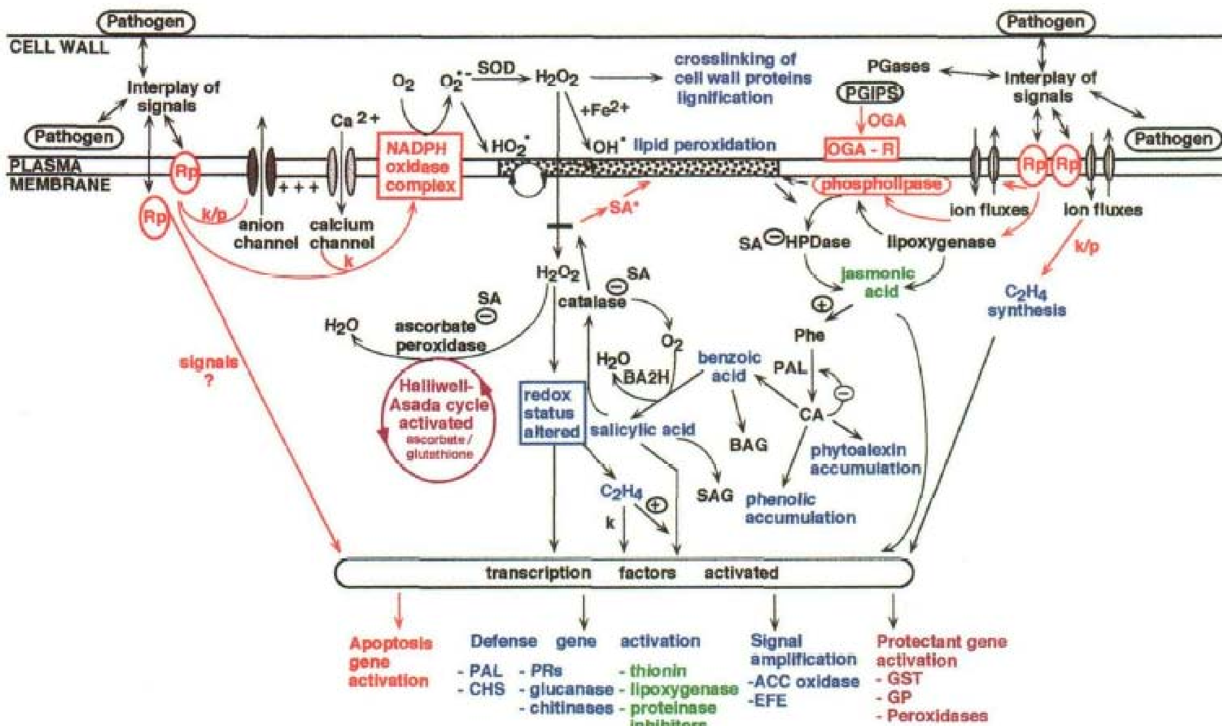


Figure 2.3. Complexity of signalling events controlling activation of defence responses. Abbreviations: ACC oxidase, 1-aminocyclopropane-1-carboxylate oxidase; BAG, benzole acid glucoside; BA2H, benzole acid-2 hydroxylase; CA, cinnamic acid; CHS, chalcone synthase; EFE, ethylene-forming enzyme; HO_2, hydroperoxyl radical; HPDase, hydroxyperoxide dehydrase; GP, glutathione peroxidase; GST, glutathione S-transferase; k, kinase; O_2~, superoxide anion; OH^-, hydroxyl radical; OGA and OGA-R, oligalacturonide fragments and receptor; p, phosphatase; PAL, phenylalanine ammonia-lyase; PGases, polygalacturonases; PGIPS, plant polygalacturonic acid inhibitor proteins; Phe, phenylalanine; PR, pathogenesis related; Rp, plant receptor protein; SA and SAG, salicylic acid and salicylic acid glucoside; SA*, SA radical; and SOD, superoxide dismutase (Hammond-Kosack and Jones, 1996).

pathogens (Pieterse *et al.*, 2001).

The plant defence response includes the synthesis of various chemicals and enzymes that allow the plant to survive the attack. Included are anti-microbial phytoalexins (Keen, 1999), protease inhibitors (Lorito *et al.*, 1994), lytic enzymes such as chitinases and glucanases which attack the pathogen cell wall (Lamb *et al.*, 1989; Ryan and Jagendorf, 1995) and other chemicals such as cyanogenic glycosides and glucosinolates (Osbourn, 1996). The latter occurs as inactive precursors of secondary metabolites that have antifungal activity that are produced in response to tissue damage or pathogenic attack.

Induced resistance is a state of enhanced defensive capability deployed by a plant when appropriately stimulated (Kuc, 1982). This includes the activation of latent resistance mechanisms that are expressed upon repetitive inoculation with a pathogen (van Loon, 1997). Induced resistance occurs naturally due to limited infection by a pathogen, especially when the plant develops a HR. It can also be induced by certain chemicals, non-pathogens, avirulent forms of pathogens, incompatible races of pathogens or by virulent pathogens under circumstances where infection is delayed due to environmental conditions (Ryals *et al.*, 1994; Hahn, 1996; van Loon *et al.*, 1998). The HR can also be induced by elicitors, chemicals that have the ability to activate a signalling cascade that could lead to the activation of SAR (Ryals *et al.*, 1996).

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

Induced resistance develops at the point of attack is known as localized acquired resistance (LAR). LAR occurs when only the tissue exposed to the pathogen or chemical becomes resistant (Ross, 1961). SAR and LAR are similar in that they are effective against a range of pathogens. They differ in that the signal that distributes

the enhanced defensive ability throughout the plant during SAR seems to be lacking in LAR (van Loon *et al.*, 1998).

2.8. Hypersensitive response

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

The HR consists of three different phases namely, the induction phase, the latent phase and the presentation or collapse phase (Jabs and Slusarenko, 2000). In the induction phase, *avr* gene expression is activated in the pathogen and the *avr* products are transported into the host cell. This phase involves a rapid reaction to close the wound thereby protecting the plant from losing cellular components and restricting micro-organisms from entering the plant tissue. During the latent phase, macroscopic symptoms and membrane damage associated with the HR occur. Photosynthetic protein synthesis is also inhibited by arresting the translation of nuclear encoded photosynthetic genes (Jabs and Slusarenko, 2000). In the final phase, the infected host cells will collapse and die, thereby restricting the flow of nutrients towards the pathogen.

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

2.8.1. Reactive oxygen species (ROS)

ROS play an important role in the early signalling of biotic and abiotic stresses (Mittler, 2002). ROS that are involved in plant-pathogen interactions include nitric oxide (NO) (Durner *et al.*, 1998), superoxide radical (O_2^-), H_2O_2 and the hydroxyl

radical (OH^-) (Wojtaszek, 1997). Enzymes generating ROS during the defence response include NADPH oxidase (Lamb and Dixon, 1997), peroxidase (Benhamou, 1996), oxalate oxidase and amine oxidase (Bolwell and Wojtaszek, 1997). The production of ROS is pH-dependent and shows optimal production at a neutral to basic pH (Bolwell and Wojtaszek, 1997). H_2O_2 also controls the influx of Ca^{2+} in the cell. The increase of Ca^{2+} concentration is shown to be an important factor in the development of reactive oxygen intermediate (ROI) mediated cell death (Levine *et al.*, 1996).

NO is a gaseous free radical with a short half-life that is synthesised by nitric oxide synthase (NOS) in plants, animals and microbes (Durner *et al*., 1998). NO diffuses through biological membranes and may play a role in intra- and intercellular signalling (Beligni and Lamattina, 2001). The function of NO in plants has not been characterised, but evidence of the role NO plays in plant defence (Durner *et al*., 1998; Wang and Wu, 2005) as well as growth and development has been emerging (Kopyra and Gwózdz, 2004). It co-operates with ROI to induce HR and the expression of various defence related genes including the *PR* genes (Delledonne *et al*., 1998, 2002). A substantial rise in NO was detected when *Arabidopsis* plants were wounded (Huang *et al*., 2004) suggesting that NO may possibly be involved in JA-induced or mediated defence responses in plants.

2.8.2. Salicylic acid

SA is a phenolic acid and plays a role during signalling in the primary defence response against pathogens. Application of SA to plants induces the expression of *SAR* related genes (Sticher *et al.*, 1997). The role of SA in signalling during the defence response was elucidated by using *NahG* transformants (Delaney *et al.*, 1994). Plants over-expressing the *NahG* gene coding for salicylate hydroxylase, which converts SA to an inactive catechol, showed enhanced susceptibility to pathogen attack (Ryals *et al.*, 1995). The *NahG* plants defective in SA signalling have an altered defence pathway that is independent of the accumulation of SA (Heck *et al*., 2003; van Wees and Glazebrook, 2003). Reduced drought tolerance and delayed

leaf chlorosis during senescence were observed in *NahG* transgenic *Arabidopsis* plants (Morris *et al*., 2000; Chini *et al*., 2004). Other mutants, including *sid1*, *sid2* and *pad4* affecting SA signalling, also showed susceptibility to pathogen infection. These mutants are defective in SA accumulation during the response to pathogen infection (Zhou *et al.*, 1998; Nawrath and Métraux, 1999). This confirms that SA is important for basic resistance against different types of pathogens (Pieterse *et al.*, 2001).

2.8.3. Jasmonic acid

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

2.8.4. Ethylene

ET is a gaseous plant hormone that plays a role in various developmental processes (Zhou and Thornburg, 1999). ET is synthesized from *S*-adenosyl-L-methionine via 1-aminocyclopropane-1-carboxylic acid (ACC) and plays an important role in various plant disease resistance pathways (Zhou and Thornburg, 1999; Guo and Ecker, 2004). Plants deficient in ET signalling show either increased susceptibility or increased resistance (Wang *et al.*, 2002). ET seems to suppress symptom development during necrotrophic pathogen infection, but enhances the cell death caused by a different type of pathogen infection. An example of this is soybean mutants with reduced ET

sensitivity that produce less severe chlorotic symptoms when challenged with the virulent *Pseudomonas syringae* pv *glycinea* and *Phytophthora sojae* strains (Hoffman *et al.*, 1999), whereas virulent strains of the fungi *Septoria glycines* and *Rhizoctonia solani* cause more severe symptoms (Hoffman *et al.*, 1999).

2.8.5. Pathogenesis-related proteins

Both pathogen- and SA-induced resistance are associated with the induced expression of several families of *PR* genes during the HR (Table 2.3). The induction of *PR* gene expression is regularly linked to necrotizing infections giving rise to SAR, and has been used as a marker of the induced defensive state (Ward *et al.*, 1991a; Uknes *et al.*, 1992). PR proteins play a major role in the defence response in many plants under stress and are detected in plants after exposure to insects (Bronner *et al.*, 1991; van der Westhuizen and Pretorius, 1995; Broderick *et al.*, 1997; van der Westhuizen *et al.*, 1998). The accumulation of PR proteins during the onset and maintenance of SAR is thought to be responsible for the enhanced resistance of the uninfected plant tissues so that they are referred to as SAR proteins. The *PR4* gene in wheat is an example of a gene that is expressed when the plant is exposed to chemical activators of SAR and wounding (Bertini *et al.*, 2003). Two other important PR proteins are β-1,3-glucanases and chitinases. During plant defence, β-1,3-glucanases and chitinases are believed to respond by degrading hyphal walls of the pathogen (Leah *et al*., 1991; Jach *et al*., 1995; Lawrence *et al*., 1996; White *et al*., 1996).

2.8.5.1. β-1,3-glucanases

β-1,3-glucanases form part of the PR2 protein family that is able to catalyse endo-type hydrolytic cleavage of the 1,3-β-D-glucosidic linkages in β-1,3-glucans (Leubner-Metzger and Meins, 1999). It is suggested to play a role in the response of plants to pathogen attack (Côte *et al*., 1991; Ward *et al*., 1991a) and other stimuli (Memelink *et al*., 1990). β-1,3-glucanases are divided into four classes. Class I is produced as a

Table 2.3. The families of pathogenesis-related proteins (modified and updated from van Loon and van Strien, 1999).

Family	Type Member	Properties	Gene Symbols
PR1	Tobacco PR1a	antifungal, 14-17 kD	*Ypr1*
PR2	Tobacco PR2	class I, II, and III endo-beta-1,3-glucanases, 25-35 kD	*Ypr2,* [*Gns2* ('*Glb*')]
PR3	Tobacco P, Q	class I, II, IV, V, VI, and VII endochitinases, about 30 kD	*Ypr3, Chia*
PR4	Tobacco R	antifungal, *win*-like proteins, endochitinase activity, similar to prohevein C-terminal domain, 13-19 kD	*Ypr4, Chid*
PR5	Tobacco S	antifungal, thaumatin-like proteins, osmotins, zeamatins, permeatins, similar to alpha-amylase/trypsin inhibitors	*Ypr5*
PR6	Tomato Inhibitor I	protease inhibitors, 6-13 kD	*Ypr6, Pis* ('*Pin*')
PR7	Tomato P_{69}	endoproteinase	*Ypr7*
PR8	Cucumber chitinase	class III chitinases, chitinase/lysozyme	*Ypr8, Chib*
PR9	Tobacco 'lignin-forming peroxidase'	peroxidases, peroxidase-like proteins	*Ypr9, Prx*
PR10	Parsley 'PR1'	ribonucleases, Bet v 1-related proteins	*Ypr10*
PR11	Tobacco class V chitinase	endochitinase activity, type I	*Ypr11, Chic*
PR12	Radish Rs-AFP3	plant defensins	*Ypr12*
PR13	*Arabidopsis* THI2.1	thionin	*Ypr13, Thi*
PR14	Barley LTP4	Non-specific lipid transfer proteins (ns-LTPs)	*Ypr14, Ltp*
PR15	barley OxOa (germin)	oxalate oxidase	
PR16	barley OxOLP	oxalate-oxidase-like proteins	
PR17	tobacco PRp27	unknown	

pre-protein with an N-terminal hydrophobic signal peptide which is co-translationally removed and a C-terminal extention that is N-glycosylated at a single site. The proteins in this class are localized in the cell vacuole. Class II, III and IV are acidic proteins lacking the C-terminal extension present in the class I enzymes and are secreted into the extracellular space (van Loon and van Strien, 1999).

2.8.5.2. Chitinases

Plant chitinases are suggested to be involved in plant disease resistance during pathogen infection (Cheng *et al.*, 2002). Chitinases catalyses the hydrolysis of chitin which is a linear polymer of β-1,4-linked *N*-acetylglucosamine residues (Khan and Shih, 2004). Chitinases have been found to be activated by fungal infection and plant activators such as INA and BTH which induce SAR (Busam *et al.*, 1997).

2.8.6. Programmed cell death

Programmed cell death is an active process occurring in response to environmental stresses and pathogen infection (Jabs and Slusarenko, 2000; Greenberg and Yao, 2004). Programmed cell death involves chromatin aggregation, cytoplasmic and nuclear condensation and fragmentation of the cytoplasm and nucleus into membrane-bound vesicles (Jabs and Slusarenko, 2000). Membrane damage is the first sign of programmed cell death. Cells that are killed usually autofluoresce and become dark brown due to the accumulation and oxidation of phenolic compounds (Heath, 2000). The role of programmed cell death during pathogenesis is to limit the spread of disease after the induction of HR at the site of infection (Greenberg, 1996; Lam *et al.*, 2001; Greenberg and Yao, 2004). There are two different mechanisms involved in cell death occurring during the compatible and incompatible interactions respectively (Greenberg, 1997). The mechanism by which cell death occurs in susceptible plants is not fully understood, but it is thought that a toxin produced by the pathogen may directly kill the plant cells. In the resistant interaction, HR is induced and rapid cell death occurs (Greenberg, 1997).

2.9. Systemic acquired resistance

SAR is a secondary response characterised by the accumulation of SA and PR proteins (Ward *et al.*, 1991b; Uknes *et al.*, 1992; Ryals *et al.*, 1996; Sticher *et al.*, 1997). Various compounds have the ability to activate SAR, namely JA, ethylene, SA and systemin (Sticher *et al.*, 1997; van Loon, 2000). SA accumulation occurs both locally and, at lower levels, systemically parallel with the development of SAR. Exogenous application of SA to wild-type plants at certain levels also induces SAR in several plants species, while excessive levels leads to phytotoxicity (Ward *et al*., 1991b; Delaney *et al.*, 1994; Chen *et al.*, 1995; Ryals *et al.*, 1996). Biochemical and morphological changes due to SAR will in the end lead to the whole plant being resistant to a broad spectrum of pathogens (Sticher *et al.*, 1997; Durrant and Dong, 2004).

2.10. Wheat and leaf rust

2.10.1. Wheat

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

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

Wheat was one of the first cereals that were domesticated. Its cultivation began in the Neolithic period. Bread wheat was grown in the Nile valley by 5000 B.C. with apparent later cultivation in other regions (e.g., the Indus and Euphrates valleys by

4000 B.C., China by 2500 B.C. and England by 2000 B.C.). This indicates that cultivation spread from Mediterranean centres of domestication. The civilizations of Western Asia and Europe have been largely based on wheat, while rice has been more important in Eastern Asia.

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

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

Wheat is constantly under threat from a variety of organisms, including the Russian wheat aphid (Walters *et al*., 1980), viruses (Truol *et al*., 2004), bacteria (Duveiller *et al*., 1992) and fungi (Marsalis and Goldberg, 2006).

2.10.2. Leaf rust

Wheat leaf (or brown) rust, caused by *Puccinia triticina* is the most widespread and regularly occurring rust on wheat (Kolmer, 1996) and is responsible for 10-15% annual yield losses and a decrease in grain quality (Šlikova *et al.*, 2003). Leaf rust are reddish-orange spore masses which are carried by wind. The spore masses occurring on the leaf are called pustules or uredinia. Leaf rust is mostly found on the leaves but can also be found on glumes and awns (McMullen and Rasmussen, 2002).

The life cycle of the rust fungi may require two different host plants and consists of up to five spore stages (Fig. 2.4). The pathogen will infect a primary alternate host, for example, meadow rue. This infection will result in circular, yellow-to-red coloured pustules on the underside of the leaves. The spores (aeciospore) produced from these hosts will in turn infect the wheat plants. The spores will develop from aeciospores to uredospores, then to teliospores which will further develop into basidiospores. These spores will then infect the primary alternate host again. Uredospores also have the ability to infect the same plants, which may lead to an epidemic.

2.10.3. The interaction between wheat and *P. triticina*

Various factors need to be present for *P. triticina* infection to occur namely viable spores, susceptible or moderately susceptible wheat plants, moisture on the leaves and favourable temperatures. Under favourable environmental conditions, rust spores germinate and penetrate the wheat leaf. Newly produced spores are wind-blown to other wheat leaves or fields (McMullen and Rasmussen, 2002).

Resistant and susceptible plants respond differently to infection. There are various levels of infection (Fig 2.5). The resistant variety has the ability to retard the fungus by depriving it of nutrients thereby stopping infection. Resistant varieties may develop yellowish-white specks at the site of infection, due to HR. The moderately resistant variety develops small reddish-orange pustules surrounded by a yellow-white ring. Susceptible varieties do not have the ability to retard fungal growth allowing the fungus to produce large pustules (Kolmer, 1996; McMullen and Rasmussen, 2002).

Genetic resistance is the most economical and preferable method of reducing yield losses due to *P. triticina* infection and can be fully utilized by knowing the identity of resistance genes in commonly used parental germplasm and released cultivars. Identification of the leaf rust resistance genes allows for efficient incorporation of different genes into germplasm pools (Kolmer, 1996).

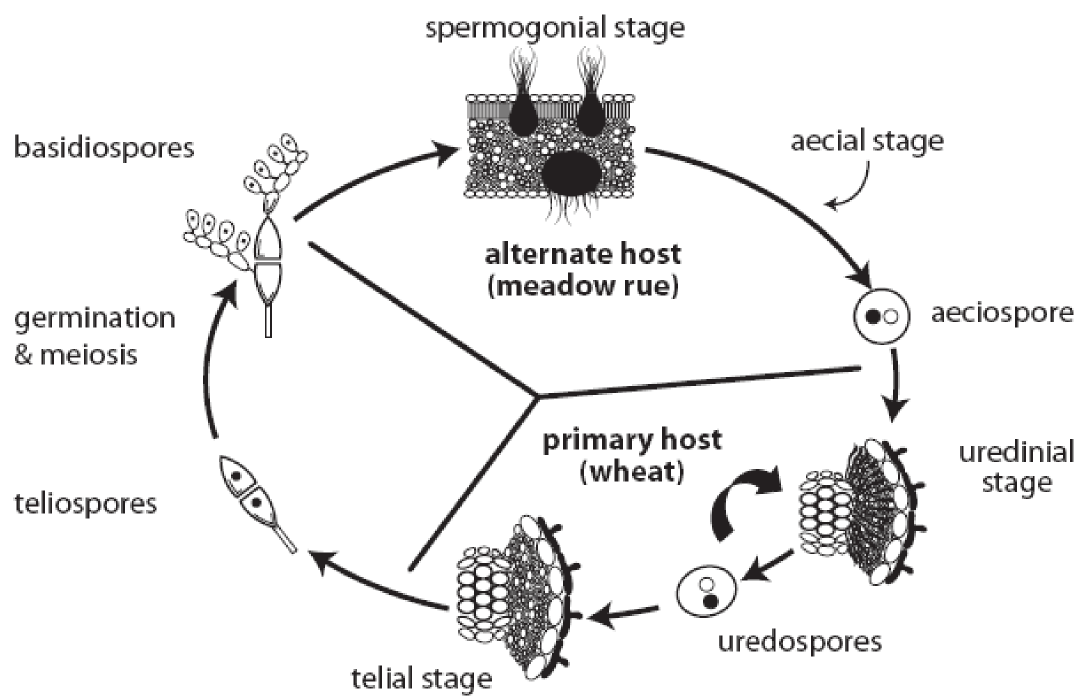


Figure 2.4. Life cycle of *P. triticina* showing the primary and alternate hosts (Marsalis and Goldberg, 2006).

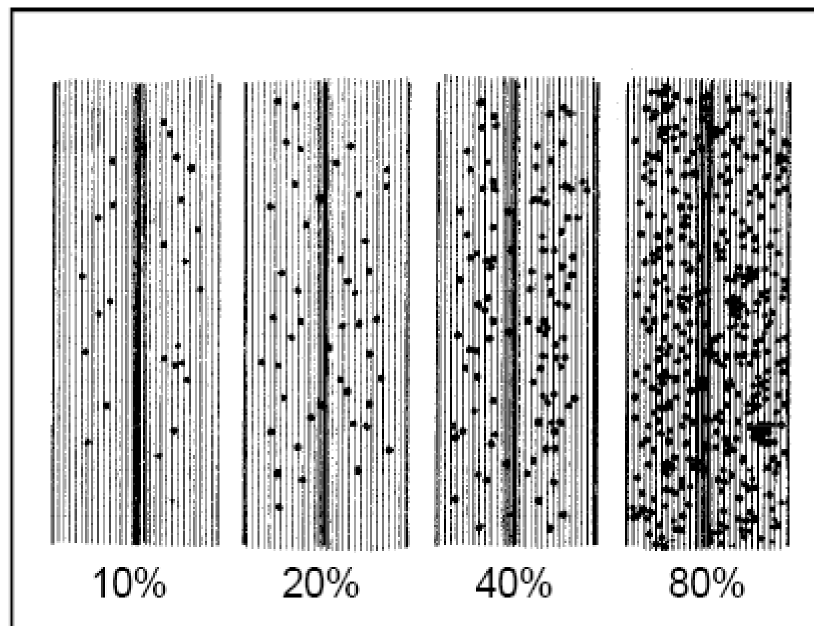


Figure 2.5. Percentage severity of leaf rust (McMullen and Rasmussen, 2002).

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

The resistance gene *Lr*34 is located at chromosome 7D and is the only effective resistance gene in the Canadian cultivar Glenlea and the American hard red winter wheat Sturdy, that has been resistant to leaf rust since its release in 1972 (Kerber and Aung, 1999). The presence of the *Lr*34 gene in the field leads to variable pustule size and low percentages of infection (Rubiales and Niks, 1995). In the wheat variety Thatcher, *Lr*34 exhibited "slow rusting" resistance and had a severity that was 50% lower than Thatcher without *Lr34*. *Lr*34 can be detected in the seedling stage, but it is best expressed in adult plants (Singh and Huerta-Espino, 2003).

Chapter 3

Isolation of cDNAs involved in the defence response of *Triticum aestivum* against *Puccinia triticina*

3.1. Introduction

Plants are constantly under both environmental and pathogenic stress. These stress situations induce a cascade of signals allowing the plant to react accordingly. The activation of an appropriate defence response involves a complex set of reactions (Brands and Ho, 2002; Suzuki *et al*., 2005; Meyer *et al*., 2006; Haydon and Cobbett, 2007). These mechanisms enable the plant to adapt and survive.

One of the responses that are induced upon pathogen infection is the HR. This involves the recognition of the pathogenic *avr* gene product by the plant's *R* gene product in a resistance response (Heath, 2000). In a susceptible response, either the *R* or *avr* gene is absent (Flor, 1956).

The defence mechanisms also include the ability to activate systemic responses like the defence related SAR. SAR involves the induction of the resistance response in the whole plant once a localized infection has taken place (Ryals *et al*., 1996). An increase in SA levels induces the expression of the pathogenesis-related genes, which play a key role in SAR (Sticher *et al.*, 1997).

Since both the HR and SAR are inducible, the inducible expressions of multiple genes are implicated. Several methods can be used to isolate these genes. Included are differential display reverse transcription polymerase chain reaction (DDRT-PCR) (Bauer *et al*., 1993), microarray analysis (Wang *et al*., 2005) and suppression subtractive hybridization (SSH) (Gibly *et al*., 2004; Shi *et al*., 2005). These methods allow for the isolation of cDNA fragments representing genes playing a role under certain conditions. SSH is a polymerase chain reaction (PCR) based technique (Diatchenko *et al*., 1996). This technique allows the isolation cDNAs that are differentially expressed by selectively removing cDNAs that are common to both control and experimental samples. A post hybridization PCR step amplifies cDNAs unique to the experimental sample.

SSH was successfully used to identify 38 genes playing a role in the response of tobacco plants against short term sulphur deficit (Wawrzyńska *et al*., 2005). Wang *et al*. (2007) isolated a large number of differentially expressed genes, including genes playing a role in photosynthesis, from *Puccinellia tenuiflora* that was saline-alkali stressed. Xiong *et al*. (2001) identified 34 differentially expressed genes from rice treated with JA, BTH, and/or rice blast infection. In another study, Neu *et al*. (2003) isolated 75 cDNA clones from *P. triticina* infected wheat. Various other studies have yielded genes playing a role in different plant-pathogen interactions (Shi *et al*., 2005; Wang *et al*., 2005).

Puccinia triticina is an important pathogen of wheat. Around 50 different resistance genes are currently present in different wheat cultivars (Feuillet *et al*., 2003). *Lr*34 is a slow rusting gene that has been associated with durable leaf rust resistance and is located on chromosome 7D (Dyck *et al*., 1966). The presence of the *Lr*34 gene in cultivars causes an increased latency period and decreased infection frequency and uredium size (Drijepondt and Pretorius, 1989). The expression of *Lr*34 is effective in the adult plant compared to seedlings (Rubiales and Niks, 1995).

