Biochemical and molecular analysis of the early response of *Triticum aestivum* infected with *Puccinia striiformis* f.sp. *tritici*.

By

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Dissertation submitted in fulfilment of requirements

for the degree

Magister Scientae

in the Faculty of Natural and Agricultural Sciences,

Department of Plant Sciences (Botany),

University of the Free State

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List of Abbreviations

ABA	Abscisic acid
AFR	Ascorbate free radical
AFRR	Ascorbate free radical reductase
APS	Ammoniumperoxodisulfate
APX	Ascorbate peroxidases
ASC	Ascorbate
ATP	Adenine triphosphate
Avr	Avirulence
BH_4	Tetrahydrobioterpin
BR	Brassinosteroid
BSA	Bovine serum albumin
BTH	Benzothiadiazole
CAT	Catalase
CC	Coiled-coil
CDPK	Calcium-dependent protein kinases
CR	Control resistant
CS	Control susceptible
DAG	Diacylglycerol
DAO	Diamine oxidase
DDRT–PCR	Differential display reverse transcription PCR
DEPC	Diethyl pyrocarbonate
DHA	Dehydroascorbic acid
DHAR	Dehydroascorbic acid reductase
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dnOPDA	Dinor oxo-phytodienoic acid
dNTP	Deoxy nucleotide triphosphate
d.p.i.	Days post inoculation
DPI	Diphenylene iodonium
DTT	Dithiotreitol
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylenebis(oxyethylenenitrilo) tetraacetic acid

EGF	Epidermal growth factor
eNOS	Endothelial NOS
FAD	Flavin adenine dinucleotide
FMN	Flavin mono nucleotide
GM	Germinating medium
GPX	Glutathione peroxidase
GSH	Glutathione
GSSG	Glutathione disulfide
GST	Glutatione S-transferase
H_2O_2	Hydrogen peroxide
h.p.i.	Hours post inoculation
HR	Hypersensitive response
ICS	Isochorismate synthase
IL-1R	Interleukin-1-receptor proteins
INA	2,6-dichloroisonicotinic acid
iNOS	Inducible NOS
IPL	Isochorismate pyrovate lyase
IPTG	Isopropylthio β-D galactoside
IR	infected resistance
IS	Infected susceptible
JA	Jasmonic acid
KAPP	Kinase associated protein phosphatases
KI	Kinase interacting
LB	Luria bertani
LOX	Lipoxygenase
LR	Local resistance
LRR	Leucine rich repeats
LZ	Leucine zipper
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MBP	Myelin basic protein
MeJa	Methyl jasmonate
MeSA	Methyl salicylic acid

NB–ARC	nucleotide binding in APAF–1, R–gene products, and CED–4
NBS	Nucleotide binding sites
NBT	4-Nitro-blue tetrazolium
NDP	Nucleotide diphosphate
nNOS	Neuronal NOS
NO	Nitric oxide
NO_2	Nitrogen dioxide
NOS	NO synthase
NR	Nitrate reductase
O_2^-	Superoxide anion
$OONO^-$	Peroxynitrite
OH^-	Hydroxyl radical
OPDA	12-oxo-phytodienoic acid
ORF	Open reading frame
PA	Phosphatidic acid
PAL	Phenylalanine ammonia lyase
PAO	Polyamine oxidase
PCD	Programmed cell death
PCR	Polymerase chain reaction
Phox	Phagocyte oxidase
PKC	Protein kinase C
PLA2	Phospholipase A2
PLC	Phospholipase C
PLD	Phospholipase D
PM	Plasma membrane
PMSF	Phenylmethylsulfonylfluoride
POD	Peroxidase
PPase	Serine/threonine protein phosphatases
PR	Pathogenesis related
PS-I	Photosystem I
PTPase	Protein tyrosine phosphatases
PVP	Polyvinylpyrolidone
R-gene	Resistance gene

RLCK	Receptor-like cytoplasmic kinases
RLK	Receptor-like protein kinases
RNA	Ribonucleic acid
ROS	Reactive oxygen species
R–protein	Resistance protein
RPK	Receptor protein kinases
RWA	Russian wheat aphid
SA	Salicylic acid
SABP	Salicylic acid binding protein
SAR	Systemic acquired resistance
SDS	Sodium dodecyl sulfate
SLSG	S-locus specific glycoprotein
SOD	Superoxide dismutase
S-RLK	S-domain class RLK
TCA	Trichloroacetic acid
TEMED	N, N, N`, N`- Tetramethylethylendiamine
TIR	Toll- and Interleukin-1-receptor proteins
TM	Transmembrane
TMV	Tobacco mosaic virus
TNFR	Tumor necrosis factor RLK
TRIS	Tris (hydroxymethyl) aminomethane
Triton X-100	Polyoxyethylene octyl phenyl ether
TWEEN 20	Polyoxyethylensorbitanmonolaurat
WAK's	Wall associated kinases
X–Gal	5-Bromo-4-Chloro-3-indolyl-β-D- Galactopyranoside
XOR	Xanthine oxidoreductase

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Acknowledgements

I would like to acknowledge and express my sincere appreciation to the following persons and institutions:

My studyleader, Dr. B. Visser: For the opportunity to work in his laboratory and the support and guidance during my study.