To obtain a better understanding of the molecular mechanisms involved in the interaction between wheat and *P. triticina*, SSH was used to isolate cDNAs that are involved in the defence response, since this technique allows the cloning of genes with low induction levels. This will ensure a better understanding of the mechanisms involved in the early recognition of the pathogen by the plant.

3.2. Materials and methods

3.2.1. Biological Material

3.2.1.1. Fungal pathogen

Plants were inoculated with *Puccinia triticina* pathotype UVPrt9.

3.2.1.2. Cultivation of wheat

Seeds of the *P. triticina* susceptible Thatcher and resistant Thatcher+*Lr*34 wheat cultivars were planted in cones containing a sterilized soil:peatmoss mixture (1:1 w/w) and grown in the greenhouse at ±24°C with a 16 h day/8 h night cycle. The plants were fertilised daily with 2% (w/v) Multifeed. Susceptible Karee plants were similarly grown in pots for the augmentation of *P. triticina* spores.

3.2.1.3. Infection of wheat with *P. triticina*

Previous studies by Dyck (1987) tested infection at 10°C for *P. triticina*, but Drijepondt and Pretorius (1989) found that a higher temperature was more efficient. Adult Thatcher and Thatcher+*Lr*34 plants were sprayed with freshly harvested *P. triticina* spores (0.6 mg spores.ml^{-1}) that were multiplied on the susceptible Karee cultivar. The spores were resuspended in distilled water containing a drop of polyoxyethylene sorbitan monolaurate (Tween™ 20). Control plants were mock infected with water. The plants were left to dry and then placed in a dark dew-simulation cabinet at 18 to 20°C for 12 h to facilitate spore germination. After the plants were dried, they were transferred to the greenhouse. Time 0 h tissue was harvested prior to placing the plants in the dew-simulation chamber. Flag leaves from infected adult plants were harvested at 3 h intervals for 30 h. The plant material was quick frozen using liquid nitrogen and stored at -80°C. The frozen tissue was ground to a fine powder in liquid nitrogen.

3.2.2. Methods

3.2.2.1. Total RNA isolation

Distilled water was treated to remove RNase by adding 0.1% (v/v) dimethyl pyrocarbonate (DMPC), leaving it overnight at room temperature and autoclaving it the following morning. All buffers were prepared using the DMPC treated water. All apparatus used for RNA extraction were washed with soap, then with 10% (w/v) sodium dodecyl sulfate (SDS) and finally rinsed in DMPC water. The glassware was baked at 260°C for 16 h.

Total RNA was isolated from leaf tissue using Eurozol (Euroclone) according to Chomczynski and Sacchi (1987). Briefly, 100 mg ground plant material was resuspended in Eurozol, incubated at room temperature for 5 min, where after chloroform was added and the solution mixed by vortexing. After precipitating the total RNA from the cleared supernatant, RNA was dissolved in DMPC treated water and stored at -20°C. The concentration of the RNA was finally determined (Sambrook *et al.*, 2000).

To evaluate the quantity and quality of the extracted RNA, 500 ng total RNA from each treatment was separated on a 1% (w/v) agarose gel prepared in 0.5X TAE (20 mM Tris(hydroxymethyl)-amino-methane-hydrochloric acid (Tris-HCl) pH 8.0, 0.5 mM ethylenediamine tetraacetic acid (EDTA), 10 mM acetic acid) (Sambrook *et al.*, 2000). Ethidium bromide (EtBr) was added to a final concentration of 0.2 $\mu g.ml^{-1}$ to allow the visualization of the separated RNA. The running buffer used was a 0.5X TAE solution. The RNA was diluted in RNA loading buffer to a final concentration of 0.04% (w/v) Bromophenol blue, 2.5% (w/v) Ficoll, loaded on the gel and separated at 10 $V.cm^{-1}$ for 1 h. The RNA was visualized under ultraviolet light (UV) illumination (302 nm) and the gel photographed.

3.2.2.2. Suppression subtractive hybridization

Two RNA pools were prepared. For the tester sample, 30 µg total RNA isolated from infected resistant wheat harvested at 9 and 12 hours post inoculation (hpi) respectively, was mixed (Fig 3.1). The driver sample was prepared using the same time intervals from the uninfected resistant wheat. These mixtures were used to purify total mRNA using the mRNA Capture Kit (Roche) according to the manufacturer's specifications. The mRNA was finally dissolved in 100 µl DMPC treated water. The sample were lyopholized and resuspended in 4 µl DMPC treated water. A SSH reaction was performed using the PCR-Select cDNA Subtraction Kit (Clontech) using the purified tester and driver mRNA samples, according to the manufacturer's specifications. The subtraction reactions and subsequent PCR reactions were done essentially as described in the supplied protocol. The enriched cDNA fragments that were finally obtained thus represented genes that were differentially expressed due to the infection of the plants.

3.2.2.3. Sub-cloning of amplified cDNA fragments

The subtracted cDNA fragments (3.2.2.2) were cloned into the pGemT-Easy vector (Promega) and transferred to ultra-competent *Escherichia coli* (JM109) cells (Promega) according to the manufacturer's specifications. The transformation mixture was plated onto Luria-Bertani (LB) plates (1% (w/v) Tryptone, 0.5% (w/v) Yeast-extract, 1% (w/v) NaCl, 1.5% (w/v) Agar) containing 50 µg.ml^{-1} ampicillin to select for transformed colonies (Sambrook *et al*., 2000). Alpha-complementation was used to select for colonies harbouring recombinant plasmids by adding 250 µg.ml^{-1} 5-bromo-4-chloro-3-indolyl-β-$_D$-galactopyranoside (X-gal) and 250 µg.ml^{-1} isopropyl-β-$_D$-thiogalactopyranoside (IPTG) to the LB plates (Sambrook *et al*., 2000). White colonies containing putative recombinant plasmids were transferred to a new LB plate containing 50 µg.ml^{-1} ampicillin.

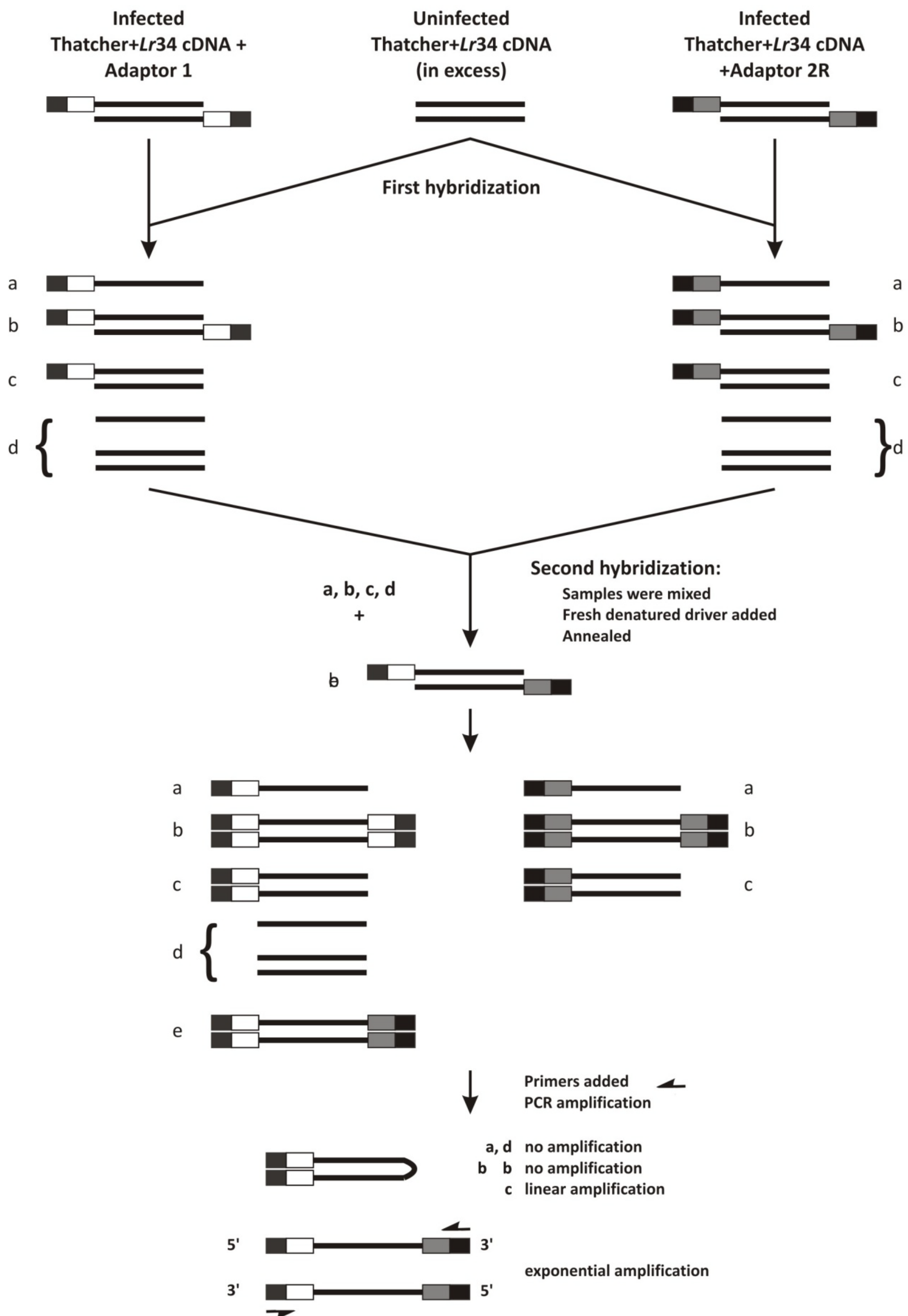


Figure 3.1. A schematic representation of SSH (adapted from Clontech).

3.2.2.4. Selection of recombinant plasmids

Individual white colonies were grown overnight in 5 ml LB medium (1% (w/v) Tryptone, 0.5% (w/v) Yeast-extract, 1% (w/v) NaCl) containing 50 $\mu g.ml^{-1}$ ampicillin and the plasmid DNA extracted according to Sambrook *et al.* (2000). Two microliter of the purified plasmid DNA was digested with 10 U *Eco*RI in the presence of 5 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, 10 mM NaCl, 0.1 mM dithioerythritol (DTE) at 37°C for 1 h. The digested plasmid DNA was separated on a 1% (w/v) agarose gel (3.2.2.1) and recombinant plasmids identified by the presence of an insert.

3.2.2.5. Expression analysis of the SSH library

The inserts of all the recombinant plasmids were amplified using T7 (5' - TAA TAC GAC TCA CTA TAG GG - 3') and SP6 (5' - TAT TTA GGT GAC ACT ATA G - 3') primers. Each PCR reaction consisted of 1 µl plasmid DNA, 50 pmol of each primer, 0.2 mM deoxynucleotide triphosphates (dNTP's), 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$ and 2.5 U Taq polymerase (Sigma-Aldrich) in a 100 µl reaction.

The amplification regime was as follows: one cycle at 94°C for two min, 30 cycles at 94°C for 30 sec, 60°C for one min and 72°C for four min followed by a final cycle of 72°C for 7 min. The success of the amplification was tested by separating 8 µl PCR product on a 2% (w/v) agarose gel (3.2.2.1). To remove unincorporated nucleotides and salts, amplified cDNA fragments were purified according to McPherson and Møller, (2006).

A reverse Northern blot was performed to confirm the differential expression patterns of the cloned cDNA fragments. To each purified PCR fragment, dimethyl sulfoxide (DMSO) was added to a final concentration of 50% (v/v). The cDNA fragments were transferred to a Hybond XL nylon membrane using a slot blot apparatus after the membrane was pretreated with 3X SSC (45 mM NaCl, 4.5 mM sodium citrate pH 7.0). A cloned *Arabidopsis* actin gene (Accession number: AY086557) was similarly amplified and transferred to each individual membrane to act as a positive hybridization control. Following the transfer of the cDNA inserts,

the individual wells were washed 3 times with 500 µl 3X SSC and the membrane dried for 15 min at 75°C.

The first strand cDNA probes used for the reverse Northern blot were prepared using 1 µg total RNA isolated from infected Thatcher+*Lr*34 plants harvested at different time intervals (0 h, 6 h, 9 h and 15 h) and 25 pmol Bovis 21 primer (5' - TTT TTT TTT TTT TTT TVN – 3'). The reactions were placed at 70°C for 5 min and then on ice for 5 min. A mix consisting of 200 U Improm-II Reverse Transcriptase (Promega), 10 µCi [α-^{32}P]-deoxycytidine triphosphate (dCTP), 1X ImProm-II reaction buffer, 3 mM $MgCl_2$ and 0.5 mM dNTP's was added to the reaction. The reaction was left at 25°C for 5 min and then 42°C for 60 min for the extension of the first strand cDNA. The ImProm-II Reverse Transcriptase was inactivated by incubation at 70°C for 15 min.

Twenty five ml hybridization solution (50% (v/v) formamide, 5X SSPE pH 7.4 (750 mM NaCl, 50 mM $NaH_2PO_4.H_2O$, 6.25 mM EDTA), 0.5% (w/v) SDS, 5X Denharts (0.05% (w/v) polyvinylpyrrolidone (PVP), 0.05% (w/v) ficoll, 0.05% (w/v) bovine serum albumin (BSA)), 0.1 µg.ml^{-1} salmon sperm DNA) was added to the nylon membranes which were pre-hybridized for two hours at 42°C. The probes were denatured by placing them in a water bath at 95°C for 5 min after which they were added to the individual membranes and left to hybridize overnight at 42°C. The following morning the membranes were washed twice with 20 ml wash buffer 1 (300 mM NaCl, 30 mM sodium citrate, 0.1% (w/v) SDS) for five min at room temperature, followed by two washes with 20 ml wash buffer 2 (75 mM NaCl, 7.5 mM sodium citrate, 0.1% (w/v) SDS) for 15 min at 60°C. The membranes were then screened with a Personal molecular imager system (Bio-Rad Laboratories). Using the appropriate computer software, the hybridization signals were quantified and normalized against that of the actin gene. These values were then expressed relative to that of time 0 to indicate whether the expression of the cDNA fragments were induced or repressed.

Each of the clones that showed induced expression values greater than 2, was sequenced using a BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems)

using the T7 primer according to the manufacturers specifications. The sequenced DNA was purified using SigmaSpin post-reaction purification columns (Sigma) according to the manufacturer's specifications and then separated on a 6% (w/v) denaturing polyacrylamide gel. The obtained nucleotide sequences were compared to those included in several databases using the Basic Local Alignment Search Tool (BLAST) tool (http://wheat.pw.usda.gov/GG2/blast.shtml at GrainGenes and http://www.ncbi.nlm.nih.gov/blast/Blast.cgi at NCBI). Complete BLAST analysis of blastn, blastx and blastn against dbEST were performed. The alignments were performed using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/) and the contigs were assembled with Chromas Pro (Technelysium).

3.3. Results

3.3.1. *Puccinia triticina* infection

In order to identify differentially expressed genes following infection with *P. triticina*, adult wheat plants were inoculated with fresh urediospores. Since the flag leaves were harvested before any visible signs of infection were present, some infected plants were left in the greenhouse until rust pustules were visible to confirm the success of the infection. One week after inoculation, the inoculated susceptible Thatcher plants showed brown fungal pustules on the leaf surface (Fig 3.2). The inoculated Thatcher+*Lr*34 showed localized lesions where infection was terminated. These necrotic lesions represented cells that were killed as part of the HR of the plant defence response. These results confirmed the effective infection of the wheat.

3.3.2. Construction of a SSH library from *P. triticina* infected resistant wheat

Infected Thatcher+*Lr*34 flag leaves were harvested and total RNA extracted. Before SSH was performed, the quality and the quantity of the extracted RNA was verified. For each RNA extract, 200 ng total RNA was separated on an agarose gel (Fig 3.3). On the gel, the characteristic banding pattern of the ribosomal RNA was visible. The bands were sharp and not broken down. The smear in the background represented the mRNA. Furthermore, the concentration of all the samples was very similar. Since both the quality and quantity of the extracted RNA was good, it was used for the construction of the SSH library.

SSH was performed to obtain a subtracted cDNA library for the infected resistant wheat. The SSH comprised of three steps. The first step was to generate cDNA fragments and ligate adaptors to both tester and driver cDNA. The second step involved the hybridization of the tester cDNA with the driver cDNA and the PCR amplification of the subtracted cDNA. This first PCR reaction did not show

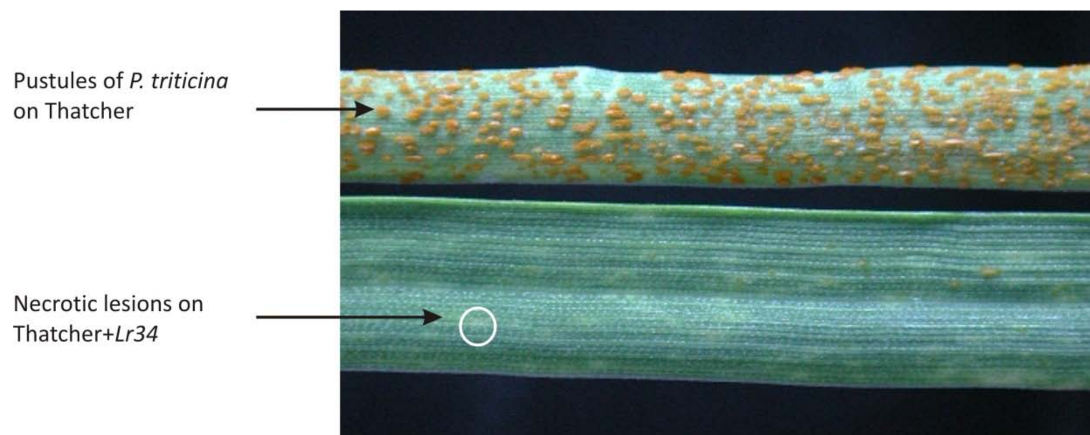


Figure 3.2. Infection of Thatcher and Thatcher+*Lr*34 wheat with *P. triticina*. Indicated on top is an infected Thatcher leaf and on the bottom that of infected Thatcher+*Lr*34.

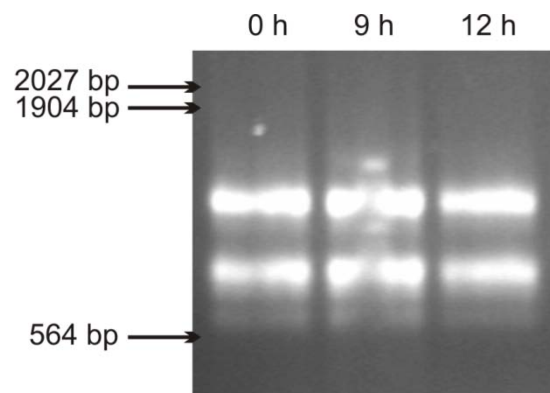


Figure 3.3. Total RNA extracted from infected Thatcher+*Lr*34 plants. The time intervals and molecular size markers are as indicated.

any visible amplification products (results not shown). However it was stated in the manual to continue with the procedure nevertheless. The third step involved further enrichment of the differentially expressed sequences by a second hybridization with excess driver cDNA. This second PCR did show a faint amplification product with a smear (results not shown). The amplified subtracted cDNA fragments were cloned into pGEM-T Easy and colonies harbouring recombinant plasmids were selected using α-complementation. Blue colonies retained their ability to utilize X-gal as substrate, because of an intact *LacZ'* gene present on the non-recombinant plasmid. This ability was lost in the white colonies due to the presence of an insert within the polycloning site of the plasmid vector, leading to a defective *LacZ'* gene whose encoded product was unable to utilize X-gal. A total of 278 white colonies were selected. To confirm that the majority of the white colonies did indeed contain recombinant plasmids, 22 clones were screened for the presence of an insert by digesting the insert from the plasmid using *Eco*RI (Fig 3.4). The single 3 kb fragment present on the gel represented the digested plasmid DNA, while the smaller fragments represented the cloned inserts. All 22 of the digested plasmids were recombinants. Some clones had double bands, probably due to an internal *EcoR*I restriction site within the insert. The sizes of the cloned cDNA fragments fell within the range of sizes that were predicted by the manufacturers.

3.3.3. Screening and analysis of the SSH library

To determine the expression levels of the various clones as well as to eliminate false positives, a reverse Northern blot was performed. For the reverse Northern Blot, the inserts of all plasmids were amplified using the T7 and SP6 primers. Amplification from a non-recombinant plasmid yielded no discernable fragment, while the amplified fragments from the recombinant plasmids were in the order of 500 bp (Fig 3.5). The amplified fragments were transferred onto nylon membranes using a slot blot system. Total mRNA isolated from different time intervals of infected Thatcher+*Lr*34 tissue was reverse transcribed to cDNA, thereby generating the respective cDNA probes. After hybridization, the expression levels of the individual clones were calculated. cDNA clones showing induction levels of two and higher

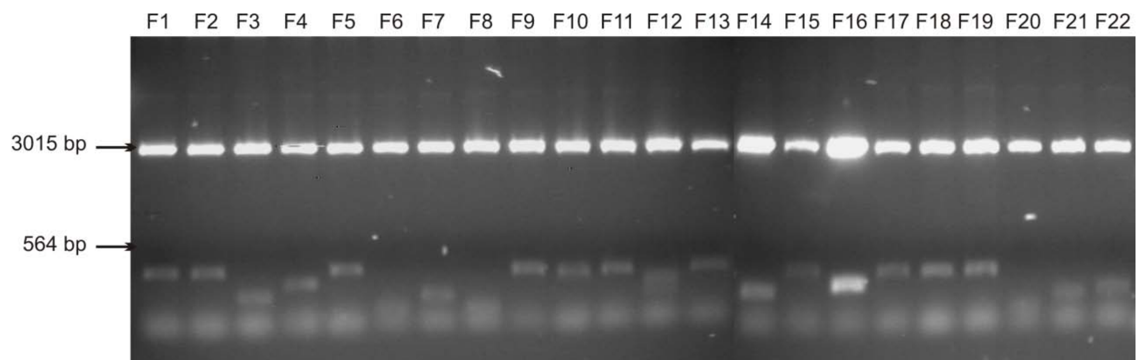


Figure 3.4. *Eco*RI digestion of 22 selected plasmid DNAs. The clone names, as well as the sizes of the DNA fragments, are as indicated.

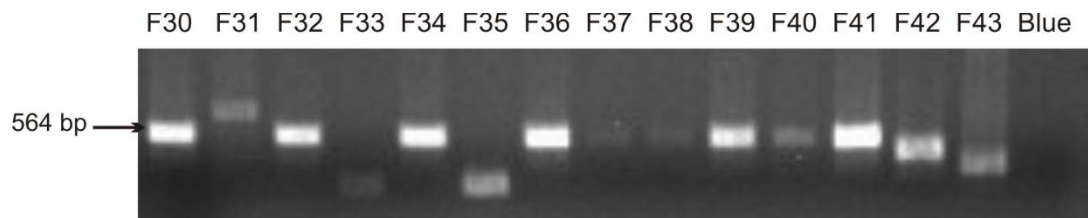


Figure 3.5. PCR amplification of inserts from selected recombinant plasmids. The clone names as well as the sizes of the DNA fragments are as indicated.

compared to 0 hpi were re-probed to confirm its accuracy.

Sixty eight cDNA clones showed induced expression levels of two or higher compared to 0 hpi. Of the 68 cDNA clones, two clones, LRW014 and LRW203 showed a substantial induction when compared to 0 hpi (Fig 3.6).

The 68 cDNA clones were sequenced and then used in a BLAST analysis to identify them (Table 3.1). Indicated in the table are the sequences that the clones shared similarity with, as well as the induced expression levels and the time of highest expression.

Most of the clones showed the highest expression levels at 15 hpi but for LRW268 it was at 9 hpi and for LRW014, LRW020, LRW030, LRW033, LRW035, LRW036, LRW037, LRW038, LRW041, LRW042, LRW043, LRW044, LRW068 and LRW203 the expression peaked at 6 hpi. This indicated that wheat reacted very swiftly to the presence of the pathogen. Based on these results, the isolated sequences where then placed into 5 groups (Table 3.2).

Group one consisted of 38 clones which formed 3 contigs that were similar to different regions of the *psbA* chloroplast gene from *Hordeum vulgare* (Accession number: EF115541, e-values: $8e^{-173}$, $5e^{-175}$ and $2e^{-140}$). A fourth contig consisting of 2 clones showed similarity to the *T. aestivum psbA* chloroplast gene (Accession number: AB042240, e-value: $9e^{-97}$). These homologies were extremely high so it can be assumed that these contigs do indeed form part of the chloroplast *psbA* gene.

Group two consisting of two contigs of 3 clones each, showed identity to a RuBisCO large subunit gene from *T. aestivum* (Accession number: AY328025, e-value: $3e^{-126}$ and $2e^{-112}$). The similarity of these contigs to the large subunit of RuBisCO was significant.

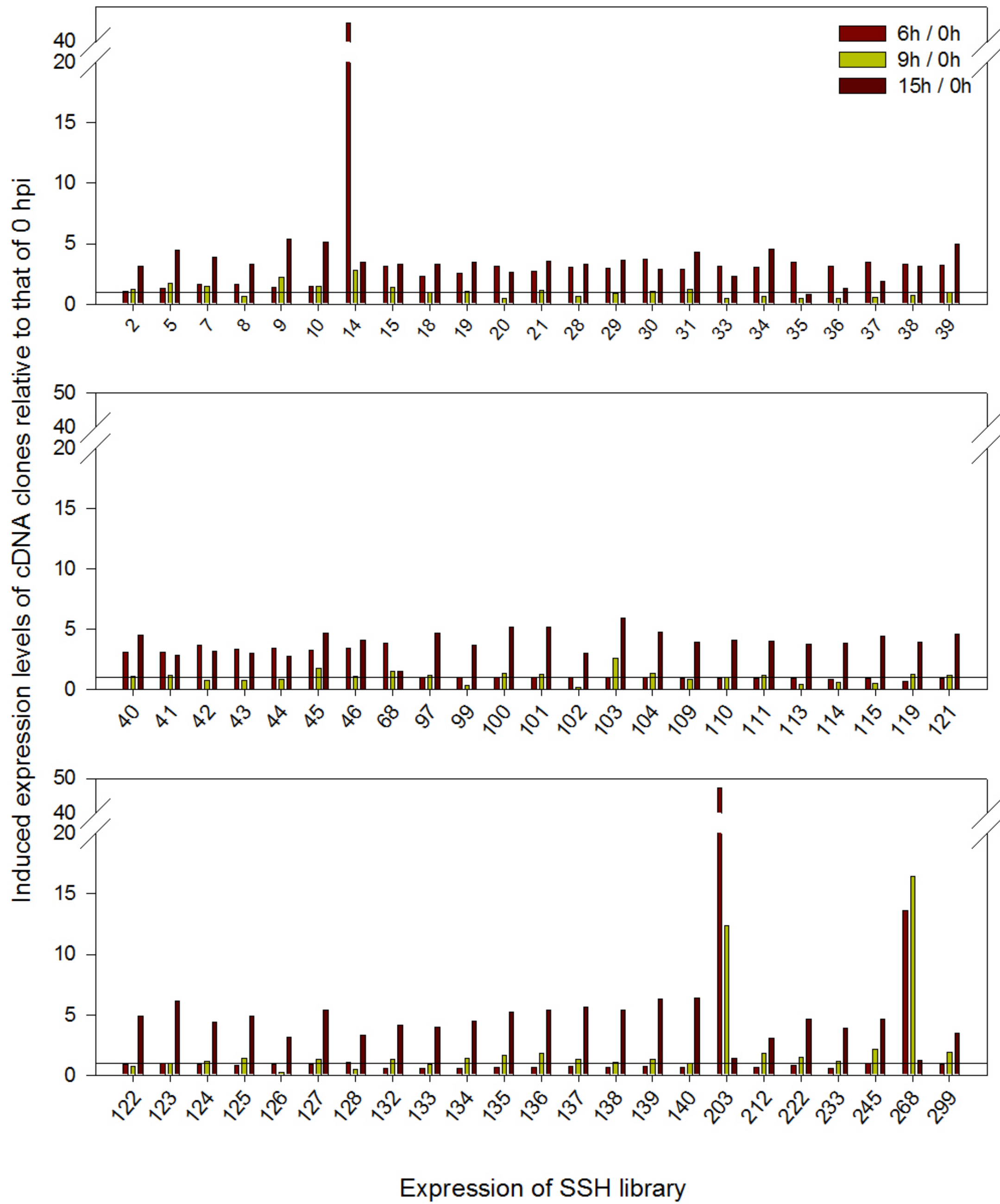


Figure 3.6. Reverse Northern blot analysis of cDNA clones present in the SSH library. The time intervals and clone names are as indicated. All the values were normalised against the actin control and finally against the value of 0 hpi.

Table 3.1. BLAST analysis and identification of SSH generated cDNA clones.

Name	Induction Level Relative to zero hour	Accession Number	e-value	Description	Highest Induced (hpi)
LRW2	3.1	CG673494	$1e^{-63}$	*Aegilops tauschii* genomic clone	15
LRW5	4.5	CG676903	$2e^{-69}$	*Aegilops tauschii* genomic clone	15
LRW7	3.9	BE430218	$3e^{-59}$	*Triticum aestivum* cDNA clone	15
LRW8	3.3	CA746565	$1e^{-06}$	*Triticum aestivum* cDNA clone	15
LRW9	5.4	CG676903	$1e^{-67}$	*Aegilops tauschii* genomic clone	15
LRW10	5.1	X07521, M38374, NC_002762	$1e^{-72}$	Barley chloroplast psbA gene, *Triticum aestivum* chloroplast	15
LRW14	42.7	CK172295	$1e^{-07}$	TaLt6 *Triticum aestivum* cDNA clone	6
LRW15	3.3	NC_002762, AB042240, CG676903	$7e^{-67}$	*Triticum aestivum* chloroplast, *Aegilops tauschii* genomic clone	15
LRW18	3.3	CG676903, X07521, M38374	$8e^{-72}$	*Aegilops tauschii* genomic clone, Barley chloroplast psbA gene	15
LRW19	3.5	CG676903	$1e^{-40}$	*Aegilops tauschii* genomic clone	15
LRW20	3.1	CK158525	$7e^{-21}$	TaLt5 *Triticum aestivum* cDNA clone	6
LRW21	3.5	DR092344	$3e^{-45}$	DDRT-RCHW *Triticum aestivum* cDNA	15
LRW28	3.1	NC_002762, AB042240	$5e^{-54}$	*Triticum aestivum* cDNA clone	15
LRW29	3.6	CG673494	$5e^{-59}$	*Aegilops tauschii* genomic clone	15
LRW30	3.7	DR092344	$2e^{-59}$	DDRT-RCHW *Triticum aestivum* cDNA	6
LRW31	4.3	CG676903, X07521, M38374	$2e^{-73}$	*Aegilops tauschii* genomic clone, Barley chloroplast psbA gene	15
LRW33	3.1	CK157348	$3e^{-40}$	TaLt4 *Triticum aestivum* cDNA clone	6
LRW34	4.5	CA739119	$2e^{-46}$	*Triticum aestivum* cDNA clone	15
LRW35	3.4	BJ250276	$4e^{-38}$	*Triticum aestivum* cDNA clone	6
LRW36	3.2	CG676903	$2e^{-73}$	*Aegilops tauschii* genomic clone	6
LRW37	3.5	CK152242	$9e^{-23}$	TaLt3 *Triticum aestivum* cDNA clone	6
LRW38	3.3	CG676903	$8e^{-42}$	*Aegilops tauschii* genomic clone	6
LRW39	4.9	CA738383	$1e^{-65}$	*Triticum aestivum* cDNA clone	15
LRW40	4.5	AJ602817	4e-61	*Triticum aestivum* cDNA clone	15
LRW41	3.1	CD928574	$1e^{-49}$	*Triticum aestivum* cDNA clone	6
LRW42	3.6	NC_002762, AB042240, CG676903	$2e^{-60}$	*Triticum aestivum* chloroplast, *Aegilops tauschii* genomic clone	6
LRW43	3.3	NC_002762, AB042240, X13327	$2e^{-67}$	*Triticum aestivum* chloroplast, *Secale cereale* chloroplast psbA gene	6
LRW44	3.4	CK167474	$1e^{-73}$	TaLt7 *Triticum aestivum* cDNA clone	6
LRW45	4.7	AJ602817	$4e^{-54}$	*Triticum aestivum* cDNA clone	15
LRW46	4.1	NC_002762, AB042240	$8e^{-61}$	*Triticum aestivum* cDNA clone	15
LRW68	3.8	CK170042	$2e^{-47}$	TaLt6 *Triticum aestivum* cDNA clone	6
LRW97	4.7	CG676903	$2e^{-58}$	*Aegilops tauschii* genomic clone	15
LRW99	3.7	BE438132	$3e^{-15}$	*Hordeum vulgare* cDNA clone	15
LRW100	5.1	NC_002762, AB042240, CG676903	$3e^{-64}$	*Triticum aestivum* chloroplast, *Aegilops tauschii* genomic clone	15
LRW101	5.1	CD892856	$2e^{-11}$	*Triticum aestivum* cDNA clone	15
LRW102	3.0	CG676903	$1e^{-67}$	*Aegilops tauschii* genomic clone	15
LRW103	5.9	AJ602817	$3e^{-61}$	*Triticum aestivum* cDNA clone	15
LRW109	3.9	NC_002762, AB042240, CG676903	$5e^{-64}$	*Triticum aestivum* chloroplast, *Aegilops tauschii* genomic clone	15
LRW110	4.1	CG673494	$2e^{-54}$	*Aegilops tauschii* genomic clone	15
LRW111	4.0	NC_002762	$1e^{-67}$	*Triticum aestivum* cDNA clone	15
LRW113	3.7	BE421429	$4e^{-46}$	Hordeum vulgare cDNA clone	15
LRW114	3.8	CA738383	$4e^{-52}$	*Triticum aestivum* cDNA clone	15
LRW115	4.4	CA739204, CA739047, CA738686	$2e^{-51}$	*Triticum aestivum* cDNA clone	15
LRW119	3.9	AJ602817	$5e^{-65}$	*Triticum aestivum* cDNA clone	15
LRW121	4.6	AJ602817	$5e^{-64}$	*Triticum aestivum* cDNA clone	15
LRW122	4.9	CA738383	$2e^{-59}$	*Triticum aestivum* cDNA clone	15
LRW123	6.1	CK156106	$2e^{-22}$	TaLt4 Triticum aestivum cDNA clone	15
LRW124	4.4	CD892856	$5e^{-64}$	*Triticum aestivum* cDNA clone	15
LRW125	4.9	NC_002762, AB042240	$1e^{-05}$	*Triticum aestivum* chloroplast	15
LRW126	3.1	DR092344	$4e^{-41}$	DDRT-RCHW *Triticum aestivum* cDNA	15
LRW127	5.3	NC_002762, AB042240, CG676903	$3e^{-65}$	*Triticum aestivum* chloroplast, *Aegilops tauschii* genomic clone	15
LRW128	3.3	CK156306	$1e^{-46}$	TaLt4 *Triticum aestivum* cDNA clone	15
LRW132	4.1	AJ602817	$8e^{-44}$	*Triticum aestivum* cDNA clone	15
LRW133	4.0	NC_002762, AB042240, CG676903	$2e^{-64}$	*Triticum aestivum* chloroplast, *Aegilops tauschii* genomic clone	15
LRW134	4.5	NC_002762, AB042240, CG676903	$2e^{-63}$	*Triticum aestivum* chloroplast, *Aegilops tauschii* genomic clone	15
LRW135	5.2	NC_002762, AB042240, CG676903	$2e^{-48}$	*Triticum aestivum* chloroplast, *Aegilops tauschii* genomic clone	15
LRW136	5.4	NC_002762, AB042240, CG673494	$7e^{-31}$	*Triticum aestivum* chloroplast, *Aegilops tauschii* genomic clone	15
LRW137	5.6	NC_002762, AB042240, CG673494	$2e^{-27}$	*Triticum aestivum* chloroplast, *Aegilops tauschii* genomic clone	15
LRW138	5.3	AJ602817	$4e^{-65}$	*Triticum aestivum* cDNA clone	15
LRW139	6.3	NC_002762, AB042240, CG676903	$1e^{-55}$	*Triticum aestivum* chloroplast, *Aegilops tauschii* genomic clone	15
LRW140	6.4	CA737819	$2e^{-27}$	*Triticum aestivum* cDNA clone	15
LRW203	47.3	DR092344	$1e^{-23}$	DDRT-RCHW *Triticum aestivum* cDNA	6
LRW212	3.1	CK170779	$2e^{-35}$	TaLt6 *Triticum aestivum* cDNA clone	15
LRW222	4.6	CA669912, AJ610914	$6e^{-59}$	*Triticum aestivum* cDNA clone, *Triticum turgidum* cDNA clone	15
LRW233	3.9	BJ250276	$5e^{-40}$	*Triticum aestivum* cDNA clone	15
LRW245	4.6	AJ602817	$7e^{-68}$	*Triticum aestivum* cDNA clone	15
LRW268	16.5	BJ247731	$2e^{-46}$	*Triticum aestivum* cDNA clone	9
LRW299	3.4	CK172098	$1e^{-46}$	TaLt6 *Triticum aestivum* cDNA clone	15

Table 3.2. Compilation of contigs represented by individual SSH generated cDNA clones.

Type	*Description*	*Clones*	*Induction Levels*	*Highest Induction times*	*Accession number*	*e-value*
Group 1	*psbA* Contig 1	LRW009	5.4	15	EF115541.1	$8e^{-173}$
		LRW010	5.1	15		
		LRW015	3.3	15		
		LRW018	3.3	15		
		LRW031	4.3	15		
		LRW127	5.3	15		
		LRW133	4.0	15		
		LRW134	4.5	15		
		LRW245	4.6	15		
	psbA Contig 2	LRW002	3.1	15	EF115541.1	$5e^{-175}$
		LRW028	3.1	15		
		LRW029	3.6	15		
		LRW038	4.1	6		
		LRW040	4.5	15		
		LRW042	3.6	6		
		LRW043	3.3	6		
		LRW045	4.7	15		
		LRW046	4.1	15		
		LRW097	4.7	15		
		LRW100	5.1	15		
		LRW102	3.0	15		
		LRW103	5.9	15		
		LRW109	3.9	15		
		LRW110	4.1	15		
		LRW111	4.0	15		
		LRW119	3.9	15		
		LRW121	4.6	15		
		LRW125	4.9	15		
		LRW132	4.1	15		
		LRW135	5.2	15		
		LRW136	5.4	15		
		LRW137	5.6	15		
		LRW138	5.3	15		
		LRW139	6.3	15		
	psbA Contig 3	LRW099	3.7	15	AB042240.3	$9e^{-97}$
		LRW124	4.4	15		
	psbA Contig 4	LRW005	4.5	15	EF115541.1	$2e^{-140}$
		LRW019	3.5	15		
Group 2	RuBisCO LSU Contig 1	LRW039	4.9	15	AY328025.1	$3e^{-126}$
		LRW115	4.4	15		
		LRW122	4.9	15		
	RuBisCO LSU Contig 2	LRW034	4.5	15	AY328025.1	$2e^{-112}$
		LRW114	3.8	15		
		LRW140	6.4	15		
Group 3	EST Contig 1	LRW030	3.7	6	DW283687.1	$5e^{-06}$
		LRW126	3.1	15		
	EST Contig 2	LRW020	3.1	6	DW394669.1	$2e^{-22}$
		LRW033	3.1	6		
		LRW037	3.5	6		
		LRW068	3.8	6		
	EST Contig 3	LRW035	3.4	6	BJ250276.1	$3e^{-112}$
		LRW233	3.9	15		
	EST Contig 4	LRW123	6.1	15	FD473424	$3e^{-85}$
		LRW212	3.1	15		
	EST Contig 5	LRW007	3.9	15	CJ918877.1	$1e^{-75}$
		LRW021	3.5	15		
Group 4	Showing similarity to published ESTs	LRW036	3.2	6	DY543205.1	$2e^{-104}$
		LRW041	3.1	6	CJ919932.1	$1e^{-120}$
		LRW113	3.7	15	CJ943396.1	$2e^{-77}$
		LRW222	4.6	15	CJ956114.1	$1e^{-136}$
		LRW268	16.5	9	CA616342.1	$4e^{-94}$
		LRW299	3.4	15	CJ963548.1	$2e^{-113}$
Group 5	Not showing significant similarity to published genes	LRW008	3.3	15		
		LRW014	42.7	6		
		LRW044	3.4	6		
		LRW101	5.1	15		
		LRW128	3.3	15		
		LRW203	47.3	6		

Group three consisted of generated contigs that were similar to expressed sequence tags (ESTs) generated from plants that were subjected to different stress conditions. The first contig consisted of LRW030 and LRW126. This contig showed identity to an EST that was isolated from a subtractive cDNA library for *Euprymna scolopes* (Accession number: DW283687.1, e-value: $5e^{-06}$). LRW020, LRW033, LRW037 and LRW068 formed another contig showing similarity to an EST from the liver of *Rattus norvegicus* (Accession number: DW394669.1, e-value: $2e^{-22}$). The contig consisting of LRW035 and LRW233 showed similarity to an EST from wheat (Accession number: BJ250276, e-value: $3e^{-112}$).

LRW123 and LRW212 formed a short contig showing similarity to a wheat EST that was isolated during water stress (Accession number: FD473424, e-value: $3e^{-85}$). The contig of LRW007 and LRW021 showed similarity to an EST isolated from wheat infected with *P. triticina* (Accession number: CJ918877.1, e-value: $1e^{-75}$).

In group four 6 individual cDNA clones showed high similarity to published ESTs. LRW036 was 548 bp in length and its highest induction level was 3.2 fold at 6 hpi. It showed sequence similarity to a cDNA clone isolated from a *T. aestivum* unstressed seedling shoot cDNA library (Accession number: BE426806, e-value: e^{-100}) and an EST isolated from young spikes of wheat (Accession number: DY543205, e-value: $2e^{-104}$).

LRW041 was 585 bp in length and its highest induction level of 3.1 occurred at 6 hpi. It showed sequence similarity to an EST isolated from wheat infected with *P. triticina* (Accession number: CJ919932, e-value: $1e^{-120}$).

Clone LRW113 was 546 bp in length and its highest induction level was 3.7 fold at 15 hpi. It showed sequence similarity to an EST isolated from wheat infected with powdery mildew (Accession number: CJ943396, e-value: $2e^{-77}$).

Clone LRW222 was 387 bp in length and its highest induction level was 4.6 fold at 15 hpi. It showed sequence similarity to an EST isolated from wheat infected with powdery mildew (Accession number: CJ956114, e-value: $1e^{-136}$).

Clone LRW268 was 308 bp in length and its highest induction level was 16.5 fold at 9 hpi. It showed sequence similarity to an EST isolated from de-etiolated wheat (Accession number: CA616342, e-value: $4e^{-94}$).

Clone LRW299 was 431 bp in length and its highest induction level was 3.4 fold at 15 hpi. It showed sequence similarity to an EST isolated from wheat infected with powdery mildew (Accession number: CJ963548, e-value: $2e^{-113}$).

Group five consisted of clones LRW008, LRW014, LRW044, LRW101, LRW128 and LRW203 that did not show any significant similarity with any gene sequence contained within GenBank.

3.4. Discussion

Different molecular techniques like DDRT-PCR (Liang and Pardee, 1992), SSH (Lu *et al.*, 2004) and micro-array analysis (Voelckel *et al.*, 2004) have been used to identify genes involved in various plant-pathogen interactions. The aim of all these studies was to elucidate the interaction between the plant and the pathogen. Once a better understanding of this interaction is achieved, methods could be derived to improve the resistance of plants to combat potential pathogens.

It is clear from previous work that the plant-pathogen interaction is extremely complex with various factors playing a role. These factors include primary and secondary metabolism, plant defence and senescence, communication and signal transduction and cellular transport and ultrastructure (Lacock *et al.*, 2003; Lu *et al.*, 2004; Voelckel *et al.*, 2004). In addition, every study yielded a number of cloned cDNA fragments that either shared similarity with DNA sequences for which no known function has been described, or did not share similarity with any known genes at all. It is these genes that usually attract the most attention, for the fact that they are novel and the encoded proteins could perform a unique function during the plant-pathogen interaction.

SSH was used in this study to isolate cDNA clones that are differentially expressed during the early interaction between wheat and *P. triticina*. Three hundred and eleven cDNA fragments that were putatively differentially expressed in Thatcher+*Lr*34 after infection with *P. triticina* were isolated and cloned. A total of 278 different recombinant clones were obtained of which 68 showed induced expression values of two or higher (Table 3.1).

Of these 68 cDNA clones, 38 showed similarity to the *psbA* chloroplast gene from different plant species (Table 3.2). *PsbA* codes for the D1 protein that forms part of the reaction center of photosystem II (Dwivedi and Bhardwaj, 1995). The photosystem II reaction center complex binds with two quinones and functions as an electron acceptor (Malkin and Niyogi, 2000) and helps regulate redox potential

(Danon and Mayfield, 1994). Redox signalling and the oxidative burst play a role in plant defence responses (Grant and Loake, 2000) which signifies that *psbA* might play a role during the defence response of wheat.

In addition to *psbA*, another key gene whose encoded polypeptide is involved in photosynthesis, namely the large subunit of RuBisCO (*RbcL*) was also found in the pool of cloned cDNA fragments. A total of 6 clones formed two *RbcL* contigs. RuBisCO's large subunit, together with the small subunit, forms an active enzyme responsible for the binding of CO_2 during photosynthesis (Malkin and Niyogi, 2000). Together with *psbA*, the differential transcription of *RbcL* indicated that photosynthesis could form an integral part of wheat's activated defence response upon infection.

Other studies have implicated the involvement of photosynthetic genes in different plant-stress interactions (Botha *et al*., 2006; Wang *et al*., 2007). For Russian wheat aphid infested wheat, it was hypothesised that the aphid releases compounds in its saliva to break down chlorophyll (Botha *et al*., 2006). RbcL was found to be broken down in Russian wheat aphid infested susceptible plants (Van der Westhuizen and Botha, 1993). It was further found that the susceptible plants do not have a timely activation of SAR. This causes the loss of photosynthetic capacity due to chlorophyll breakdown thus decreasing energy production. In the resistant plants, the activation of SAR seems to circumvent the breakdown of photosynthetic components.

Even though Wang *et al*. (2007) did not study a plant pathogen interaction they did isolate cDNA clones from *Puccinellia tenuiflora*, a halophyte subjected to salt stress. Several of the isolated cDNA clones were involved in photosynthesis again indicating a possible role for photosynthesis in the adaptation of plants placed under stress conditions.

Since the SSH results obtained in this study corroborates the findings of previous studies (Botha *et al*., 2006; Wang *et al*., 2007) that photosynthesis plays an active role in plant defence it was decided to define its specific role in the interaction

between wheat and *P. triticina*. More specifically two aspects will be investigated (Chapter 5). For the first, the photosynthetic capacity of resistant and susceptible cultivars will be compared following infection. Secondly, the role of light as an integral part of photosynthesis during the plant defence response will be analysed.

In a separate part of the study, two of the isolated cDNA clones (Table 3.2) will be investigated to confirm their role in the defence of wheat upon *P. triticina* infection (Chapter 4). Of the two selected cDNA clones, LRW268 showed a significant increase in gene expression very early after infection. Furthermore, this clone showed similarity to an EST that was originally isolated from light treated wheat, again emphasising the possible role of photosynthesis during plant defence. At this stage the identity of the cDNA is still unknown.

Even though the expression levels of LRW222 was not as strong as that of LRW268 the interest in this clone was due to the fact that it showed similarity to an EST isolated from wheat infected with powdery mildew. It was decided to investigate the role of LRW222 during the defence response as it could perform a universal function during the different plant-pathogen interactions.

It was decided not to include LRW014 and LRW203 for further analysis despite both genes showing significant induced expression levels. The reason for this was twofold. Firstly, the very high induction levels were basically transient during only one of the tested time intervals. This most probably indicates an experimental artefact. Secondly, both clones did not show similarity to any known published gene or EST.

During this study, only a forward SSH was performed, allowing us to clone cDNAs that were upregulated following infection. It is however acknowledged that a reverse SSH could have improved the study by identifying cDNAs that are downregulated after infection. Such a subtraction was performed, by will only be analysed in the future.

3.5. References

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Chapter 4

Characterisation of two cDNA clones isolated from *P. triticina* infected wheat

4.1. Introduction

Puccinia triticina is the most widespread and regularly occurring rust on wheat (Kolmer, 1996) and is responsible for 10-15% yield losses and a decrease in grain quality (Šlikova *et al.*, 2003). *P. triticina* causes a reddish-orange spore mass which occurs mostly on the leaves of the plant. Susceptible plants are unable to retard fungus infection thereby allowing the development of pustules. The resistant plants however impede or destroy the infecting fungus by the activation of an appropriate defence response (Kolmer, 1996; McMullen and Rasmussen, 2002).

Various signalling pathways are induced upon pathogen attack in which signalling molecules like SA, JA and ET play important roles in the primary defence of plants against pathogens (Pieterse *et al.*, 2001). Application of chemical elicitors like menadione sodium bisulphite (MSB), ET, MeJA and methyl salicylate (MeSA) also has the ability to induce defence related responses using the respective pathways (Neill *et al*., 2002; Borges *et al*., 2003; Wang and Wu, 2005). Application of a secondary signalling compound like MSB (vitamin K) has been shown to induce both local and systemic resistance against *Leptosphaeria maculans* in oilseed rape seedlings (Borges *et al*., 2003). ROS like H_2O_2 and NO which play an important role in programmed cell death, are also important in the signalling events during plant defence (Clarke *et al*., 2000; Desikan *et al*., 2000).

Protease inhibitors (PIs) are highly stable enzymes that can withstand extreme pH and high temperatures (Lorito *et al*., 1994; Dunaevsky *et al*., 2005; Fan and Wu, 2005; Habib and Khalid, 2007). One of their diverse functions is a protective role against insects and pathogenic microorganisms (Dunaevsky *et al*., 2005). PIs have been reported to influence the synthesis of chitin in fungi and reduce growth and development of herbivorous insects (Lorito *et al*., 1994). A maize PI gene showed induced expression after wounding and pathogen infection (Cordero *et al*., 1994). Overexpression of *RBBI2-3* in transgenic rice plants led to resistance of the plant against the fungal pathogen *Pyricularia oryzae* (Qu *et al*., 2003). This is an indication of the direct and important role that PIs could play during plant defence.

Plant PIs are divided into 10 classes (Fan and Wu, 2005; Habib and Khalid, 2007). One of these is the serine PIs which are mostly small water soluble proteins that range between 4-85 kDa in molecular weight. Most PIs form a protease-inhibitor complex with their target proteases thus rendering the proteases inactive (Fan and Wu, 2005). Plant PIs are mostly found in seeds of legumes and cereal grains and are double-headed with two similar domains each bearing separate reactive sites for binding the cognate proteases. Most PIs do not affect the proteases present in the seed but are extremely efficient in inhibiting proteolytic enzymes and proteases from pests (Dunaevsky *et al*., 2005). A serine PI called the Bowman-Birk serine protease inhibitor (BBI), has the ability to reduce the biomass of an infecting pathogen (Gatehouse *et al*., 1993). BBIs were first isolated from soybean seeds by Bowman in 1944 and characterised by Birk *et al*. in 1963 (Ragg *et al*., 2006). BBIs possess repetitive cysteine-rich domains with reactive sites for the trypsin or chymotrypsin proteins from the plant pathogen (Habib and Khalid, 2007).

Using a PCR based SSH approach, various gene fragments playing putative roles in the defence response of *P. triticina* infected wheat were isolated (Chapter 3). Two of these clones, *LRW268* and *LRW222*, were chosen for further analysis. *LRW268* showed similarity to various ESTs isolated from light treated plants. This cDNA clone was chosen to further elucidate the effect of light on the defence response and to determine whether this gene played a part. *LRW222* on the other hand showed similarity to a BBI protease inhibitor. Since PIs have been shown to be essential components of the plant defence response, it was decided to further investigate it. This chapter is dedicated to the initial characterisation of these two unknown genes to determine their involvement and putative roles in *P. triticina* infected wheat.

4.2. Materials and methods

4.2.1. Biological Material

4.2.1.1. Fungal pathogen

Thatcher plants were inoculated with *Puccinia triticina* pathotype UVPrt9. Avoset plants were inoculated with *Puccinia striiformis* pathotype 6E22.

4.2.1.2. Cultivation of wheat

Seeds from the *P. triticina* susceptible Thatcher and resistant Thatcher+*Lr*34 wheat cultivars were planted in cones containing a sterilized soil:peatmoss mixture (1:1 w/w) and grown in the greenhouse at ±24°C with a 16 h day/8 h night cycle. The plants were daily fertilised with 2% (w/v) Multifeed. Susceptible Karee plants used for the augmentation of *P. triticina* spores were similarly grown in pots.

Fourteen day old seedlings of *P. striiformis* susceptible Avoset-S and resistant Avoset-*Yr*1 wheat were grown under similar greenhouse conditions. The plants were fertilised with fertiliser with a N:P:K ratio of 3:2:1 that was applied to seedlings at a final concentration of 10 $g.l^{-1}$ three days before infection with *P. striiformis*.

4.2.1.3. Infection of wheat with *P. triticina* and *P. striiformis*

Adult Thatcher and Thatcher+*Lr*34 plants were sprayed with freshly harvested *P. triticina* spores (0.6 mg $spores.ml^{-1}$) that were multiplied on the susceptible Karee cultivar. The spores were resuspended in distilled water containing a drop of Tween™ 20. Control plants where mock inoculated with water. The plants were left to dry and then placed in a dark dew-simulation cabinet at 18 to 20°C for 12 h to facilitate spore germination. After the plants were dried, they were transferred to the greenhouse. Flag leaves from adult plants were harvested at 3 h intervals for 30 h. Time 0 h was harvested prior to placing the plants in the dew-simulation

chamber. The plant material was quick frozen in liquid nitrogen and stored at -80°C. The frozen tissue was ground to a fine powder in liquid nitrogen.

Fresh *P. striiformis* spores were harvested from Avoset-S seedlings and resuspended in kerosene oil to a final concentration of 22 X 10^4 spores.ml^{-1}. This was used to infect the Avoset-S and Avoset-*Yr*1 seedlings. After infection the plants were left to dry for 2 h at room temperature. Cool sterile water was sprayed on the plants to simulate high humidity conditions. The plants were then incubated for 48 h in the dark at 8°C with high humidity to allow infection. Plant tissue was collected at 6 h intervals due to *P. striiformis* being a slow rusting fungus, quick frozen in liquid nitrogen and stored at -80°C. Time 0 h was harvested prior to infection. Tissue was ground to a fine powder in liquid nitrogen.

4.2.1.4. Chemical treatments of plants

Fourteen day old Thatcher+*Lr*34 seedlings were treated with 20 mM H_2O_2 (Neill *et al*., 2002), 100 µM MeJA (Wang and Wu, 2005), 5 mM MeSA, 200 µM Menadione (Borges *et al*., 2003) or water as control. Each sample was quick-frozen in liquid nitrogen and stored at -80°C. Plant tissue was harvested at 30 min, 1 h, 2 h, 4 h, 8 h, 12 h and 24 h. Time 0 h was harvested prior to treatment. Tissue was ground to a fine powder in liquid nitrogen.

4.2.2. Methods

4.2.2.1. Total RNA isolation

All apparatus and solutions were treated prior to use (3.2.2.1). Total RNA was isolated from leaf tissue using Eurozol (Euroclone) according to Chomczynski and Sacchi (1987), the quality tested (3.2.2.1) and mRNA purified (3.2.2.2).

4.2.2.2. Presence of *LRW268* and *LRW222* in different wheat cultivars

To confirm the presence of *LRW268* and *LRW222* in wheat, genomic DNA was extracted from Avoset-S, Avoset-*Yr*1, Thatcher and various Thatcher *Lr*-containing wheat cultivars. Ten ml extraction buffer (25 mM NaCl, 5 mM Tris-HCl pH 7.8, 2.5 mM EDTA, 1 mM sodium meta-bisulfate, 1.25% (w/v) SDS, 10 $\mu g.ml^{-1}$ RNase A) was added to 1 g ground tissue and incubated at 65°C for 30 min. One volume chloroform:isoamyl alcohol (24:1) was added to each extract, the solution mixed and the tubes centrifuged at 12000 *g* for 15 min at 4°C. The upper phase was transferred to new tubes and 2 volumes 100% (v/v) ethanol were added. The precipitated DNA was scooped out and washed with 5 ml 70% (v/v) ethanol. The DNA was air dried and dissolved in 2 ml water. The concentration of the DNA was determined and expressed as $\mu g.ml^{-1}$ (Sambrook *et al.*, 2000). To evaluate the quantity and quality of the extracted DNA, 500 ng genomic DNA was separated on a 1% (w/v) agarose gel (3.2.2.1).

For the amplification of *LRW222*, Bovis 279 and Bovis 280 were used, while for the amplification of *LRW268*, Bovis 277 and Bovis 278 were used (Table 4.1). The primer sets were designed using the contigs generated for *LRW222* and *LRW268* (4.3.3, 4.3.4). Each PCR reaction consisted of 10 ng DNA, 25 pmol of each primer, 1X Kapa*Taq* ReadyMix (Kapa Biosystems). The amplification regime was as follows: one cycle at 94°C for two min, 30 cycles at 94°C for 30 sec, 62°C for 30 sec and 72°C for one min followed by a final cycle at 72°C for 10 min. The success of the amplification was tested by separating the entire reaction on a 1.5% (w/v) agarose gel (3.2.2.1).

4.2.2.3. Expression levels of *LRW222* and *LRW268*

A two step RT-PCR was used to determine the expression of *LRW222* and *LRW268*. First strand cDNA was prepared for all time intervals using 200 ng total RNA with 20 pmol Bovis 32 (Table 4.1). This was incubated at 70°C for five min and chilled on ice for another five min. To this was added 1X ImProm-II reaction buffer, 20 U ImProm-II reverse transcriptase and 3 mM $MgCl_2$. The reactions were incubated at 25°C for 5 min to allow the primers to anneal and 1 h at 42°C to allow for cDNA synthesis.

Table 4.1. Nucleotide sequences of primers used in this chapter (N = A or T or G or C and V = A or G or C).

Name	***Primers***	***Sequence***	***Annealing Temperature***
LRW222 **Set 1**	Bovis 257	5' - ACG GGA AAC ATG TAA CTG CGT - 3'	53°C
	Bovis 258	5' - GGC ATA TGA TAT GTT CCG CCT - 3'	
LRW268 **Set 1**	Bovis 127	5' - AGC ATT TTG TGA AAG CCA AAG CTG - 3'	60°C
	Bovis 128	5' - TTT TTT GAA AGT TCA AGC CAC TG - 3'	
LRW222 **Set 2**	Bovis 279	5' - ATG GGA GTC CTC TCG CAC GTC AA - 3'	62°C
	Bovis 280	5' - AGT ACT TCT TGC AGG GTC CAC CG - 3'	
LRW268 **Set 2**	Bovis 277	5' - TAC TGG ACA ATG TGG AAG CTG - 3'	62°C
	Bovis 278	5' - GCA ATG AAG CTG ACG CAC T - 3'	
18S rRNA	Bovis 26	5' - CAA CTT TCG ATG GTA GGA TAG - 3'	58°C
	Bovis 27	5' - CTC GTT AAG GGA TTT AGA TTG - 3'	
Oligo-dT primer	Bovis 32	5’ - GAA GAA TTC TCG AGC GGC CGC TTT TTT TTT TTT TTT TTT TVN - 3’	42°C
LRW268 **Nested Primer**	Bovis 218	5' - AAT AGG TCC AAT AAG ACC TCG GC - 3'	Dependant on the primer combination
RACE primer 1	Oligo-dT anchor primer	5' - GAC CAC GCG TAT CGA TGT CGA CTT TTT TTT TTT TTT TTV - 3'	
RACE primer 2	PCR anchor primer	5' - GAC CAC GCG TAT CGA TGT CGA C - 3'	

The ImProm-II reverse transcriptase was inactivated by incubation at 70°C for 15 min. Using 1 µl first strand cDNA of each time interval, the individual gene fragments were amplified with 25 pmol of each fragment specific primer and 1X Kapa*Taq* ReadyMix (Kapa Biosystems). For the amplification of *LRW222*, Bovis 257 and Bovis 258 were used, and for *LRW268* Bovis 127 and Bovis 128 (Table 4.1). A control amplification of *18S rRNA* was performed using Bovis 26 and Bovis 27 with an annealing temperature of 58°C (Table 4.1). The amplification regime for *LRW222* was as follows: 94°C for two min, 30 cycles at 94°C for 30 sec, 53°C for 30 sec and 72°C for one min followed by a final cycle at 72°C for 10 min. The same conditions were used for *LRW268*, but the annealing temperature was at 60°C. After amplification the products were separated on a 1.5% (w/v) agarose gel (3.2.2.1).

4.2.2.4. Obtaining the full length *LRW268* gene using Rapid amplification of cDNA ends (RACE)

To obtain the full length *LRW268* gene, 5'- and 3'-RACE were performed using the the 5'/3' 2nd generation RACE kit (Roche) according to the manufacturer's specifications. Two microgram total RNA isolated from infected Thatcher+*Lr*34 tissue harvested at 9 hpi was used for the RACE. The first strand cDNA synthesis was performed using Bovis 128 (Fig 4.7). After the cDNA synthesis, a poly-A tail was added on the 3'-end to this cDNA fragment. For the first PCR step of 5'-RACE, Bovis 128 was used as reverse primer 1 in combination with the supplied Oligo-dT anchor primer (Table 4.1). For the second nested PCR step, Bovis 218 was used as the reverse primer 2 in combination with the supplied PCR anchor primer (Table 4.1).

Of the second nested 5'-RACE PCR reaction, 5 µl was cloned into pGemT-Easy (Promega) according to the manufacturer's instructions. The ligation mixture was transferred to ultra competent *E. coli* (JM109) cells (Promega) according to the manufacturers' conditions. The reactions were incubated and plated and recombinant plasmids were selected for using α-complementation (3.2.2.3).

White colonies containing putative recombinant plasmids were inoculated in 5 ml LB medium containing 50 $\mu g.ml^{-1}$ ampicillin and grown overnight at 37°C. Plasmid DNA was

extracted from the *E. coli* cells according to Sambrook *et al.* (2000). The prescence of the inserts was confirmed by PCR amplification using the PCR anchor primer and Bovis 218. The PCR reaction consisted of 1 µl purified plasmid DNA, 25 pmol of each primer and 1X Kapa*Taq* ReadyMix (Kapa Biosystems). The amplification regime was as follows: one cycle at 94°C for one min, 30 cycles at 94°C for 15 sec, 53°C for 30 sec and 72°C for one min followed by a final cycle at 72°C for 10 min. The amplification reaction was separated on a 1.5% (w/v) agarose gel (3.2.2.1).

RNA isolated from infected Thatcher+*Lr*34 tissue harvested at 9 hpi was used with Bovis 32 to synthesize first strand cDNA for 3'-RACE (Fig 4.7). Bovis 127 was used in combination with the supplied Oligo-dT anchor primer for the first PCR step. The second nested PCR reaction used Bovis 127 and the PCR anchor primer. The amplified 3'-RACE fragment was cloned into pGemT-Easy as described (3.2.2.3). The inserts were similarly amplified from recombinant plasmids using the PCR anchor primer and Bovis 127, with the exception that an annealing temperature of 56°C was used.

Six recombinant plasmids of various sizes from the 5'-RACE and six from the 3'-RACE pools were selected and sequenced with a BigDye terminator v3.1 cycle sequencing kit (Applied Biosystems). The sequencing of 5'-RACE fragments was performed using Bovis 218 while for the 3'-RACE fragments Bovis 127 was used. Sequences were aligned to the original *LRW268* sequence to locate the regions that overlapped. Sequences were translated to amino acid sequences using the ExPASy translate tool (http://au.expasy.org/tools/dna.html). The longest open reading frame (ORF) was used for a BLAST search on the GenBank website to search for similarities on DNA and protein level with all known genes.

4.2.2.5. Virtual gene walking of *LRW222* and *LRW268*

Since there is a large database of ESTs available (http://www.ncbi.nlm.nih.gov/sites/entrez?db=nucest), a contig for both *LRW222* and *LRW268* was built by searching the GenBank database using BLAST with the original *LRW222* and *LRW268* sequences. The sequence of the EST with the best similarity was first

obtained. An alignment of the original sequence and the EST sequence was performed with ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/). A second BLAST search was then done using the first EST sequence. This was continued until the longest contig could be assembled using ChromasPro (Technelysium). The sequence was then translated using ExPASy (http://ca.expasy.org/tools/dna.html), and then used in a BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) search. The ORF was finally searched for functional sites (http://elm.eu.org/).

4.3. Results

4.3.1. Presence of LRW222 and LRW268 in wheat

To determine the presence of *LRW222* and *LRW268* in the various wheat cultivars, a PCR using primers designed for each individual clone, was performed. This PCR yielded fragments in all the tested cultivars (Fig 4.1). All the cultivars contained the expected 217 bp *LRW222* fragment and 242 bp *LRW268* fragment. No other fragments were amplified using these primer combinations.

4.3.2. Analysis of LRW222 and LRW268 gene expression

To compare the effect different *Puccinia* spp. have on wheat, the expression of *LRW222* and *LRW268* was tested on wheat infected with *P. triticina* and *P. striiformis* respectively (Fig 4.2). The expression of *LRW222* in *P. triticina* inoculated plants was induced at 9 hpi in the infected susceptible Thatcher plants (Fig 4.2a). The expression then remained relatively constant till 30 hpi. In the infected resistant Thatcher+*Lr*34 cultivar, a low level of induction occurred at 3 hpi with a more pronounced increase from 15 hpi peaking at 21 hpi and then dropping to a lower level at 27 hpi. The expression of *LRW222* in the *P. striiformis* infected susceptible Avoset-S plants was relatively constant until 48 hpi were it started to decline (Fig 4.2b). The levels were higher in the infected resistant plants and reach a peak at 36 hpi whereafter it decreased to 72 hpi. The basal levels of *LRW222* expression in the *P. striiformis* infected Avoset plants were in general higher than that of *P. triticina* infected plants.

Treatment of seedling Thatcher+*Lr*34 plants with chemical elicitors was used to further elucidate the role *LRW222* could play in plant defence response. Plants that were mock treated with H_2O showed relative constant expression of *LRW222* (Fig 4.3a). Plants treated with H_2O_2 showed an initial induction of expression 30 min after treatment and a secondary induction 4 hpi (Fig 4.3b). The MeSA treated plants showed a constitutive expression of *LRW222* (Fig 4.3c), while in MeJA treated plants, *LRW222* showed a strong increase in expression at 2 hpi that remained constant thereafter (Fig 4.3d). Plants treated with menadione showed an initial repression of *LRW222* expression after 30 min whereafter the

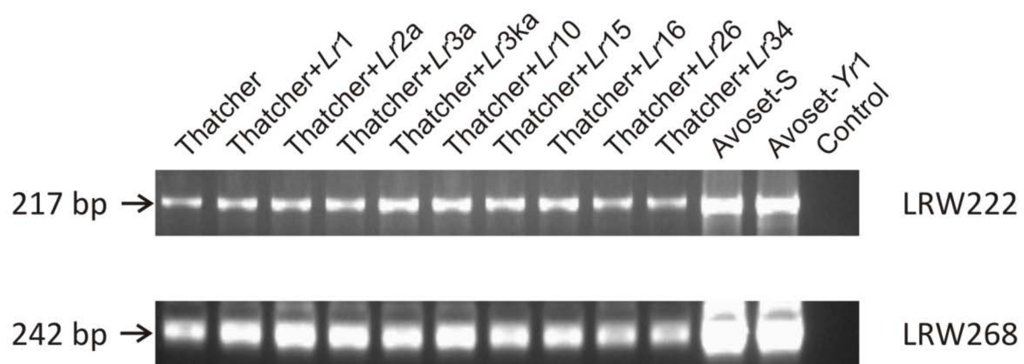

Figure 4.1. The presence of *LRW222* and *LRW268* in different wheat cultivars. The names of the different cultivars as well as the fragment sizes are as indicated.

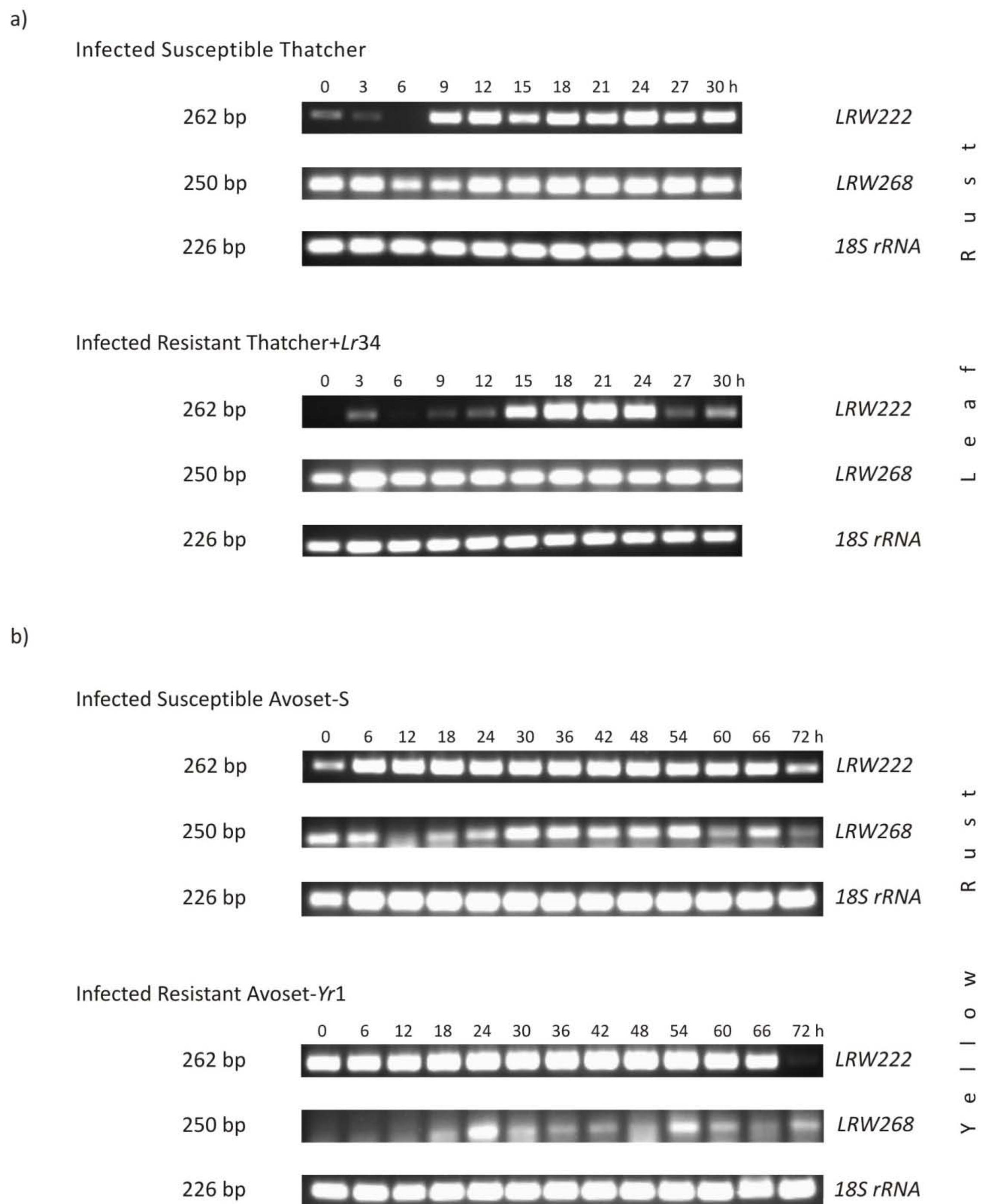

Figure 4.2. Expression analysis of *LRW222* and *LRW268* in susceptible and resistant wheat infected with (a) *P. triticina* and (b) *P. striiformis*.

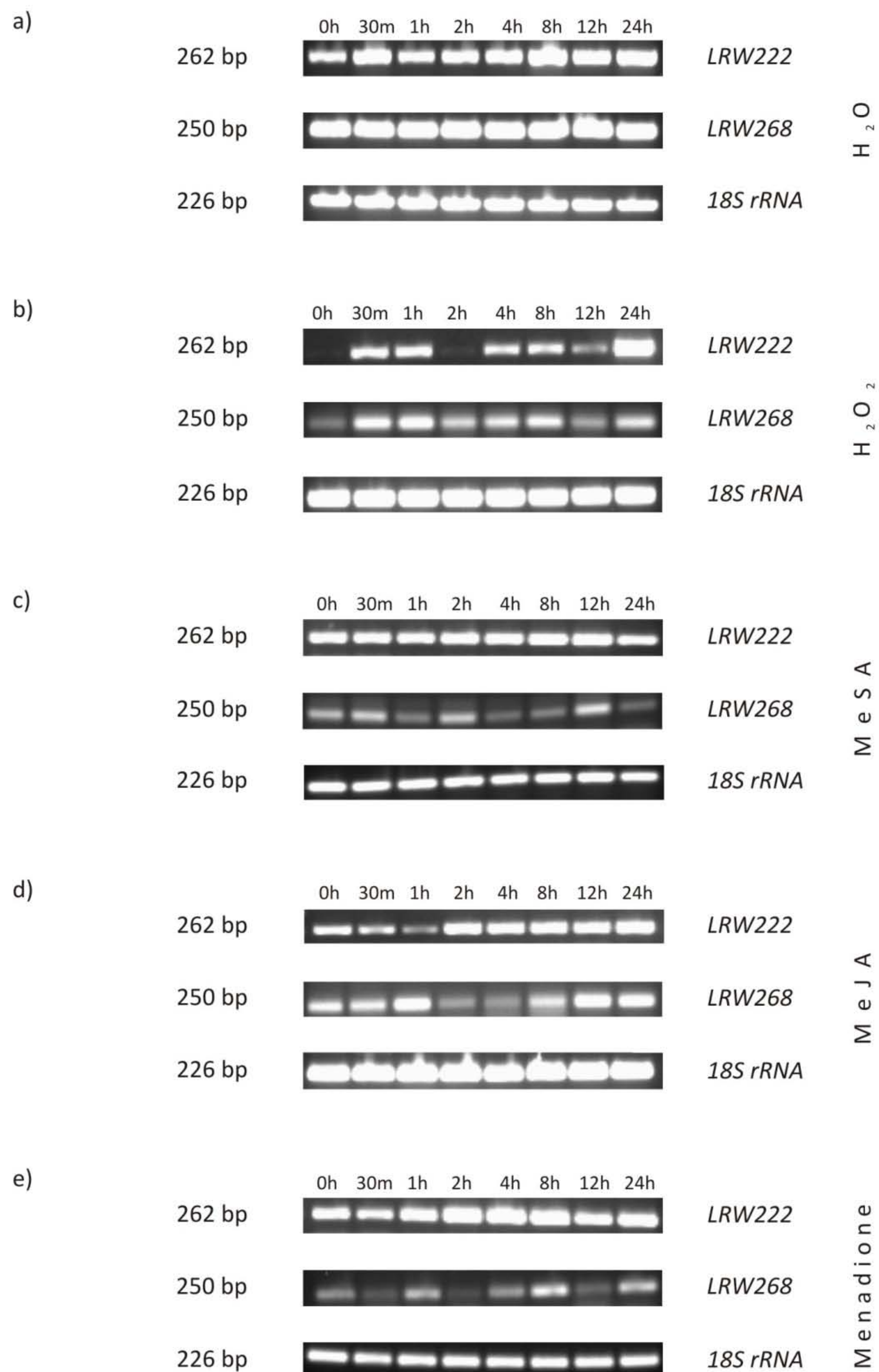

Figure 4.3. Expression analysis of *LRW222* and *LRW268* in Thatcher+*Lr*34 seedlings with different chemicals.

The expression of *LRW268* in *P. triticina* infected plants was relatively constant other than the slight decrease in expression at 6 and 9 hpi for infected susceptible plants (Fig 4.2a). A constant expression was observed in the infected resistant plants except for 3 hpi showing an increase in expression (Fig 4.2a). There was a more drastic effect in the *P. striiformis* infected wheat. *LRW268* levels in infected susceptible plants were initially slightly repressed but were then induced after 12 hpi, reaching a peak at 30 hpi. The infected resistant plants showed a significant induction in the expression level of *LRW268* at 24 hpi while a secondary less prominent induction occurred at 54 hpi.

Plants that were mock treated with H_2O showed constitutive for *LRW268* expression (Fig 4.3a). Treatment with H_2O_2 produced an initial induction of expression 30 min after treatment with a secondary induction 8 hpi (Fig 4.3b). The MeSA inoculated plants showed only a slight induction around 12 hpi (Fig 4.3c). The expression of *LRW268* in MeJA inoculated plants had an initial induction as early as 1 hpi with the secondary response initiated at 8 hpi (Fig 4.3d). Plants treated with menadione showed repressed expression levels of *LRW268* 30 min after treatement with an increase at 1 hpi (Fig 4.3e). Expression then decreased until peaking at 8 hpi.

4.3.3. Sequence analysis of LRW222

Seeing that *LRW222* levels are significantly influenced by the inoculation of wheat by *P. triticina* as well as different chemical elicitors, it was pertinent that more sequence information be obtained for this cDNA clone. The original sequence was used for a BLAST search on GenBank for ESTs that showed similarity. *LRW222* showed similarity to a large array of ESTs but a *T. aestivum* EST (Accession number: CJ955962, e-value: $1e^{-136}$) showed nearly 100% identity (Fig 4.4). The sequence of this *T. aestivum* EST was used for another BLAST search during which CJ955962 was significantly similar to a *Triticum turgidum* subsp. *durum* EST (Accession number: AM285535, e-value: 0.0) (Fig 4.5a). The three sequences were then used to construct a contig based on their similar regions (Fig 4.5b). The contig was 668 bp in length with a 270 bp ORF which translated to a 89 amino acid sequence (Fig 4.6a) with a molecular weight of 9.5 kDa (Fig 4.6b). This is the longest contig sequence that could be obtained. When the LRW222 polypeptide (Fig 4.6a) was used for a

```
LRW222          ------------------------------------------------------------
CJ955962        ACACAAGTGAAGTGAGAGCTTTGCGCTGAAAGTTCATCAGCAACTATGAAGGGCACCAAG 60

LRW222          ------------------------------------------------------------
CJ955962        CTCGCGGCGATCCTGATCCTCCAGGCCGTCCTGGTCATGGGAGTCCTCTCGCACGTCAAC 120

LRW222          ------------------------------------------------------------
CJ955962        GCCGACTTCTTCCCCAAGTGCTGCAACAACTGCGGGTCCTTCTCGGGGGTCGACGTCTGC 180

LRW222          ------------------------------------------------------------
CJ955962        GACGACGCCCACCCCAAGTGTCCCAAGGGCTGCTCGGCGTGCCGCGTGGTGTCGACGAGC 240

LRW222          ------------------------------------------------------------
CJ955962        CCCGAAATGTGGCGCTGCGCGGATATGAAATCCACCGTCGACGGCACCTGCGGTGGACCC 300

LRW222          ----------------CGGTTCATGCCGGCATATGATATGTTCCGCCTAAAATAAAATAA 44
CJ955962        TGCAAGAAGTACTGATCGGTTCATGCCGGCATATGATATGTTCCGCCTAAAATTAAATAA 360
                                ************************************* ******

LRW222          AAGCTCGGACGAGATGAGCAGCGTCATCGTGCCTATGCGTGTGTGTGTGACCAACAAATA 104
CJ955962        AAGCTCGGACGAGATGAGCAGCGTCATCGTGCCTATGCGTGTGTGTGTGACCAACAAATA 420
                ************************************************************

LRW222          TGTATACCAGTATGTCGTGTCTTCGTCGCTGTGTTTTCTCTCTCGAGAAAAAGTTGGTGT 164
CJ955962        TGTATACCAGTATGTCGTGTCTTCGTCGCTGTGTTTTCTCTCTCGAGAAAAAGTTGGTGT 480
                ************************************************************

LRW222          TGCTTGTTGTTCAGCTATCCGGAATATCCTCCGTGAATAAACACCTGTAGTGATTGTGCG 224
CJ955962        TGCTTGTTGTTCAGCTATCCGGAATATCCTCCGTGAATAAACACCTGTAGTGATTGTGCG 540
                ************************************************************

LRW222          TGGCTGCCAATAATGTGAGTTGNGTNGTCCACGCAGTTACATGTTTCCCGTTGTATCC-- 282
CJ955962        TGGCTGCCAATAATGTGAGTTCTCTTGTCCACGCAGTTACATGTTTCCCGTTGTATTTAT 600
                *********************   * ******************************

LRW222          -------------------------------------------------
Cj955962        ATACTCTCCGATATGATATTTGTTGGCGTGTTAAGCAGTAAAAAAAAAA 649
```

Figure 4.4. Alignment of cDNA clone *LRW222* with *T. aestivum* EST1 (GenBank accession nr: CJ955962). Consensus sequences are indicated with an (*).

a)

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AM285535        GAATTCGGCACGAGGTACTCCTCCACACAAGTGAAGTGAGAGCTTTGCGCTGAAAGTTCA 60
CJ955962        ------------------------ACACAAGTGAAGTGAGAGCTTTGCGCTGAAAGTTCA 36
                                        ************************************

AM285535        TCAGCAACTATGAAGGGCACCAAGCTCGCGGCGATCCTGATCCTCCAGGCCGTCCTGGTC 120
CJ955962        TCAGCAACTATGAAGGGCACCAAGCTCGCGGCGATCCTGATCCTCCAGGCCGTCCTGGTC 96
                ************************************************************

AM285535        ATGGGAGTCCTCTCGCACGTCAACGCCGACTTCTTCCCCAAGTGCTGCAACAACTGCAGG 180
CJ955962        ATGGGAGTCCTCTCGCACGTCAACGCCGACTTCTTCCCCAAGTGCTGCAACAACTGCGGG 156
                ********************************************************* **

AM285535        TCCTTCTCGGGGGTCGACGTCTGCGACGACGCCCACCCCAAGTGTCCCAAGGGCTGCTCG 240
CJ955962        TCCTTCTCGGGGGTCGACGTCTGCGACGACGCCCACCCCAAGTGTCCCAAGGGCTGCTCG 216
                ************************************************************

AM285535        GCGTGCCGCGTGGTGTCGACGAGCCCCGAAATGTGGCGCTGCGCGGATATGAAATCCACC 300
CJ955962        GCGTGCCGCGTGGTGTCGACGAGCCCCGAAATGTGGCGCTGCGCGGATATGAAATCCACC 276
                ************************************************************

AM285535        GTCGACGGCACCTGCGGTGGACCCTGCAAGAAGTACTGATCGGTTCATGCCGGCATATGA 360
CJ955962        GTCGACGGCACCTGCGGTGGACCCTGCAAGAAGTACTGATCGGTTCATGCCGGCATATGA 336
                ************************************************************

AM285535        TATGTTCCGCCTAAAATTAAATAAAAGCTCGGACGAGATGAGCAGCGTCATCGTGCCTAT 420
CJ955962        TATGTTCCGCCTAAAATTAAATAAAAGCTCGGACGAGATGAGCAGCGTCATCGTGCCTAT 396
                ************************************************************

AM285535        GCGTGTGTGTGTGACCAACAAATATGTATACCAGTATGTCGTGTCTTCGTCGCTGTGTTT 480
CJ955962        GCGTGTGTGTGTGACCAACAAATATGTATACCAGTATGTCGTGTCTTCGTCGCTGTGTTT 456
                ************************************************************

AM285535        TCTCTCTCGAGAAAAAGTTGGTGTTGCTTGTTGTTCAGCTATCCGGAATATCCTCCGTGA 540
CJ955962        TCTCTCTCGAGAAAAAGTTGGTGTTGCTTGTTGTTCAGCTATCCGGAATATCCTCCGTGA 516
                ************************************************************

AM285535        ATAAACACCTGTAGTGATTGTGCGTGGCTGCCAATAATGTGAGTTCTCTTGTCCACGCAG 600
CJ955962        ATAAACACCTGTAGTGATTGTGCGTGGCTGCCAATAATGTGAGTTCTCTTGTCCACGCAG 576
                ************************************************************

AM285535        TTACATGTTTCCCGTTGTATTTATATACTCTCCGATATGATATTTGTTGGCGTGTTAAGC 660
CJ955962        TTACATGTTTCCCGTTGTATTTATATACTCTCCGATATGATATTTGTTGGCGTGTTAAGC 636
                ************************************************************

AM285535        AGTATCTT----- 668
CJ955962        AGTAAAAAAAAAA 649
                ****
```

b)

Figure 4.5. Construction of a contig for *LRW222*. Indicated in a) is the alignment of *T. aestivum* ESTs CJ955962 and AM285535 and b) is the contig map. Consensus sequences are indicated with an (*).

a) 5'-GAATTCGGCACGAGGTACTCCTCCACACAAGTGAAGTGAGAGCTTTGCGCTGAAAGTTCATCAGCAACT**ATG**AAGGGCA
CCAAGCTCGCGGCGATCCTGATCCTCCAGGCCGTCCTGGTCATGGGAGTCCTCTCGCACGTCAACGCCGACTTCTTCCCCA
AGTGCTGCAACAACTGCAGGTCCTTCTCGGGGGTCGACGTCTGCGACGACGCCCACCCCAAGTGTCCCAAGGGCTGCTC
GGCGTGCCGCGTGGTGTCGACGAGCCCCGAAATGTGGCGCTGCGCGGATATGAAATCCACCGTCGACGGCACCTGCGGT
GGACCCTGCAAGAAGTAC**TGA**TCGGTTCATGCCGGCATATGATATGTTCCGCCTAAAATTAAATAAAAGCTCGGACGAGAT
GAGCAGCGTCATCGTGCCTATGCGTGTGTGTGTGACCAACAAATATGTATACCAGTATGTCGTGTCTTCGTCGCTGTGTTTT
CTCTCTCGAGAAAAAGTTGGTGTTGCTTGTTGTTCAGCTATCCGGAATATCCTCCGTGAATAAACACCTGTAGTGATTGTGC
GTGGCTGCCAATAATGTGAGTTCTCTTGTCCACGCAGTTACATGTTTCCCGTTGTATTTATATACTCTCCGATATGATATTTGT
TGGCGTGTTAAGCAGTATCTT-3'

b) MKGTKLAAIL ILQAVLVMGV LSHVNADFFP KCCNNCRSFS GVDVCDDAHP KCPKGCSACR VVSTSPEMWR CADMKSTVDG
TCGGPCKKY

c)

```
AAA50850          MKGTKLAAILILQAVLVMGLLSHVNADFFPKCCNNCRSFSGVDVCDDAHPKCPQGCSACR 60
LRW222_contig     MKGTKLAAILILQAVLVMGVLSHVNADFFPKCCNNCRSFSGVDVCDDAHPKCPKGCSACR 60
                  *******************:*********************************:******

AAA50850          VVSTSPEMWRCADMKSTVDGTCGGPCKKY 89
LRW222_contig     VVSTSPEMWRCADMKSTVDGTCGGPCKKY 89
                  *****************************
```

Figure 4.6. Analysis of the *LRW222* contig. Indicated in a) is the contig sequence of *LRW222*, in b) LRW222 polypeptide sequence and in c) the amino acid similarity between LRW222 and Wali5. Start and stop codons are indicated in bold. ORF has been underlined. Consensus sequences are indicated with an (*) while (:) indicates conservative substitutions.

BLAST search, it showed the best similarity to Wali5 protein (Accession number: AAA50850, e-value: $8e^{-45}$) from *T. aestivum* as well as other protease inhibitors from other organisms (Table 4.2). An amino acid alignment of Wali5 and LRW222 indicated that the proteins were nearly identical (Fig 4.6c). A BowB conserved domain was detected within the coding region. Proteins containing this domain form part of the Bowman-Birk type of proteinase inhibitor groups.

The LRW222 polypeptide sequence (Fig 4.6b) was further analysed for functional domains (Table 4.3). Present was a 14-3-3 ligand binding site between position 60 and 65. This portion mediates signal transduction by binding to phosphoserine or phosphothreonine containing motifs from other proteins. Between 62 and 68, a FHA phosphopeptide ligand was found, which is a signal transduction module recognising phosphothreonine containing peptides on the ligand proteins. Between amino acid 62 and 68, a MAPK phosphorylation site was found where MAPK phosphorylates substrates. A MAPK docking motif was present between amino acid 2 and 12. This motif helps regulate interactions between PI and components of the MAPK cascade. A CK2 phosphorylation site present between 61 and 67, is recognised by CK2 for Ser/Thr phosphorylation. The GSK3 phosphorylation recognition site between 74 and 81, on the other hand, is recognised by GSK3 for Ser/Thr phosphorylation.

4.3.4. Sequence analysis of LRW268

To obtain the full length gene of clone *LRW268*, 5'- and 3'-RACE were performed. The first gradient PCR was performed to obtain the optimal amplification for the Bovis 218 and Oligo-dT anchor primer pair (Fig 4.7a). The optimum temperature was 56.3°C due to the fact that the fragment of interest, which was larger than 500 bp, was the most distinct (Fig 4.7a). The second set of nested primers was used to increase the specificity of the PCR. This PCR yielded a product smaller than 500 bp. These fragments were then cloned into pGEM-T Easy and recombinant plasmids were selected using α-complementation.

Twenty recombinant plasmids were isolated and the inserts amplified with gene specific primers. Six recombinant cDNA clones of the 5'-RACE reaction were selected and

Table 4.2. Amino acid BLAST analysis of LRW222.

Accession number	***Description***	***Organism***	***e-value***
AAA50850	Wali5 protein	*Triticum aestivum*	$8e^{-45}$
CAA88619	Putative protease inhibitor	*Hordeum vulgare*	$4e^{-40}$
AAS49905	Putative proteinase inhibitor-related protein	*Triticum aestivum*	$2e^{-39}$
AAC37417	Wali6 protein	*Triticum aestivum*	$3e^{-22}$
AAA50848	Wali3 protein	*Triticum aestivum*	$6e^{-21}$
NP001041929	Putative wound-induced protease inhibitor	*Oryza sativa*	$1e^{-08}$
NP001041928	Putative wound-induced protease inhibitor	*Oryza sativa*	$2e^{-08}$
AAS17863	Wound-induced protease inhibitor	*Zea diploperennis*	0.004
AAL01296	Wound-induced protease inhibitor	*Zea mays*	0.004
AAL01267	Wound-induced protease inhibitor	*Zea luxurians*	0.005

Table 4.3. Putative functional sites present on the polypeptide of the *LRW222* contig.

Name	***Description***	***Position***
14-3-3 ligand	14-3-3 proteins interact with phosphoserine or phosphothreonine containing motifs	60-65
FHA phosphopeptide ligand	Signal transduction module which recognizes phosphothreonine containing peptides on the ligand proteins	62-68
MAPK docking motif	Docking motifs that help to regulate specific interactions in the MAPK cascade	2-12
CK2 phosphorylation site	Motif recognised by CK2 for Ser/Thr phosphorylation	61-67
GSK3 phosphorylation recognition site	Site recognised by GSK3 for Ser/Thr Phosphorylation	74-81
MAPK phosphorylation site	Site at which MAP Kinase phosphorylates substrates Proline-Directed Kinase phosphorylation site	62-68

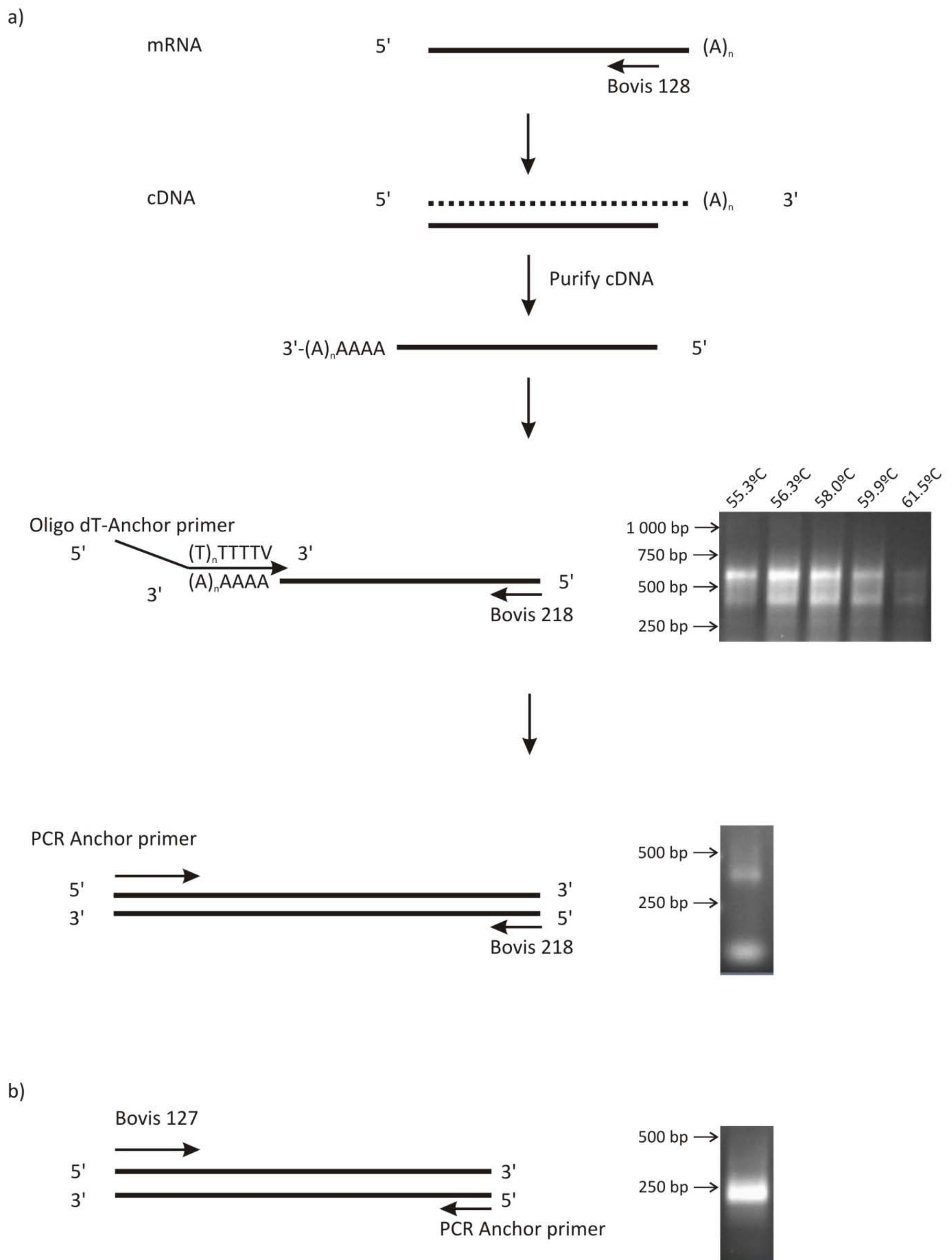

Figure 4.7. A schematic representation of a) 5’-RACE and b) 3’-RACE. Diagrams and DNA sizes are as indicated.

sequenced. After the obtained sequences were analysed, it was noticed that all were different and shared no similarity. In addition, no overlapping areas were found between the clones and *LRW268*. The sequencing results were inconclusive. The 3'-RACE did yield a 165 bp fragment when sequenced (Fig 4.7b). This sequence showed similarity with the original *LRW268* sequence indicating that the original *LRW268* sequence represented the 3'-end of the gene (Fig 4.8).

Further analysis of the original sequence of *LRW268* was performed by building a contig using similar EST sequences on GenBank. When the *LRW268* sequence was used in a BLAST search, a 553 bp *T. aestivum* EST showed good similarity (Accession number: CJ944189, e-value: $2e^{-87}$) (Fig 4.9). A second BLAST search was then performed using EST1 which generated a second *T. aestivum* cDNA clone that was 812 bp long (Fig 4.10a). This time the similarity was across 543 bp with an e-value of 0.0, thus leading to the conclusion that the three cDNA clones were the same. The contig was compiled using the two published sequences (Fig 4.10b).

The nucleotide sequence of the contig after assembly yielded a 291 bp ORF (Fig 4.11a) which when translated rendered a 96 amino acid sequence (Fig 4.11b). This polypeptide was similar to an *Oryza sativa* hypothetical protein (Accession number: NP_001052832, e-value: $3e^{-41}$) (Fig 4.11c and Table 4.4). Even though the similarity between LRW268 and the SLL1 protein (Accession number: ABD78324) was not the highest, the e-value of $5e^{-26}$ was still significant.

A functional site analysis of the amino acid sequence of the *LRW268* contig showed a FHA ligand binding site between position 16 and 22 (Table 4.5) (4.3.3). A MAPK docking motif was present between amino acid 7 and 15. A CK2 phosphorylation site was present between amino acid 15 and 21 as well as a GSK3 phosphorylation recognition site between 20 and 27 (4.3.3). A USP7 binding motif which is found on USP7 targeting substrates was located between amino acid 20 and 24. Amino acid 36 to 42 showed a PKA protein kinase_1 and 2 motif involved with PKA Ser/Thr phosphorylation and protein kinase cell signalling.

```
LRW268          TTATTGGACCTATTTTGAATTTAGCATTTTGTGAAAGCCAAAGCTGTCACTTCACTTGAC 60
3'-RACE268      -----------------------------------------------------CCTTCAT 7
                                                                     *** *

LRW268          TATTGTTGTTTCGTGTTACTCCATTTGCTCTTAGCAGATGATCTGAATCTGTAAGCTCGT 120
3'-RACE268      GAATGTTGTT-CGTGT-ACTC-ATTTGCTCTTAGCAGATGATCTGAATCTGTAAGTTCGT 64
                 * ******* ***** **** ********************************* ****

LRW268          TTCTTTACGGCACTG-CTTTCTGTTAATAATACAGTGG-CTTGAACTTTCA--------- 169
3'-RACE268      TTCTTTACGGCACTGGCTTTCTGTTAATAATACAGTGGGCTTGAACTTTCNCAGGCTGTG 124
                *************** ********************** ***********

LRW268          -AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA--- 206
3'-RACE268      GAGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 165
                 * ***********************************
```

Figure 4.8. The alignment of *LRW268* with the sequence of the 3'-RACE fragment. Consensus sequences are indicated with an (*).

```
LRW268          ------------------------------------------------------------
CJ944189        AACGCACCCACCACCACAACTCCGGCGGCGGCGACCAGACGAACACGAGCACCAAGGCGA 60

LRW268          ------------------------------------------------------------
CJ944189        TGGCGACTGCTCTCAACCGCGGCCTCCGCTCCGGGATCCGCCTCCTCGCCACCGGCGCCG 120

LRW268          ------------------------------------------------------------
CJ944189        AGGCCTCCAAGCCAGCTTCACGTGGATTCCATGCTACCGGCGTGAAGAGGATGTCAGGGC 180

LRW268          ------------------------------------------------------------
CJ944189        ACGGCCATGATGAGCCATACTACCTCCACGCCAAGCACATGTACAACTTGCACAGGATGA 240

LRW268          ------------------------------------------------------------
CJ944189        AGCATCAGAAGCTGACTGCATGGACTTCGGTGCTGGGAGCCGTAAGCATTGGTATCGGTG 300

LRW268          ------------------------------------------------------------
CJ944189        TCCCGGTCTTCGCGGTCGTCTTCCAGCAAAAGAAGACCTCCTCCGGATGAGCTCACATAC 360

LRW268          ---------------TTATTGGACCTATTTTGAATTTAGCATTTTGTGAAAGCCAAAGCT 45
CJ944189        CCTCCCAGGGCCTACTTATTGGACCTATTTTGAATTTAGCATTTTGTGAAAGCCAAAGCT 420
                               *********************************************

LRW268          GTCACTTCACTTGACTATTGTTGTTTCGTGTTACTCCATTTGCTCTTAGCAGATGATCTG 105
CJ944189        GTCACTTCACTTGACTATTGTTGTTTCGTGTTACTCCATTTGCTCTTAGCAGATGATCTG 480
                ************************************************************

LRW268          AATCTGTAAGCTCGTTTCTTTACGGCACTGCTTTCTGTTAATAATACAGTGGCTTGAACT 165
CJ944189        AATCTGTAAGCTCGTTTCTTTACGGCACTGCTTTCTGTTAATAATACAGTGGCTTGAACT 540
                ************************************************************

LRW268          TTCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 206
CJ944189        TTCAAAAAAAAAA---------------------------- 553
                *************
```

Figure 4.9. Alignment of cDNA clone *LRW268* with *T. aestivum* EST1 (GenBank accession nr: CJ944189). Consensus sequences are indicated with an (*).

a)

```
CV764795        CCACGCGTCCGATCACCCCCAAAACGCACCCACCACCACAACTCCGGCGGCGGCGACCAG 60
CJ944189        ----------------------AACGCACCCACCACCACAACTCCGGCGGCGGCGACCAG 38
                                      **************************************

CV764795        ACGAACACGAGCACCAAGGCGATGGCGACTGCTCTCAACCGCGGCCTCCGCTCCGGGATC 120
CJ944189        ACGAACACGAGCACCAAGGCGATGGCGACTGCTCTCAACCGCGGCCTCCGCTCCGGGATC 98
                ************************************************************

CV764795        CGCCTCCTCGCCACCGGCGCCGAGGCCTCCAAGCCAGCTTCACGTGGATTCCATGCTACC 180
CJ944189        CGCCTCCTCGCCACCGGCGCCGAGGCCTCCAAGCCAGCTTCACGTGGATTCCATGCTACC 158
                ************************************************************

CV764795        GGCGTGAAGAGGATGTCAGGGCACGGCCATGATGAGCCATACTACCTCCACGCCAAGCAC 240
CJ944189        GGCGTGAAGAGGATGTCAGGGCACGGCCATGATGAGCCATACTACCTCCACGCCAAGCAC 218
                ************************************************************

CV764795        ATGTACAACTTGCACAGGATGAAGCATCAGAAGCTGACTGCATGGACTTCGGTGCTGGGA 300
CJ944189        ATGTACAACTTGCACAGGATGAAGCATCAGAAGCTGACTGCATGGACTTCGGTGCTGGGA 278
                ************************************************************

CV764795        GCCGTAAGCATTGGTATCGGTGTCCCGGTCTTCGCGGTCGTCTTCCAGCAAAAGAAGACC 360
CJ944189        GCCGTAAGCATTGGTATCGGTGTCCCGGTCTTCGCGGTCGTCTTCCAGCAAAAGAAGACC 338
                ************************************************************

CV764795        TCCTCCGGATGAGCTCACATACCCTCCCAGGGCCTACTTATTGGACCTATTTTGAATTTA 420
CJ944189        TCCTCCGGATGAGCTCACATACCCTCCCAGGGCCTACTTATTGGACCTATTTTGAATTTA 398
                ************************************************************

CV764795        GCATTTTGTGAAAGCCAAAGCTGTCACTTCACTTGACTATTGTTGTTTCGTGTTACTCCA 480
CJ944189        GCATTTTGTGAAAGCCAAAGCTGTCACTTCACTTGACTATTGTTGTTTCGTGTTACTCCA 458
                ************************************************************

CV764795        TTTGCTCTTAGCAGATGATCTGAATCTGTAAGCTCGTTTCTTTACGGCACTGCTTTCTGT 540
CJ944189        TTTGCTCTTAGCAGATGATCTGAATCTGTAAGCTCGTTTCTTTACGGCACTGCTTTCTGT 518
                ************************************************************

CV764795        TAATAATACAGTGGCTTGAACTTTCCAGGCTGTGGAGAACCCTGCAGTGTGAAAAA 596
CJ944189        TAATAATACAGTGGCTTGAACTTTCAAAA----AAAAAA----------------- 553
                ************************* *        * **
```

b)

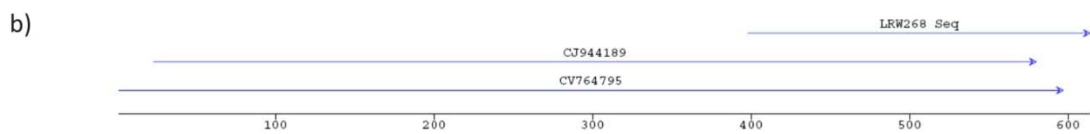

Figure 4.10. Sequence analysis of *LRW268* where a) is the alignment of *T. aestivum* ESTs CJ944189 and CV764795 and b) the contig map. Consensus sequences are indicated with an (*).

a) 5'-CCACGCGTCCGATCACCCCCAAAACGCACCCACCACCACAACTCCGGCGGCGGCGACCAGACGAACACGAGCACCAAG
GCG<u>**ATG**GCGACTGCTCTCAACCGCGGCCTCCGCTCCGGGATCCGCCTCCTCGCCACCGGCGCCGAGGCCTCCAAGCCAG</u>
<u>CTTCACGTGGATTCCATGCTACCGGCGTGAAGAGGATGTCAGGGCACGGCCATGATGAGCCATACTACCTCCACGCCAAG</u>
<u>CACATGTACAACTTGCACAGGATGAAGCATCAGAAGCTGACTGCATGGACTTCGGTGCTGGGAGCCGTAAGCATTGGTAT</u>
<u>CGGTGTCCCGGTCTTCGCGGTCGTCTTCCAGCAAAAGAAGACCTCCTCCGGA**TGA**</u>GCTCACATACCCTCCCAGGGCCTAC
TTATTGGACCTATTTTGAATTTAGCATTTTGTGAAAGCCAAAGCTGTCACTTCACTTGACTATTGTTGTTTCGTGTTACTCCA
TTTGCTCTTAGCAGATGATCTGAATCTGTAAGCTCGTTTCTTTACGGCACTGCTTTCTGTTAATAATACAGTGGCTTGAACTT
TCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA-3'

b) MATALNRGLR SGIRLLATGA EASKPASRGF HATGVKRMSG HGHDEPYYLH AKHMYNLHRM KHQKLTAWTS VLGAVSIGIG
VPVFAVVFQQ KKTSSG

c)

```
NP_001052832       MATATALNRGLRSGIRLLATGAEASKTDSRGFHATGVKRMGGHGHDEPYYLHAKHMYNLH 60
LRW268_contig      --MATALNRGLRSGIRLLATGAEASKPASRGFHATGVKRMSGHGHDEPYYLHAKHMYNLH 58
                      ***********************. ************.*******************

NP_001052832       RMKHQKPKVYLSVLGAVGIGIAVPVYAVVFQQKKTASG 98
LRW268_contig      RMKHQKLTAWTSVLGAVSIGIGVPVFAVVFQQKKTSSG 96
                   ****** ..: ******.***.***:*********:**
```

Figure 4.11. Sequence analysis of the *LRW268* contig where a) is the contig sequence of *LRW268*, b) is the amino acid sequence for *LRW268* and c) shows the amino acid similarity. Start and stop codons are indicated in bold. The ORF has been underlined. Consensus sequences are indicated with an (*) while (:) indicates conservative substitutions and (.) semiconservative substitutions.

Table 4.4. BLAST analysis of the LRW268 polypeptide.

Accession number	***Description***	***Organism***	***e-value***
NP_001052832	Os04g0432600	*Oryza sativa*	$3e^{-41}$
CAE03043	Hypothetical protein	*Oryza sativa*	$5e^{-29}$
EAY94176	Hypothetical protein OsI_015409	*Oryza sativa*	$2e^{-28}$
ABD78324	SLL1 protein	*Primula vulgaris*	$5e^{-26}$
ABK92617	Unknown	*Populus trichocarpa*	$1e^{-25}$
ABD78323	SLL1 protein	*Primula vulgaris*	$3e^{-25}$
ABD78312	SLL1 protein	*Primula vulgaris*	$3e^{-25}$
ABD78325	SLL1 protein	*Primula vulgaris*	$3e^{-25}$
ABD78313	SLL1 protein	*Primula vulgaris*	$2e^{-24}$
ABD78311	SLL1 protein	*Primula vulgaris*	$2e^{-24}$

Table 4.5. Putative functional sites present on the polypeptide of the *LRW268* contig.

Name	***Description***	***Position***
FHA phosphopeptide ligand	Signal transduction module which recognizes phosphothreonine containing peptides on the ligand proteins	16-22
MAPK docking motif	Docking motifs that help to regulate specific interactions in the MAPK cascade	7-15
USP7 binding motif	Targeting motif found in USP7 substrates, docking to the NTD domain	20-24
CK2 phosphorylation site	Motif recognised by CK2 for Ser/Thr phosphorylation	15-21
GSK3 phosphorylation recognition site	Site recognised by GSK3 for Ser/Thr Phosphorylation	20-27
PKA is a protein kinase_1	PKA is a protein kinase involved in cell signalling	36-42
PKA is a protein kinase_2	Motif recognized by PKA for Ser/Thr phosphorylation	36-42

4.4. Discussion

SSH is a widely used technique to generate cDNA libraries representing different plant-pathogen interactions. This PCR based technique allows for the isolation of cDNAs that are differentially expressed (Diatchenko *et al*., 1996). Various studies have successfully utilised this technique to identify genes playing a role during plant defence (Xiong *et al*., 2001; Bittner-Eddy *et al*., 2003; Vieira Dos Santos *et al*., 2003).

To better understand the interaction between *T. aestivum* and *P. triticina*, two cDNA clones that were isolated using SSH (Chapter 3) were further characterised. A more detailed understanding of the cDNA clones was achieved by treating wheat with various chemical elicitors playing a crucial role in the plant's defence mechanism. These include H_2O_2, menadione, MeSA and MeJA (Neill *et al*., 2002; Borges *et al*., 2003; Wang and Wu, 2005). Application of H_2O_2 and menadione to the plants will mimic the effect of oxidative stress, while the application of MeSA and MeJA will reveal whether the encoded proteins are involved in either salicylic acid and/or jasmonic acid mediated defence responses.

Since *LRW268*, a novel gene induced at an early stage after wheat was infected with *P. striiformis*, could play a role in the recognition of the pathogen or form part of the defence response, an attempt was made to obtain the full length gene. With the RACE a few complications were incurred. Of the 20 5'-RACE clones and 21 3'-RACE clones that were isolated, 6 clones of each were sequenced. The 3'-RACE clones when sequenced, were identical to the *LRW268* sequence, leading to the assumption that the *LRW268* sequence represented the 3'-end of the *LRW268* gene. The 5'-RACE clones however did not show similarity to the original *LRW268* sequence. Even though only 6 of the 20 clones were sequenced, neither of the sequences showed any similarity with the original *LRW268* sequence nor did it show similarity with each other. It was therefore decided to use ESTs to construct the 5'-end of the gene. The resulting contig yielded a 96 amino acid polypeptide. This showed similarity with an *O. sativa* hypothetical protein (Accession number: NP_001052832, e-value: $3e^{-41}$) as well as SLL1 proteins (Table 4.4). *SLL1* is *S* locus linked and is an anther specific gene

(Yu *et al*., 1996). The *S* locus is involved in self-incompatibility which involves two genes, the *S*-locus glycoprotein (SLG) and the *S* receptor kinase (SRK) (Stein *et al*., 1991; Cui *et al*., 1999).

Plant receptor-like protein kinases (RLKs) such as SRK, are involved in cell signalling and is ligand specific (Becraft, 2002). The RLKs span the plasma membrane and is able to detect and bind extracellular ligands (Haffani *et al*., 2004). This leads to autophosphorylation of the RLK after the RLK has dimerized (Torii, 2000). SLL1 is assumed to function as an intermediate between the RLK and a MAPK cascade (Yu *et al*., 1996). Activation of an RLK triggers the initiation of a protein phosphorylation cascade leading to changes in gene expression and other cellular functions (Becraft, 2002). Since the LRW268 showed such good similarity with SLL1, it could indicate that LRW268 could function as a RLK interacting protein. The presence of several binding and phosphorylation sites (Table 4.5) further strengthened the hypothesis that LRW268 could be involved in cell signalling. The fact that a MAPK docking site was found, also supports the view that the encoded protein could link the signals generated by an RLK to a MAPK signalling cascade.

Further analysis into the function of *LRW268* was done with RT-PCR. This showed that *LRW268* gene expression was differentially regulated in *P. striiformis* (Fig 4.2a) infected wheat showing a more pronounced response in the infected resistant plants at 24 hpi (Fig 4.2b). The infected susceptible plants had a more moderate expression level of *LRW268*. This showed a definite involvement of *LRW268* in *P. striiformis* infected Avoset-*Yr*1 resistant plants. Thatcher+*Lr*34 seedlings treated with chemicals indicated that *LRW268* expression is regulated by both the JA pathway and oxidative stress (Fig 4.6b and 4.6d). This was an indication that the gene forms part of a general defence response of wheat upon infection. This was further strengthened by the fact that *LRW268* occurred in all the Thatcher and Thatcher+*Lr* lines as well as in Avoset-S and Avoset-*Yr*1. Thus, it is assumed that LRW268 could play a role in the general defence signalling of wheat upon infection, most probably as a signalling partner for a general defence related RLK. Several general defence related RLKs have already been identified in plants. These include SFR2, BRI1, ELIP, LRPKm1 and

PR5K (Wang *et al*., 1996; Li and Chory, 1997; Pastuglia *et al*., 1997; Komjanc *et al*., 1999; Takemoto *et al*., 2000). In a number of cases, such interacting proteins were already indentified (Martin *et al*., 1993). Their precise roles are still unclear, but they are definitely needed for efficient signalling. Future research would aim to confirm the involvement of LRW268 with a RLK as part of the activated defence response in wheat.

To further understand *LRW222*, a longer cDNA sequence was required. A BLAST search yielded a contig with a 270 bp ORF which encoded a 89 amino acid polypeptide. This SSH cDNA clone had a putitive BowB conserved domain present. BowB domains are present in the Bowman-Birk type serine proteinase inhibitor family (Habib and Khalid, 2007). These PIs have been found to play a role in the plant's defence against insects and pathogenic microorganisms (Lorito *et al*., 1994; Dunaevsky *et al*., 2005; Ragg *et al*., 2006). Lorito *et al*. (1994) showed inhibition of spore germination of *Botrytis cinerea* and germ tube elongation of *Fusarium solani* by serine PIs. Transgenic *Saccharum offıcinarum* constitutively expressing BBIs retarded larvae growth of *Diatraea saccharalis* (Falco and Silva-Filho, 2003).

Analysis of *LRW222* showed a definite opposite expression profile in wheat that were infected with *P. triticina* and *P. striiformis*. The *P. striiformis* infected plants showed a strong but constitutive expression of *LRW222*. However, the *P. triticina* infected plants showed a strong induction in both the infected resistant and susceptible plants (Fig 4.2a). From the expression profiles, it can be assumed that *LRW222* is strongly linked to the establishment of resistance, since its expression is induced early after infection and then switched off at 24 hpi when the resistance response is presumably established. In contrast, in the susceptible wheat where no resistance can be established, the expression of the gene is induced for the whole duration of the study, indicating a valiant but futile attempt of the plant to overcome the infection. Induced expression after H_2O_2 and menadione treatment was observed signifying that the gene is regulated by oxidative stress (Fig 4.6b and e). MeJA treated plants showed an induction of *LRW222* expression signifying that *LRW222* plays a putative role in the JA mediated resistance. Since LRW222 shows a high

similarity to PIs the induced expression profile in MeJA treated plants can be explained by the fact that PIs are considered wound-induced proteins. Zhao *et al*. (1996) found that wounding or treatment with MeJA induced similar expression profiles of PIs. Similar to *LRW268*, *LRW222* amplification occurred in all the Thatcher and Thatcher+*Lr* lines as well as in Avoset-S and Avoset-*Yr*1, again indicating that LRW222 forms part of the general defence response. The presence of a BowB domain in LRW222 signifies that it is a PI and PIs have been found in previous studies to inhibit proteases from infecting pathogens (Eckelkamp *et al*., 1993; Lorito *et al*. 1994; Falco and Silva-Filho, 2003) implicating a role for *LRW222* in the plant defence response. The presence of a MAPK docking motif in the polypeptide however poses a very interesting question! Is LRW268 a general PI that targets the pathogen itself after being exported outside the cell or does it function within the cell as part of the signal transduction machinery? Or does it have a dual function? Research into the location of the enzyme would help solve this question.

Future research will focus on both the encoded proteins to both characterise them and finally propose a role for each during the wheat/*P. triticina* interaction.

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Chapter 5

The effect of light on plant defence of wheat infected with *P. triticina*

5.1. Introduction

The defence response of a plant is extremely complex. Different pathways play a role in the regulation of the defence response utilising signalling molecules like SA, JA and ET (Feys and Parker, 2000). Upon initiation of the defence response, plants activate various components of the defence response. Included are amongst others ROS production (Mittler, 2002) and PR protein synthesis (Van Loon *et al*., 2006). The typical response during an incompatible plant-pathogen interaction is localized cell death which forms part of the hypersensitive response (Heath, 2000) with a subsequenct activation of SAR (Van Loon, 2000). During the compatible interaction, the pathogen overruns the plant due to an insufficient or inadequate defence response.

Photosynthesis occurring in the chloroplasts provides plants with energy rich components by capturing the radiation energy of light into organic molecules, using carbon dioxide and water (Malkin and Niyogi, 2000). These energy rich compounds are used to drive normal growth and metabolic processes. Light energy absorbed by the photosynthetic pigments is thus converted to stored energy (Whitmarsh and Govindjee, 1999).

Photosynthesis consists of two phases, the light-dependent and the light-independent reactions. The light-dependent phase requires photosystem I (PSI) and photosystem II (PSII) located in the thylakoid membrane of the chloroplast. Light (optimal wavelength of 680 nm) excites an electron of PSII which is accepted by pheophytin which passes into an electron transport system (Mauseth, 1995). The electron is then transferred via a series of molecules which act as electron acceptors to PSI. In the process, protons are pumped across the thylakoid membrane into the lumen which is then used to drive photophosphorylation (ATP production). Light (optimal wavelength of 700 nm) also excites PSI to release another electron which is now sent down another electron transport chain where it is ultimately used to reduce $NADP^+$ to NADPH.

The light-independent reactions, occuring in the chloroplast stroma, utilize the products from the light-dependent reactions (ATP and NADPH) to drive the synthesis of organic sugars using the Calvin-Benson cycle, where the key enzyme RuBisCO is involved.

The plant's photosynthetic capacity, the ability to photosynthesise optimally, is compromised upon pathogen infection and environmental stress (Grobbelaar and Mohn, 2002; Ribeiro *et al*., 2003; Thomson *et al*., 2003; Bonfig *et al*., 2006; Berger *et al*., 2007). Grobbelaar and Mohn (2002) observed that ozone stress affected photosynthesis in *Helianthus annuus*. The resistant and susceptible plants did however react differently to the ozone stress. Treated resistant plants were able to repair the plastoquinone molecule (Q_A) which was impaired due to the ozone stress. Q_A is an electron acceptor of PSII in the electron transport chain. The susceptible plants did not show the repair of Q_A. The resistant cultivar also showed higher levels of electron transport and photochemical quenching. This demonstrated that the resistant cultivar was able to withstand strenuous conditions better compared to the susceptible cultivar.

Nitrogen-deficient plants also showed greater susceptibility to photoinhibition, as well as a decrease in quantum yield of PSII (Lu and Zhang, 2000). The portion of open PSII reaction centres were also reduced in nitrogen-deficient plants and the plants were therefore unable to proceed with optimum photosynthesis causing a decrease in electron transport leading to a decline of ATP and NADPH levels.

Recent research have shown that the photosynthetic pathway plays an indirect role in plant-pathogen interactions since pathogen infection affects the photosynthetic capacity of the plant (Ribeiro *et al*., 2003; Zeier *et al*., 2004; Bonfig *et al*., 2006; Berger *et al*., 2007). When *Arabidopsis* plants were infected with *P. syringae*, the quantum yield which is an indication of the efficiency of PSII, declined after 3 h (Bonfig *et al*., 2006). The effects of the infection on photosynthesis were more prominent after 24 hpi. Ribeiro *et al*. (2003) showed that infection of *Citrus sinensis* plants with *Xylella fastidiosa* caused a lower level of open PSII reaction centres (q_P)

compared to the uninfected plants leading to decreased photosynthesis. The non-photochemical fluorescence quenching (NPQ) showed an increase in infected plants indicating that more energy was dispersed as heat.

In addition to affecting photosynthesis, pathogens or stress also targets either the photosynthetic machinery (Botha *et al*., 2006) or individual components thereof (Van der Westhuizen and Botha, 1993). Chloroplasts were assumed to be targeted by enzymes present in the saliva of Russian wheat aphid during infestation (Botha *et al*., 2006). A key component of photosynthesis, RuBisCO, is also targeted where the large subunit was degraded in the susceptible plants (Van der Westhuizen and Botha, 1993). Plants react to this by elevated expression of genes coding for proteins involved in metabolic processes and photosynthesis (Neu *et al*., 2003; Lu *et al.*, 2004).

Light has been shown to regulate SA levels (Zeier *et al*., 2004). Chandra-Shekara *et al*. (2006) showed that the HR occurring in resistant *Arabidopsis* when infected with Turnip crinkle virus is light dependent. The absence of light caused reduced levels of SA glucoside thereby resulting in susceptibility.

Combined, the results implicate light and photosynthesis to be crucial members of the general plant defence response. Since a large number of cDNA clones which were isolated with SSH during this study showed similarity to photosynthetic genes (Chapter 3), it was crucial to determine the roles of light and photosynthesis on wheat's disease resistance mechanism after *P. triticina* infection. The hypothesis that light is essential for an efficient defence response in wheat infected with *P. triticina* will be tested.

5.2. Materials and methods

5.2.1. Biological Material

5.2.1.1. Fungal pathogen

Plants were inoculated with *Puccinia triticina* pathotype UVPrt9.

5.2.1.2. Cultivation of wheat

Seeds from the susceptible Thatcher and resistant Thatcher+*Lr*34 wheat cultivars were planted and grown as described (3.2.1.2).

5.2.1.3. Infection of wheat with *P. triticina*

Adult Thatcher and Thatcher+*Lr*34 plants were sprayed with freshly harvested *P. triticina* spores (0.6 mg spores.ml^{-1}) that were multiplied on the susceptible Karee cultivar. The spores were resuspended in distilled water containing a drop of Tween™ 20. Control plants where mock infected with water. 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. After the plants were dried, half the plants of each treatment were transferred to the greenhouse. The remaining plants were placed in a dark cabinet at ±24°C for another 24 h. Afterwards, these plants were also moved to the greenhouse. Time 0 h harvesting and fluorescence measurements were performed prior to placing the plants in the dew-simulation chamber. Flag leaves from adult plants were harvested at 12 h intervals for 120 h. The plant material was quick frozen using liquid nitrogen and stored at -80°C. The frozen tissue was ground to a fine powder in liquid nitrogen. Leaves were collected and photographed one week after inoculation to determine the efficiency of the infection.

5.2.2. Methods

5.2.2.1. Chlorophyll a fluorescence

Chlorophyll a fluorescence measurements were taken at 24 h intervals from 0 h just before inoculation until 168 h when pustules were visible on the infected susceptible plants. Three flag leaves of plants of all treatments were selected for chlorophyll fluorescence measurements using a FMS-2 fluorometer from Hansatech, UK. Measurements were taken daily at noon until pustules were visible on the leaves. When measurements were taken, the leaf areas were dark adapted for 5 min. The ground state fluorescence (F_0) was first measured, whereafter a single saturating light flash was applied for 0.7 sec. Maximum fluorescence of a dark adapted sample (F_m) was then measured. After 20 sec, the actinic light source (70 µmol.m^{-2}.s^{-1}) of the fluorometer was switched on, followed by a series of saturating flashes where minimum (F_s) and maximum steady state fluorescence ($F_{m'}$) were measured. From these measurements, the maximum quantum yield of PSII (F_v/F_m = $\frac{F_m - F_0}{F_m}$), the quantum yield of PSII (Φ_{PSII} = $\frac{F_{m'} - F_s}{F_{m'}}$), the proportion of open PSII reaction centres (q_P = $\frac{F_{m'} - F_s}{F_{m'} - F_0}$) and non-photochemical quenching (NPQ = $\frac{F_m - F_{m'}}{F_{m'}}$) were calculated (Maxwell and Johnson, 2000).

5.2.2.2. Fluorescense microscopy

Three flag leaves were harvested from control light and 40 h dark treated infected Thatcher and Thatcher+*Lr*34 plants at 48 hpi. The leaves were fixed in a mixture of ethanol/dichloromethane (3:1) containing 0.15% (v/v) trichloroacetic acid. 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 until examined using an Olympus AX70 microscope. Different infection structures were counted for each treatment and the average number of each infection structure expressed relative to the total number of counted structures for that treatment.

5.2.2.3. Expression analysis of photosynthetic and defence related genes

All apparatus and solutions that were used for RNA extraction were treated as described (3.2.2.1). Total RNA was isolated from leaf tissue as described (3.2.2.1) and mRNA purified (3.2.2.2).

Primers were designed for the various genes using Web Primer (http://seq.yeastgenome.org/cgi-bin/web-primer). All the isolated RNA samples were converted to cDNA using 200 ng total RNA and 25 µM Bovis 32 (Table 4.1) and the ImProm-II Reverse Transcription System (Promega) according to the manufacturer's specifications. Each subsequent PCR reaction consisted of 1 µl cDNA, 25 pmol of each primer and 1X Kapa*Taq* ReadyMix (Kapa Biosystems). A control amplification of *18S rRNA* using Bovis 26 and Bovis 27 (Table 4.1) was also performed for each treatment.

The amplification regime was as follows: 94°C for two min, 30 cycles of 94°C for 30 sec, gene specific annealing temperature (Table 5.1) for 30 sec and 72°C for two min followed by a final cycle of 72°C for 10 min. The amplified fragments were separated on a 1.5% (w/v) agarose gel (3.2.2.1).

Table 5.1. Nucleotide sequences of primers used in this study (N = A or T or G or C and V = A or G or C).

Name	***Primers***	***Sequence***	***Annealing Temperature***
RuBisCO Small Subunit *(RbcL)*	Bovis 229	5' - TAC TGG ACA ATG TGG AAG CTG - 3'	61°C
	Bovis 230	5' - GCA ATG AAG CTG ACG CAC T - 3'	
RuBisCO Large Subunit *(RbcS)*	Bovis 231	5' - TGC AGT AGC TGC CGA ATC TT - 3'	61°C
	Bovis 232	5' - TTT CTT CAC ATG TAC CCG CA - 3'	
psbA	Bovis 289	5' - ATT CCA GGC AGA GCA CAA CAT CCT - 3'	65.2°C
	Bovis 290	5' - TAC TAC AGG CCA AGC AGC CAA GAA - 3'	
psbQ	Bovis 214	5' - TCA AAG CTT GAG ATG GCG CAA ACC - 3'	65.3 °C
	Bovis 215	5' - ACG GCC ATT GCT TCT TGT CGA TGA - 3'	
Phenylalanine ammonia-lyase *(PAL)*	Bovis 210	5' - TGT CCT ACA TTG CCG GCC TTA TCA - 3'	65.3°C
	Bovis 211	5' - CGC TCA ATC GAC TTG GTG GCA AAT - 3'	
Glutathione S-transferase *(GST)*	Bovis 212	5' - AAG TAT GCG TGC CGC AAG AAC AAG - 3'	65.3°C
	Bovis 213	5' - AGA ACA CAC ACT CGT ACA GGC ACA - 3'	
β-1,3-glucanase *(PR2)*	Bovis 223	5' - TCC ACG GCG GTC AAG ATG A - 3'	61°C
	Bovis 224	5' - GGT TCT CGT TGA ACA TGG C - 3'	
Thaumatin-like protein *(PR5)*	Bovis 227	5' - TTT CTC TGG TGG TGC TCG T - 3'	61°C
	Bovis 228	5' - GAT CAT GGC CGT CGT GAA - 3'	
Peroxidase *(PR9)*	Bovis 208	5' - GCA GCT GAG CCT GAT CTG - 3'	55.4°C
	Bovis 209	5' - ATC AGA CCG TCT CCT GCG - 3'	
18s rRNA	Bovis 26	5' - CAA CTT TCG ATG GTA GGA TAG - 3'	58°C
	Bovis 27	5' - CTC GTT AAG GGA TTT AGA TTG - 3'	

5.3. Results

5.3.1. Chlorophyll a fluorescence analysis

Since the flag leaves were harvested before any infection signs were visible, some plants of all the treatments were left in the greenhouse until infection was evident. One week after inoculation, the susceptible, inoculated Thatcher plants showed brown fungal pustules on the leaf surface (Fig 5.1). The inoculated Thatcher+*Lr*34 showed localized lesions where infection was terminated. These necrotic lesions represented cells that were killed as part of the HR of the activated plant defence response. These results confirmed the effective inoculation of wheat with the leaf rust pathogen. No visible differences were evident between the control light treatment (Fig 5.1a) and the dark treated plants (Fig 5.1b).

The photosynthetic measurements of plants were taken daily at noon. F_v/F_m values are an indication of the maximum photosynthetic potential of the plant (Maxwell and Johnson, 2000). All treatments showed a gradual decrease in F_v/F_m over time (Fig 5.2a), with the dark incubated plants showing overall lower F_v/F_m values compared to the control light treated plants. All treatments showed a decrease in the F_v/F_m values at 48 hpi with the value for the dark treatment being the lowest. Uninfected plants generally had a higher F_v/F_m value compared to the infected plants. Dark incubated plants showed a bigger difference between the resistant and susceptible cultivars. During the control light treatment, the F_v/F_m values at 168 hpi did not vary much between the infected resistant and infected susceptible plants. A clear difference was however evident in the dark treated plants where the infected resistant plants (DIR) recovered much better after the 40 h dark incubation compared to the infected susceptible plants (DIS). The difference between the DIS and DIR plants at 168 hpi was nearly twice that found in the control treated plants. It is clear that infected resistant plants outperformed infected susceptible plants in both treatments.

Φ_{PSII} measures the proportion of light absorbed by chlorophyll associated with PSII

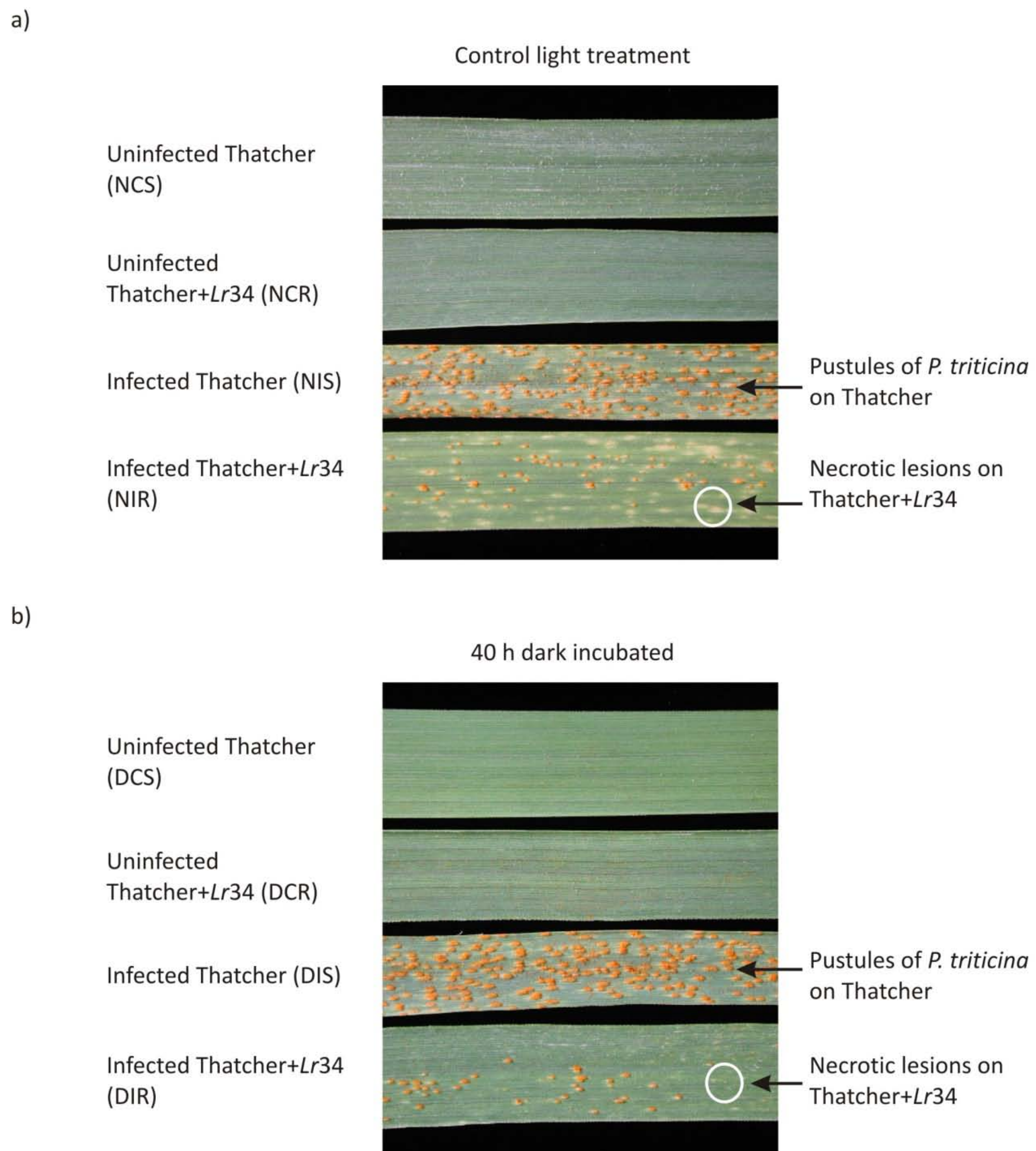

Figure 5.1. *P. triticina* infected and uninfected Thatcher and Thatcher+*Lr*34. In a) plants were placed under normal light conditions and in b) incubated for 40 h in the dark.

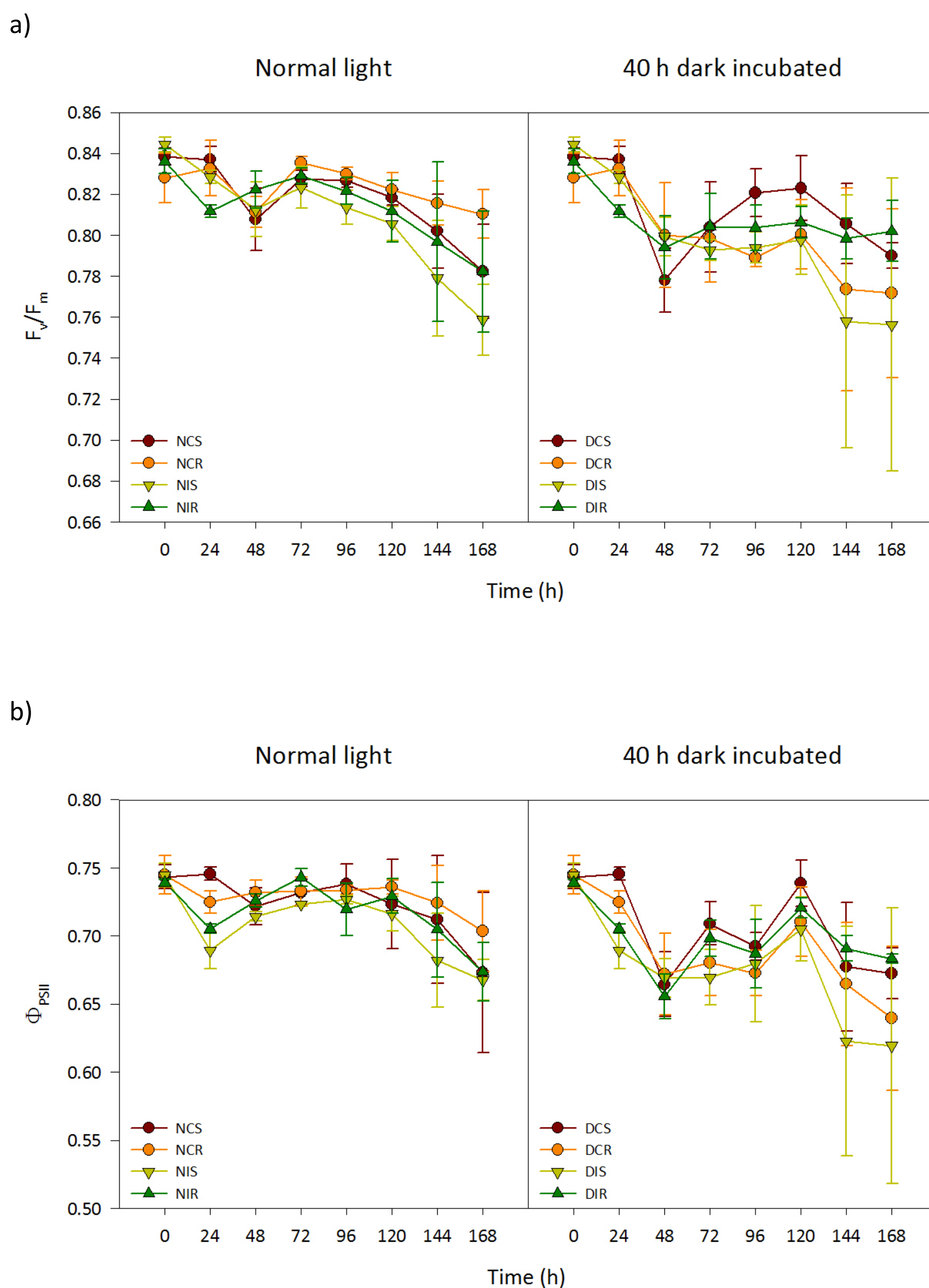

Figure 5.2. The influence of dark incubation on the photosynthetic capacity of wheat (1). In a) the maximum quantum yield and b) quantum yield at steady state for all treatments is shown. The time intervals are as indicated. Note the expanded scale of the Y-axis. Abbreviations: NCS control uninfected susceptible; NCR control uninfected resistant; NIS control infected susceptible; NIR control infected resistant; DCS dark uninfected susceptible; DCR dark uninfected resistant; DIS dark infected susceptible and DIR dark infected resistant.

that is used in photochemistry (Maxwell and Johnson, 2000). A general decrease of Φ_{PSII} occurred in plants of all treatments over time with higher values in the control treated plants compared to the 40 h dark incubated plants (Fig 5.2b). Infection caused an immediate drop in Φ_{PSII} at 24 hpi in both infected resistant and infected susceptible plants as well as in the control treatment with both plants showing a transient increase thereafter with a subsequent decrease. In the dark treatment, all plants had a much stronger decrease at 48 hpi compared to the control treatment with the infected resistant plants again showing a stronger recovery at 168 hpi. The infected susceptible plants in contrast resulted in a substantial decrease in PSII quantum yield.

The q_P gives an indication of the proportion of PSII reaction centres that are open (Maxwell and Johnson, 2000). The q_P values were higher in control plants for both cultivars for both treatments (Fig 5.3a), with infected plants generally showing lower q_P levels. As with the F_v/F_m and Φ_{PSII} values, an initial decrease in q_P values was evident in both treatments with the dark incubation having a bigger effect. This decline in the control treatment was only in the infected plants with the uninfected plants remaining unchanged. Dark incubation did however lead to lower levels of open reaction centres at 48 hpi in all treatments with a subsequent recovery thereafter. This indicated that while infection did lead to lower q_P values, the dark treatment was even more severe. Control susceptible plants resulted in a dramatic transient decrease at 144 hpi, but this was not mirrored in any of the other treatments.

NPQ measures changes in heat dissipation (Maxwell and Johnson, 2000). NPQ was lower in all normal light treated plants compared to the dark treated plants (Fig 5.3b). In addition, while the general trend of the light treated plants was constant, the dark treated plants resulted in increased heat dissipation during the latter part of the time trial. Both the NIS and NCS plants had an increase in NPQ at 24 hpi which decreased to normal levels later on. All treatments in the dark incubated plants had a drop at 120 hpi with a subsequent increase thereafter. An increase in the NPQ value of the DCS plants at 144 hpi mirrored the sharp decrease in q_P value evident in

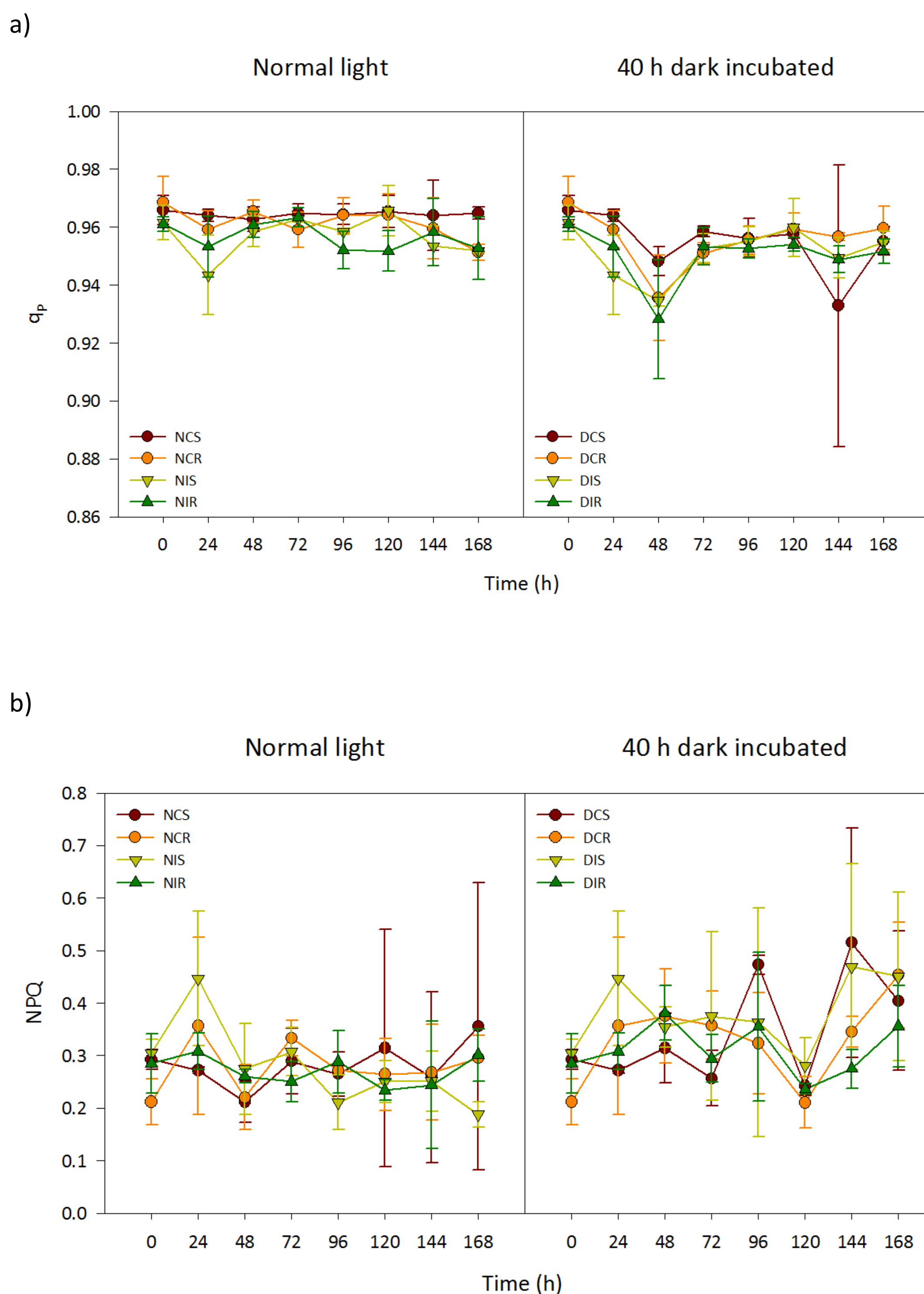

Figure 5.3. The influence of dark on the photosynthetic capacity of wheat (2). In a) photochemical and b) non-photochemical fluorescence quenching of all treatments are shown. The time intervals are as indicated. Note the expanded scale of the Y-axis. Abbreviations: NCS control uninfected susceptible; NCR control uninfected resistant; NIS control infected susceptible; NIR control infected resistant; DCS dark uninfected susceptible; DCR dark uninfected resistant; DIS dark infected susceptible and DIR dark infected resistant.

figure 5.3a.

5.3.2. Histology

To determine whether light deprivation would have an effect on the infection efficiency of *P. triticina* on Thatcher and Thatcher+*Lr*34 cultivars, flag leaves harvested at 48 hpi from normal light and 40 h dark treated plants were examined microscopically (Fig 5.4). Examples of aborted spores (Fig 5.4a), non-penetrating spores (Fig 5.4b) and small colonies (Fig 5.4c) are given in the respective pictures.

On NIS plants, 37% (± 8%) of all counted structures were small colonies with less than 6 haustorium mother cells (HMCs), 44% (± 7%) were germinated spores that showed aborted growth and 19% (± 2%) were germinated spores that did not penetrate the leaf (Fig 5.5). In contrast, on leaves of NIR plants, only 15% (± 5%) of spores developed into small colonies with less than 6 HMCs, while 68% (± 1%) showed aborted growth and 17% (± 3%) did not penetrate the leaf. These results are characteristic of resistant and susceptible plants after infection with *P. triticina*.

In the DIS plants, 27% (± 14%) of all structures were small colonies with less than 6 HMCs, 55% (± 13%) were germinated spores that showed aborted growth and 18% (± 7%) of the germinated spores did not penetrate the leaf (Fig 5.5). On leaves of DIR plants, only 20% (± 8%) of spores developed into small colonies with less than 6 HMCs, while 62% (± 13%) showed aborted growth and 18% (± 12%) did not penetrate the leaf.

5.3.3. Expression analysis

To elucidate the importance of light for the activation of defence and photosynthesis related gene expression, plant material was harvested at 12 h intervals from both inoculated and uninoculated plants that were placed under both control light and 40 h dark conditions.

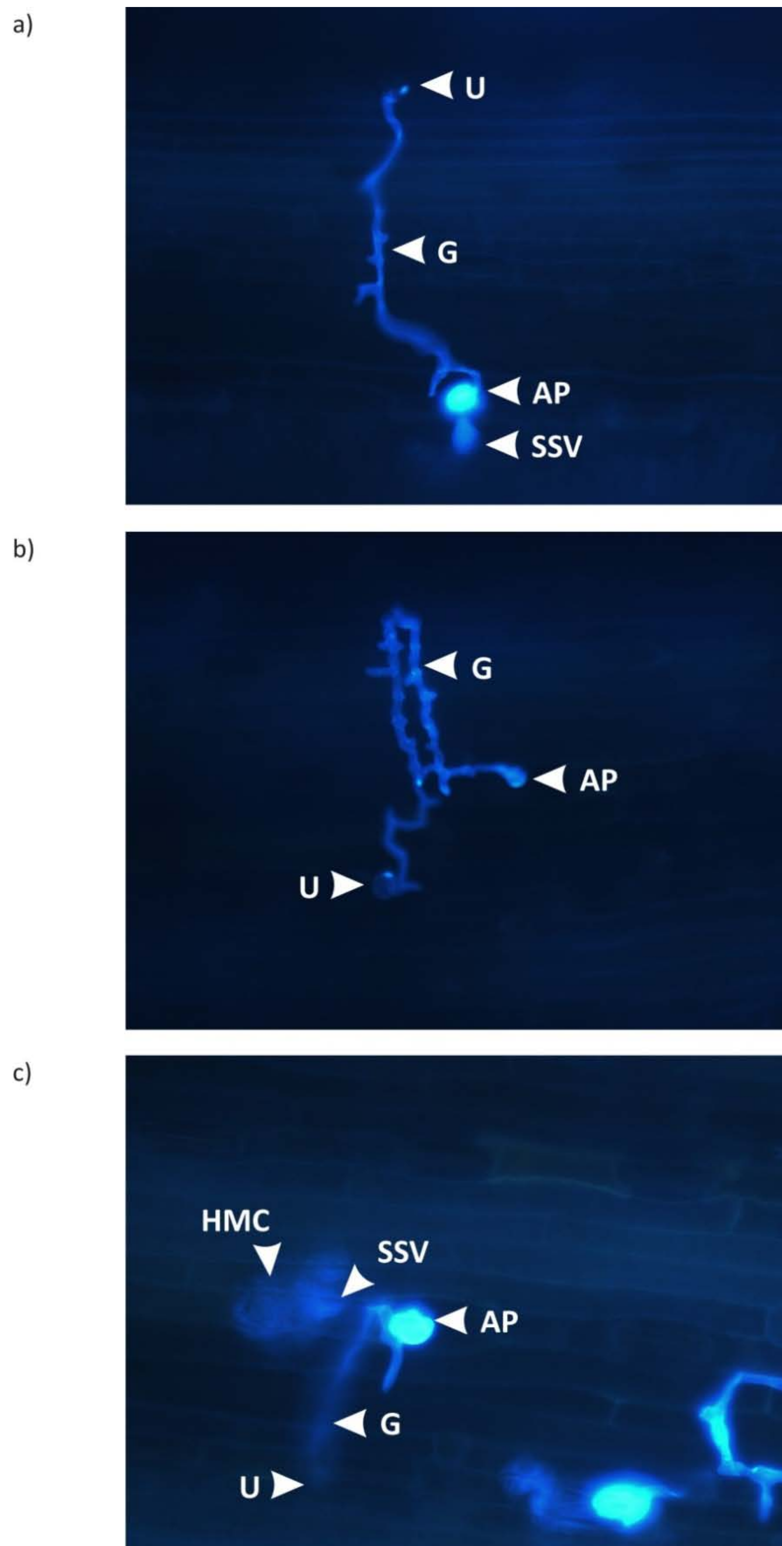

Figure 5.4. Histological analysis of *P. triticina* infection of wheat. Fluorescence microscopy images of a) an aborted substomatal vesicle, b) a non-penetrating appressorium and c) an infection site exhibiting haustorium mother cells of pathotype UVPrt9 of *P. triticina* in flag leaves 48 hpi is shown. Abbreviations: AP appressorium; G germtube; HMC haustorium mother cells; SSV substomatal vesicle and U urediospore.

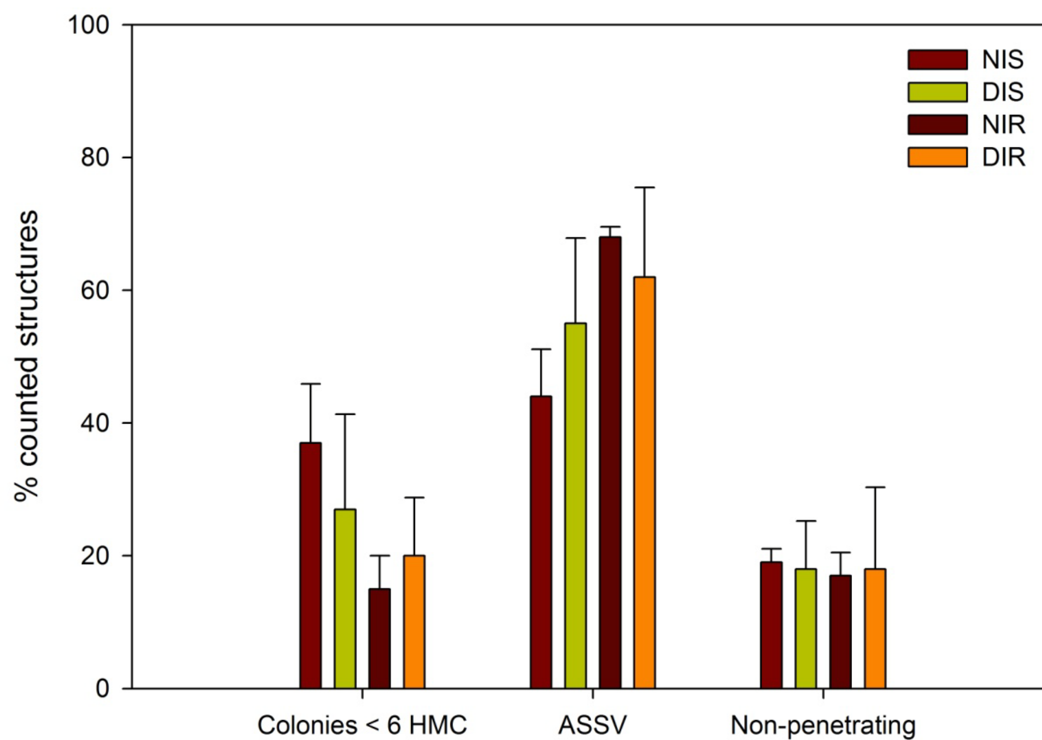

Figure 5.5. The effect of light on the formation of infection structures of *P. triticina* in wheat. Error bars indicate the standard deviation and n=3. Abbreviations: HMC haustorium mother cells and ASSV aborted substomatal vesicle. Abbreviations: NCS control uninfected susceptible; NCR control uninfected resistant; NIS control infected susceptible; NIR control infected resistant; DCS dark uninfected susceptible; DCR dark uninfected resistant; DIS dark infected susceptible and DIR dark infected resistant.

For all treatments, most of the photosynthesis related genes were constitutively expressed with the exception of *psbQ* which showed a decrease in expression at 36 hpi (Fig 5.6 and 5.7). This decrease in *psbQ* expression correlated with a harvesting time at 00:00 at night. This decrease occurred for the control light treated plants as part of the normal light regime, while for the dark treated plants, it occurred during the latter part of their 40 h dark incubation.

To determine whether the saturation of the RT-PCR reactions could have masked any small changes in the expression of these genes, a control amplification of three genes using $\frac{1}{50}$ of the initial template volume was done (Fig 5.8). Despite the dilution, the expression levels still indicated the constitutive expression of these genes, confirming the original results.

In addition to the photosynthetic genes, the expression of several defence related genes was tested. The expression of *Pr2* in NIS plants had a steady increase from 48 hpi until the end (Fig 5.6). NIR plants in contrast showed an increased expression which peaked between 48 hpi and 72 hpi with a decrease after that. DIS plants had a slightly delayed increase in expression when compared to the NIS plants (Fig 5.7). DIR plants exhibited a similar expression profile as DIS plants with an increase at 48 hpi that remained high for the duration of the study. This clearly differed from the expression of the NIR plants not exposed to the dark treatment (Fig 5.7).

Pr5 expression in the NIS plants rapidly increased, peaking at 24 hpi whereafter a decrease was observed (Fig 5.6). A similar expression pattern with minor variations was observed in the NIR plant. An interesting difference was observed in the dark treated plants. While the DIS plants had a similar profile to the NIS plants, the expression levels of *Pr5* after 48 hpi were much lower in the DIS plants (Fig 5.7). In contrast, a much stronger expression level was observed in the DIR plants for the duration of the study compared to the NIR plants. A similar expression pattern was observed for *Pr9* gene expression. To summarize, all three the *PR* gene's DIS expression showed a similar pattern as NIS albeit that *Pr5* was at a lower level (Fig

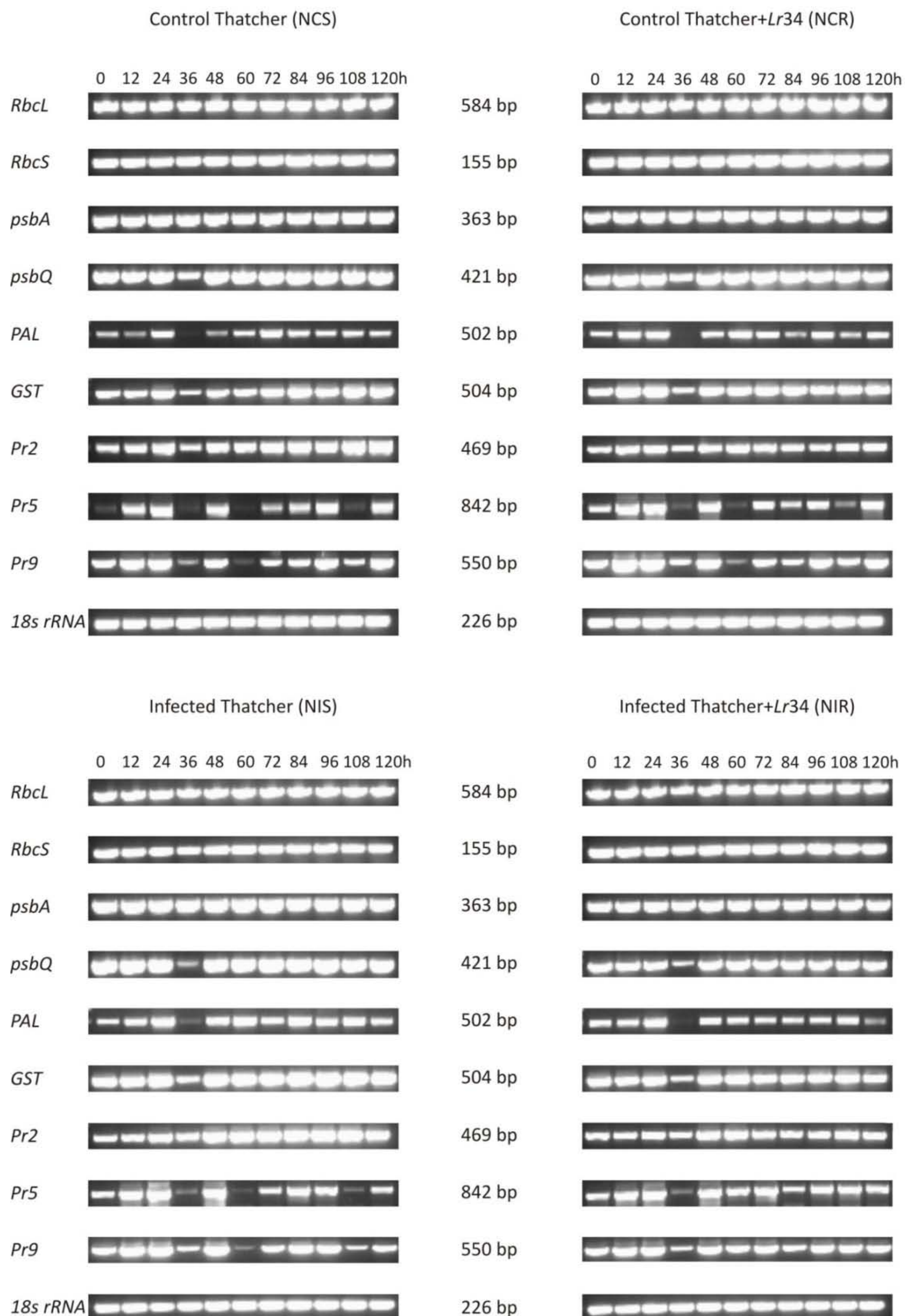

Figure 5.6. Expression of various photosynthetic and defence related genes in plants that were placed under control light conditions. Gene names, fragment sizes and time intervals are as indicated. Abbreviations: NCS control uninfected susceptible; NCR control uninfected resistant; NIS control infected susceptible; NIR control infected resistant; DCS dark uninfected susceptible; DCR dark uninfected resistant; DIS dark infected susceptible and DIR dark infected resistant.

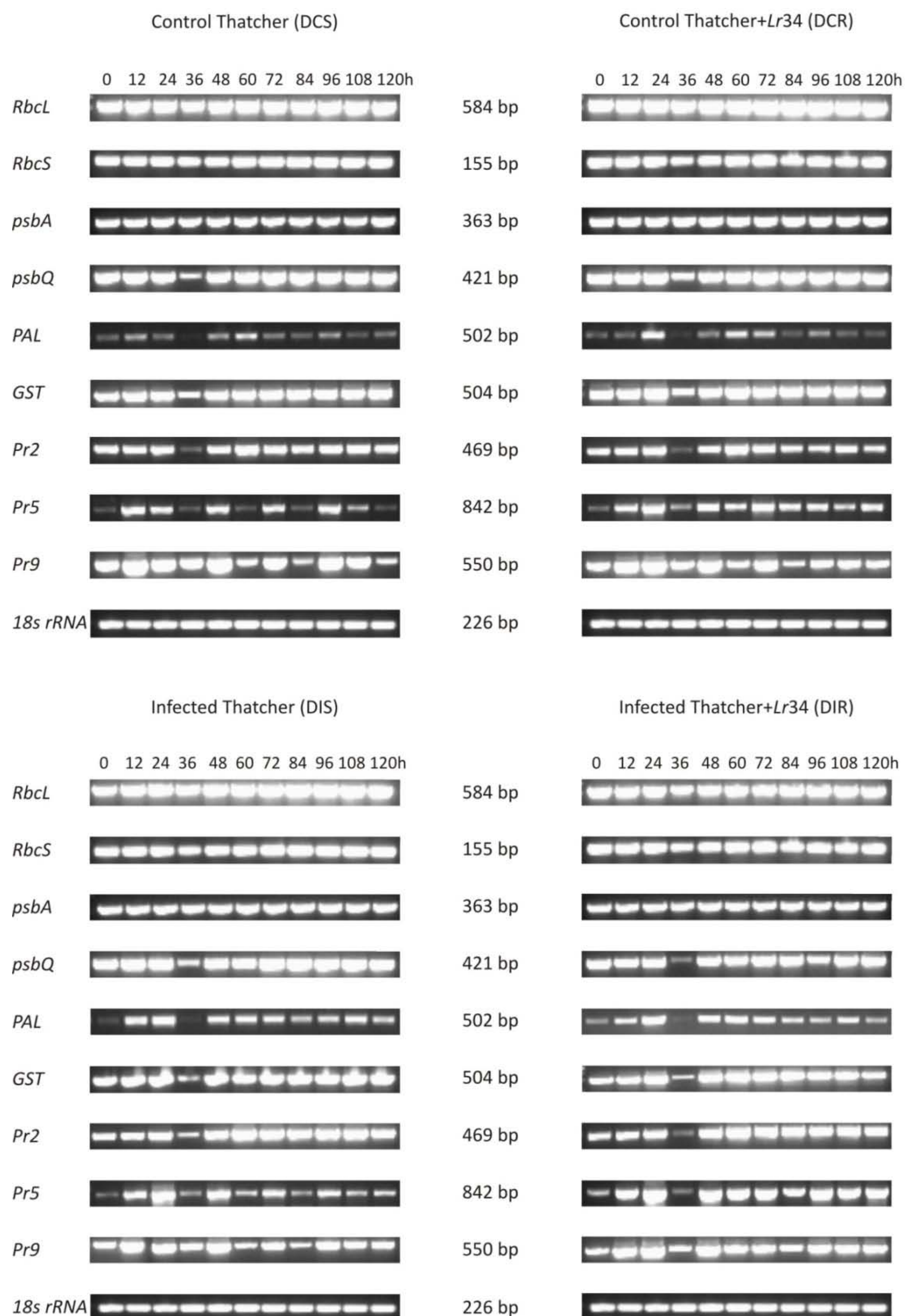

Figure 5.7. Expression of various photosynthetic and defence related genes in plants that were placed under 40 h dark conditions. The names and sizes of the amplified genes are as indicated. Abbreviations: NCS control uninfected susceptible; NCR control uninfected resistant; NIS control infected susceptible; NIR control infected resistant; DCS dark uninfected susceptible; DCR dark uninfected resistant; DIS dark infected susceptible and DIR dark infected resistant.

Figure 5.8. Expression of three selected genes after 50X dilution of the cDNA template. Time intervals are as indicated.

5.7). The DIR plants expressed *Pr5* at a higher level, but the pattern was similar to NIR.

PAL expression in NIS plants showed a strong induction which proceeded throughout the duration of the study (Fig 5.6). NIR plants also showed a strong induction in expression that decreased after 48 hpi. A strong induced expression was observed for *PAL* in DIS and DIR plants (Fig 5.7) with a subsequent decrease later. The expression of *GST* in NIS, DIS and NIR plants was constitutive (Fig 5.6 and 5.7) with an induced expression in the DIR plants.

The expression of *Pr2* in the NCS and NCR plants were similar to the NIS and NIR plants respectively (Fig 5.6), whereas the expression profiles of DCR and DCS remained more or less unchanged (Fig 5.7). In contrast, *Pr5* and *Pr9* expression in the uninfected plants had a similar expression profile as their infected counterparts for both the light control and dark treatments.

For the expression of *PAL* in the uninfected plants, normal light treated plants resulted in a similar pattern as the infected plants (Fig 5.6). The DCR plants had an induced *PAL* expression pattern, however the expression levels were much lower than the rest (Fig 5.7). The DCS plants had a delayed induction of *PAL* expression, but the levels were low. *GST* expression levels for NCS, NCR, DCS and DCR plants were induced.

5.4. Discussion

Chlorophyll fluorescence is a non-destructive method providing the opportunity to measure the changes in photosynthetic performance of a plant (Maxwell and Johnson, 2000). In this study, two different stresses were simultaneously applied to wheat to analyse the changes in photosynthesis. The first challenge was in the form of a pathogen, *P. triticina*, that was used to infect the plants. Secondly, infected plants were further dark treated to evaluate how this additional stressor affected them.

A decrease in photochemical quenching and an increase in NPQ were observed when *Arabidopsis* plants were infected with *P. syringae* (Bonfig *et al*., 2006; Berger *et al*., 2007). The application of 12-oxo-phytodienoic acid has similar affects as pathogen infection but the response appears faster than the *P. syringae* infected plants (Berger *et al*., 2007). In this current study, all treatments resulted in a decrease in photosynthetic capacity over time (Fig 5.2a). This could either be due to the age of the plants since adult plants were used or due to photoinhibition (Schreiber and Neubauer, 1990). A difference in F_v/F_m and Φ_{PSII} was observed between the resistant and susceptible plants with the resistant plants exhibiting higher quantum efficiency (Fig 5.2). All plants incubated in the dark resulted in decreased photosynthetic capacity with the infected resistant plants being able to recover better from the added stress compared to the infected susceptible plants. This additional dark stress the plants incurred showed the importance of light in the activation of an effective plant defence response.

This stronger response was however not due to the number of open PSII reaction centres as was measured with q_P (Maxwell and Johnson, 2000). While a higher q_P was visible for the uninfected plants signifying that more fluorescence was quenched in the primary photochemical reactions (Fig 5.3a), infection of both resistant and susceptible plants caused a decrease in q_P levels which was exacerbated by the dark. NIS plants had a stronger and faster decrease in q_P due to infection while the DIS plants, due to the added dark stress, had lower q_P values during the earlier stages.

Both NIS and DIS plants did however manage to recover at the later stages showing a similar pattern compared to the resistant plants. NIR plants did not have such a big drop during the earlier stages. These levels did however recover, indicating that the lower quantum efficiencies (Fig 5.2) were not due to closed reaction centres. Ribeiro *et al*. (2003) observed a lowering of q_P when *Citrus sinensis* was infected with *Xylella fastidosa* while uninfected plants had a stable pattern, indicating that infection or stress causes decreased photochemical quenching as observed in this study.

An increase in NPQ in *Arabidopsis* plants was observed when infected with *Albugo candida* (Chou *et al*., 2000) and *Botrytis cinerea* (Berger *et al*., 2004). NPQ values indicate the amount of energy lost in the form of heat which is an indication of impaired photosynthesis (Maxwell and Johnson, 2000). Increased NPQ values could be due to the stimulation of O_2-dependent electron flow when CO_2-fixation in the Calvin-Benson cycle is limited or inhibited (Schreiber and Neubauer, 1990). In both the NCS and NIS plants, constant NPQ levels were found after an initial transient increase (Fig 5.3b). Similarly NPQ levels in NCR and NIR remained more or less constant. However, when placed in the dark, all treatments had increased heat dissipation during the latter stages with the DIS plants performing worse than DIR plants.

This is an indication that susceptible plants experienced infection associated stress in combination with the dark treatment much stronger than the resistant plants. This suggests that infection places the susceptible plants under more stress causing the plants to lose more energy as heat and lowering the photosynthetic ability. NIS plants had lower NPQ values possibly due to the better repair of reaction centres that were damaged by the dark treatment at 48 hpi as indicated by the q_P values (Fig 5.3a). Infected resistant plants again show more stable and lower NPQ values signifying that it is more adapt under stress conditions. Plants with a high q_P value display a low NPQ value, indicating that q_P and NPQ are inversely proportional. Karpinski *et al*. (1997) showed that exposure of plants to excess light causes an imbalance in excited energy between PSII and PSI thereby increasing expression of peroxidase indicating generation of ROS. Dark incubation could also have similar

effects as exposure to excess light. This was observed in the dark incubated wheat where *Pr9* and *GST* expression levels (Fig 5.7) were higher than the control light treated wheat (Fig 5.6).

It can therefore be deduced that light plays a crucial role in wheat after *P. triticina* infection since plants that were deprived of light for an extended amount of time showed a decrease in energy quenched in the photochemical reactions (Fig 5.3a). Light deprivation decreased the quantum yield of PSII. This supports the theory that plants require light to have a timely activation of defence responses since less energy is utilized in photosynthesis when plants are deprived of light. It also indicated that the resistant plants benefitted from a clearly more efficient photosynthetic capacity to withstand the infection.

This enhanced defensive ability of the infected resistant plants was however not evident when the different infection structures were counted (Fig 5.5). The number of colonies with less than 6 HMC was higher in DIR compared to the NIR plants (Fig 5.5) with a lower percentage of ASSV in DIR plants compared to the NIR plants. This indicated that the dark incubation caused the Thatcher+*Lr*34 plants to be more susceptible to infection. The dark however seemed to offer the susceptible plants some relief from infecting *P. triticina* since a higher percentage of aborted spores with fewer colonies were found. Increase of ROS which is showed by the induced expression of *GST* and *Pr9* (Fig 5.7) could cause tissue damage to the plant thus leading to a decrease in viable cells where nutrients are present. This could be a reason for the fewer spores that were able to form HMC on the susceptible cultivar.

Despite the fact that a large number of photosynthesis related cDNA clones were isolated using SSH (Chapter 4), no visible differences in the expression of these genes were observed. This could have been due to low levels of fluctuation in expression, which was not evident using RT-PCR. Differences might be more evident using qRT-PCR. Therefore, the enhanced defensive capacity of the resistant plants in the form of a higher photosynthetic capacity cannot be directly linked to enhanced transcription of the tested genes. It therefore just indicates that resistant plants

make better use of an efficient photosynthetic system to generate the needed capacity to withstand infection. An assumption can however be made that plants that were incubated in the dark were placed under more stress since the photosynthetic capacity declined. The added dark incubation caused oxidative stress in the plants which is observed by increased expression of *GST* (Fig 5.7). Dark incubation has a deleterious effect on the expression of defence related genes (Chandra-Shekara *et al*., 2006). This is caused by the imbalance of energy excitation between the two photosystems leading to an increased ROS production (Karpinski *et al*., 1999). The increase of ROS can be an effect of *P. triticina* infection (Fig 5.6 and 5.7).

The induced expression of all *PR* genes in all treatments indicated an effort by the infected plants to fight off disease (Fig 5.6 and 5.7). *Pr2* expression in *Linum usitatissimum* increases resistance to *Fusarium culmorum* and *F. oxysporum* (Wróbel-Kwiatkowska *et al*., 2004). NIS plants resulted in a sustained induced expression of *Pr2*, but a transient induction at 48 to 72 hpi was evident in the NIR plants (Fig 5.6). While *Pr2* expression in DIS plants was similar to NIS plants, the DIR plants showed a higher sustained expression that differed from the NIR plants. This expression profile was similar to that of *Pr2* in the NIS plants.

Tobacco plants overexpressing *Pr5* were more resistant to *Alternaria alternata* infection (Velazhahan and Muthukrishnan, 2003). In addition, the expression of *Pr9* is important for the resistance response since plants containing an anti-sense *Pr9* gene showed an impaired oxidative burst (Bindschedler *et al*., 2006). In the current study, expression of both *Pr5* and *Pr9* was induced in the light treated plants with a subsequent decrease starting at 60 hpi (Fig 5.6). In contrast, while expression levels decreased at 60 hpi in the DIS plants higher levels of expression were found in the DIR plants after 60 hpi (Fig 5.7). Combined, the expression profiles of *Pr2*, *Pr5* and *Pr9* in the DIR plants showed elevated levels implicating a role for light in the controlled expression of these genes in the resistant cultivar. Previously *Pr5* and *Pr9* expression levels were shown to be light dependent being modulated by phytochrome (Casal *et al*., 1990; Magliano and Casal, 1998; Genoud *et al*., 2002).

DIR plants therefore seem to overcompensate for the dark by overexpressing these three genes.

Pathogen infection induces the expression of *PAL* and *GST* (Li *et al*., 2001; Dean *et al*., 2005). Tobacco plants overexpressing *GST* had an enhanced ability to withstand oxidative stress conditions (Yu *et al*., 2003). Overexpression of *PAL* in tobacco plants caused a reduced susceptibility to *Cercospora nicotianae* (Shadle *et al*., 2003). It can therefore be assumed that an increase in expression of *PAL* observed in our study (Fig 5.6 and 5.7) will allow plants to cope better with pathogen infection. *GST* expression levels on the other hand remained constitutive for both light and dark treatments. From this it can be concluded that *PAL* and *PR* gene expression is light dependent for optimal expression during pathogen infection, especially in the resistant cultivar. This hypothesis is supported by the fact that the SA signalling pathway is light dependent (Genoud *et al*., 2002; Zeier *et al*., 2004). Future research will aim to accurately quantify these differences in expression using qRT-PCR.

The fact that induced expression profiles were observed in control and dark treated uninfected plants was surprising since these plants were never exposed to the pathogen (Fig 5.1). A possible explanation could be that this was due to inter-plant communication via volatiles (Fig 5.6 and 5.7). Volatile signalling has been described in various instances (Koch *et al*., 1999; Kessler and Baldwin, 2001; Huang *et al*., 2003). Engelberth *et al*. (2004) found that when healthy plants were exposed to volatiles, JA production was induced. A previous study involving the current plant-pathogen interaction also showed that infected wheat produce volatiles that signalled its uninfected counterparts in close vicinity to activate their defence responses (JJ Appelgryn, PhD unpublished results). This was again observed in the current study with the induced expression of most of the defence related genes in the uninfected plants. The uninfected plants were primed to withstand a pathogen attack due to the expression of the *PAL* and *PR* genes (Fig 5.6 and 5.7). This induction was however not evident for *Pr2* and *PAL* in the DCS and DCR plants, indicating that the absence of light could influence volatile production and signalling.

In conclusion, the results highlighted the crucial role light plays in the defence response of wheat. The presence of light allows a better activation of the defence response and perception of defence signalling molecules. Further studies into the effect of light on other defence related genes and signalling would further clarify the effect light has on the initiation and activation of plant defence.

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Chapter 6

General discussion

Pathogens invading plants induce an array of responses. These include amongst others elevated levels of PR proteins, SA and/or JA/ET (Feys and Parker, 2000). In addition to the activation of defence responses, pathogens influence the plant's photosynthetic efficiency (Ribeiro *et al*., 2003; Thomson *et al*., 2003; Bonfig *et al*., 2006; Berger *et al*., 2007). A decrease in photosynthetic capacity of the plant is due to the inability for electrons to be transferred from PSII to PSI therefore throwing the system into disarray, causing photodamage (Karpinski *et al*., 1999).

The same effect can be achieved by exposing the plants to excess light resulting in an increase in ROS (Karpinski *et al*., 1997). ROIs and NO function in concert to initiate HR during the plant's defence response (Delledonne *et al*., 1998; Delledonne *et al*., 2002). ROS induces the expression of the plant's protective genes such as *GST* (Lamb and Dixon, 1997). Induced expression of defence related genes like the *PR* genes and the activation of enzymes such as PIs and PAL further impedes the pathogen (Lamb and Dixon, 1997; Fan and Wu, 2005; Van Loon *et al*., 2006).

The role that light plays in the plant defence response have been reported in various plant-pathogen interactions (Zeier *et al*., 2004; Chandra-Shekara *et al*., 2006). Certain defence responses are light dependent (Casal *et al*., 1990; Magliano and Casal, 1998; Genoud *et al*., 2002). The accumulation of SA and the expression of *Pr1*, *Pr5* and *Pr9* were shown to be modulated by phytochrome (Zeier *et al*., 2004; Chandra-Shekara *et al*., 2006). This was also seen in this study in the expression of *Pr5* and *Pr9* in plants of all treatments (Fig 5.6 and 5.7). The expression of both genes was influenced by the absence or presence of light, thus indicating the importance of light. Since *Pr5* and *Pr9* are both defence related, the assumption can be made that light is essential for the defence response.

Since light is proposed to play a crucial role in the defence response it is only logical that photosynthesis is an important factor that needs to be considered. The detrimental effect that stress and pathogen infection has on photosynthesis can be determined by measuring the photosynthetic capacity (Lu and Zhang, 2000; Genoud *et al*., 2002; Grobbelaar and Mohn, 2002; Ribeiro *et al*., 2003; Thomson *et al*., 2003;

Bonfig *et al*., 2006; Berger *et al*., 2007). These studies have shown a decrease in photosynthetic capacity when plants were placed under stress or infected by various pathogens. The decrease in photosynthetic capacity observed in the current study further confirms the effect of pathogen infection on photosynthesis. However, depriving plants of light for an additional 24 h has an even stronger effect on the plant's photosynthetic capacity (Fig 5.2). In situations where plants are exposed to excess light the excess excitation energy however has the potential to increase production of ROS (Karpinski *et al*., 1997, 1999). This could also be the case when plants are infected by pathogens or deprived of light. Pathogens affect photosynthetic components like RbcL (Van der Westhuizen and Botha, 1993) thus directly affecting the plant's photosynthetic capacity. A decrease in photosynthetic capacity and potential of wheat infected with *P. triticina* was observed in both infected resistant and susceptible plants (Fig 5.2). The susceptible plants were however not as fit after inoculation. Plants of all treatments which received dark treatment also resulted in a decrease in photosynthetic capacity and an increase in dissipated heat (Fig 5.3). However, resistant plants were able to handle the stress of infection and light deprivation better.

The induced expression of defence related genes in uninfected plants was possibly due to volatiles produced by the infected plants that was observed in various other studies (Koch *et al*., 1999; Kessler and Baldwin, 2001; Huang *et al*., 2003). This was for instance found in a similar study involving the same plant/pathogen interaction (JJ Appelgryn, unpublished). Contradictory to this assumption the expression of *PAL*, *GST* and the *PR* genes were not as prominent in the uninfected dark incubated plants (Fig 5.7). One can speculate whether this is due to an ineffective recognition of the volatile signals produced by the infected plants.

Cell signalling forms an important part of the activation of the plant's defence response. If no signal is activated, the plant will not be able to respond. Therefore, it is significant that a cDNA clone was isolated from infected wheat, that could play a putative role as an intermediate between a RLK and the MAPK signalling cascade (Chapter 4). This gene, *LRW268*, was present in all the tested wheat cultivars. Since

it shared similarity with SLL1 (Yu *et al*., 1996) and was isolated during a plant-pathogen interaction, it implicated that *LRW268* forms part of the general defence signaling of wheat upon infection.

PIs were shown to play an important role in the plant's defence towards attacking pathogens by targeting proteases that are secreted by pathogens to hydrolyze peptide bonds of proteins (Habib and Khalid, 2007). PI genes are mostly wound-induced and the encoded proteins bind with proteases from the infecting pathogen to inactivate them (Hermosa *et al*., 2006). PIs are able to inhibit the growth and development of attacking pathogens (Echelkamp *et al*., 1993; Lorito *et al*., 1994; Falco and Silca-Filho, 2003; Hermosa *et al*., 2006). The isolation of a PI encoding gene during this study has allowed a better understanding of the defence response of the resistant Thatcher+*Lr*34 against *P. triticina* infection (Chapter 4). The expression of PIs is also induced upon MeJA treatment (Zhao *et al*., 1996) which correlated with the MeJA mediated increase of *LRW222* expression. Since *LRW222* is present in various different cultivars, it suggests that it, similar to LRW268, plays a role in the plant's general defence response. One such a role could be in the abortion of spore germination observed in the more effective resistant plants, since this was implicated in other studies (Lorito *et al*., 1994).

While RT-PCR indicates the involvement of *LRW222* and *LRW268* in the general defence responses, it is acknowledged that more quantitative approachs such as Northern blot and qRT-PCR will be more accurate techniques to confirm induced expression. The same applies for gene expression analysis in chapter 5.

The results generated in this study show that a RLK interacting protein and a PI, which is part of the PR family, form part of the induced defence response of wheat to the infecting *P. triticina*. Light was also implicated to play a major part in this plant-pathogen interaction, and together these three factors are pieces of the bigger puzzle that help in elucidating the wheat-*P. triticina* interaction.

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Summary

The aim of this study was to study various aspects of the defence response of wheat infected by *Puccinia triticina*. SSH was used to isolate differentially expressed cDNA fragments from *P. triticina* infected wheat. Once sequenced, two cDNA clones were selected for further analysis.

One of the cDNA clones, *LRW268*, showed similarity to an EST derived from de-etiolated wheat. It showed a 16.5 fold induced expression at 9 hpi in the *P. triticina* infected wheat. In *P. striiformis* infected wheat, an induced expression was found in the resistant Avoset-*Yr*1 plants. Application of chemical elicitors showed an induction of *LRW268* expression for plants treated with H_2O_2, MeJA and menadione. To obtain the full length gene, 5'-RACE was attempted, but it was unsuccessful. However, a contig was assembled using ESTs present in the GenBank database. This yielded a 603 bp contig encoding a 96 amino acid sequence that showed good homology to a RLK interacting protein. The presence of a putative MAPK docking motif and a phosphorylation site indicated that LRW268 could play a role in cell signalling. The presence of *LRW268* in different wheat cultivars signified that it could form part of the general defence response of wheat.

The second cDNA clone, *LRW222,* which was similar to an EST from wheat infected with powdery mildew, showed a 4.6 fold induction of expression at 15 hpi in wheat infected with *P. triticina*, while the susceptible Thatcher cultivar showed an induction at an earlier time interval. *P. striiformis* infected wheat showed a more constant expression of *LRW222*. Putative induced expression of *LRW222* was observed in H_2O_2, MeJA and menadione treated wheat. Assembly of a contig using published ESTs yielded a 668 bp contig which encoded an 89 amino acid polypeptide showing homology to various wound-induced protease inhibitors. The presence of a putative MAPK docking motif on LRW222 suggested that it could be a general or pathogen specific protease inhibitor.

The effect of light on the wheat defence response was also examined. The photosynthetic capacity of all treatments was measured and fluorescence microscopy performed. Infection caused a decrease in the photosynthetic capacity

of the susceptible plants with the resistant plants showing less fluctuation. The infected resistant plants recovered faster and better than the infected resistant plants after the dark incubation. Plants that were additionally dark incubated showed a lower photosynthetic capacity compared to the control treatments. This difference in photosynthetic capacity was not observed on molecular level with photosynthesis related genes showing unaltered expression. The putative expression of certain defence related genes did however show a light dependency. An induced defence was observed in the uninfected plants, confirming a putative volatile signalling event that was detected during an earlier study involving this particular plant/pathogen interaction. The importance of light in wheat resistant towards *P. triticina* could thus be attributed to the ability of plant to photosynthesise optimally.

It must however be emphasized that the expression of all tested genes were not quantitatively determined, since end-point analysis using RT-PCR was used. Future research will include the use of techniques that allows quantitative measurement of gene expression.

Keywords: *Triticum aestivum*, *Puccinia triticina*, *Puccinia striiformis*, cell signalling, protease inhibitors, photosynthetic capacity

Opsomming

Die doel van die studie was om verskillende aspekte van die verdedigingsrespons van koring na infeksie deur *Puccinia triticina*, te ondersoek. cDNA fragmente wat na infeksie differensieël tot uiting gekom het, is met behulp van SSH geïsoleer. Nadat die klone se basisvolgorde bepaal is, is twee cDNA klone vir verdere analise gekies.

Een cDNA kloon, *LRW268*, het ooreenkoms met'n EST wat uit gedeëtioleerde koring gekloneer was, getoon. Kloon *LRW268* se uitdrukking was 9 uur na infeksie 16.5-voudig hoër. Geïnduseerde uiting was ook gevind in Avoset-*Yr*1 koring wat met *P. striiformis* geïnfekteer was. Verder het die bespuiting van koring met H_2O_2, MeJA en menadioon ook tot hoër uitdrukking gelei. Hoewel onsuksesvol, is daar met 5'-RACE probeer om die volledige geen te kloneer. Uiteindelik is 'n contig wat die volledige geen verteenwoordig, deur middel van ESTs soos gevind in die GenBank databasis, saamgestel. Die voorgestelde geen was 603 bp lank en het vir 'n polipeptied van 96 aminosure kodeer. Die polipeptied het goeie ooreenkoms met 'n proteïen wat aan RLKs bind, getoon. Aangesien die voorgestelde polipeptied moontlike MAPK-bindingsmotiewe en fosforileringssetels besit, dui dit daarop dat dit by seinoordraging betrokke kan wees. Aangesien die geen ook verder in verskillende kultivars teenwoordig is, is dit'n aanduiding dat dit moontlik 'n rol te speel het tydens die algemene verdedigingsrespons van koring.

'n Tweede cDNA kloon, *LRW222*, wat ooreenkoms toon met'n EST uit poeieragtige meeldou-geïnfekteerde koring, het'n 4.6 -voudige geïnduseerde uitdrukking na 15 uur in geïnfekteerde Thatcher+*Lr*34 koring getoon. Die geïnduseerde uitdrukking was vroeër in die vatbare Thatcher kultivar. Koring geïnfekteer met *P. striiformis* het 'n meer konstante uiting getoon. Moontlike geïnduseerde uitdrukking is gevind in koring wat behandel was met H_2O_2, MeJA en menadioon. 'n Contig saamgestel uit beskikbare ESTs was 668 bp lank en het kodeer vir 'n polipeptied van 89 aminosure. Die polipeptied het groot ooreenkomste getoon met verskeie wond-geïnduseerde protease inhibitore. Die verder teenwoordigheid van 'n moontlike MAPK bindingsmotief het weereens daarop gedui dat die gekodeerde polipeptied of 'n algemene of patogeen-spesifieke protease inhibitor kan wees.

Laastens is die invloed van lig op koring se verdedigingsrespons ook ondersoek. Die fotosintetiese kapasiteit van alle behandelings was gemeet en fluoresensiemikroskopie is gebruik om infeksiestrukture te tel. Infeksie het gelei tot ’n afname in fotosintetiese kapasiteit van vatbare koring terwyl die weerstandbiedende plante minder fluktuasies getoon het. Laasgenoemde plante het ook vinniger en beter na die donker inkubasie herstel in vergelyking met die geïnfekteerde vatbare plante. Die plante wat addisioneel aan donker blootgestel was, se fotosintetiese kapasiteit was laer as die van die kontrole plante. Die uitdrukking van verskeie verdedigingsverwante gene het ook ’n lig-afhanklikheid getoon. ’n Aangeskakelde verdedigingsrespons in die ongeïnfekteerde plante was moontlik as gevolg van seinoordraging deur vlugtige stowwe wat al voorheen in dieselfde plant/patogeen interaksie waargeneem is. ’n Effektiewe verdedigingsrespons van weerstandbiedende koring teen *P. triticina* infeksie is dus afhanklik van die plant se vermoë om optimaal te fotosintetiseer.

Dit is egter belangrik om te noem dat die uitdrukking van alle gene in die studie nie kwantitatief gemeet is nie, aangesien RT-PCR gebruik is wat net ’n eindpuntproduk lewer. Toekomstige studies sal egter van ander tegnieke gebruik maak om te verseker dat alle uitdrukkingstudies kwantitatief is.

Sleutelwoorde: *Triticum aestivum*, *Puccinia triticina*, *Puccinia striiformis*, selseinoordraging, protease inhibitore, fotosintetiese kapasiteit