My co-studyleader, Prof. A.J. van der Westhuizen: For his guidance and support during my study.

All my friends and colleagues in the lab: For their interest, company and sincere friendship.

To Anthia: For her love and support.

My parents, grandmother, brother, other family members and friends: For their loving support and understanding.

National Research Foundation (NRF): For the bursary received in support of my study.

The Department of Plant Sciences, University of the Free State: For the opportunity to do my study in this dynamic department and for the use of departmental facilities.

“If we knew what it was we were doing, it would not be called research, would it?”

Albert Einstein

“Only a life lived for others is worth living!”

Albert Einstein

CHAPTER 1

Introduction

1.1. INTRODUCTION

Puccinia striiformis (stripe rust) is a serious disease of wheat and account for large economical losses in the wheat industry world-wide. Stripe rust was first observed in South Africa during the winter of 1996 (Pretorius *et al*., 1997). In surveys conducted in the major wheat-producing areas in South Africa during 1996 and 1997, only one pathotype (6E16A) was detected (Pretorius *et al*., 1997). During the 1998 season stripe rust infection reached epidemic proportions when a second pathotype (6E22) occurred in the eastern Free State (Boshoff and Pretorius, 1999).

Certain wheat cultivars are resistant against stripe rust and therefore possess the ability to defend it against the intruding fungi (McIntosh *et al*., 1995). Resistant plants in general make use of a variety of strategies that include structural and biochemical defense mechanisms to defend themselves (Bayles *et al*., 1990). Some of these defense mechanisms can be expressed constitutively, while others may be induced upon perception of the pathogen (Hammerschmidt and Schultz, 1996).

Perception occurs via elicitors being bound by receptors in the plant, mostly receptor-like protein kinases, resulting in signal transduction cascade, which involves a cascade of phosphorylation/dephosphorylation events. Various receptor-like protein kinases [RLK's] involved in plant–pathogen interactions have been found. These include LRK10 (Feuillet *et al*., 1997) the resistance gene involved in the interaction between wheat and leaf rust (*Puccinia triticina*). Recognition of the pathogen will ultimately activate certain defense responses and lead to altered gene expression of defense related genes. These elicitors are mainly pathogen related, either being produced by the pathogen itself or through the action of pathogenesis (Yamaguchi *et al*., 2000).

The defense response of a resistant plant can be directly linked to signaling events leading to the specific defense responses. The timely response to intruding pathogens also plays a critical role in acquiring resistance (Maleck *et al*., 2000). Susceptible plants often take longer to activate their defense response after infection by a pathogen. In some instances they do not respond at all (Moerschbacher *et al*., 1999). The latter might be due to the fact that the signal transduction leading to the response is in some way blocked by the attacking pathogen (Moerschbacher *et al*., 1999).

Thus, the early signal events in response to pathogens are a major contributing factor to effective resistance in plants. The earliest signaling events leading to defense responses in plants after pathogen infection, includes a Ca^{2+} flux (Atkinson *et al*., 1996), the phosphorylation /dephosphorylation of target proteins through RLK's (Haffani *et al*., 2004), calcium dependent protein kinases [CDPK's] (Evans *et al*., 2001) and mitogen activated protein kinase [MAPK's] (Frey *et al*., 2001), the production of nitric oxide [NO] (Durner *et al*., 1998; Delledonne *et al*., 1998) and an oxidative burst (Clarke *et al*., 2000a). Both Ca^{2+} fluxes and phosphorylation /dephosphorylation events are important signals not only in the response of plants towards pathogens, but also in normal cellular functioning, while the oxidative burst and NO levels act synergistically in plant defense (Zeier *et al*., 2004)

During the oxidative burst, H_2O_2 is produced which can act as a signal to adjacent cells (Dangl and Jones, 2001). Zeier *et al*. (2004) proposed that NO also acts as an important cell-to-cell signal. The combination of the two molecules could then lead to the death of infected cells, the activation of cell wall bound peroxidases (Thordal-Christensen *et al*., 1997), phenylalanine ammonia lyase [PAL] (Desikan *et al*., 1998) and other enzymes that are involved in cellular protection (Levine *et al*., 1994).

Thus, H_2O_2 and NO could act as signal molecules, as well as be a direct response to invading pathogens by having anti-microbial functions (Wu *et al*., 1995; Delledonne *et al*., 2001). It seems therefore that NO and H_2O_2 are produced simultaneously and play a very complex role in hypersensitive cell death (De Gara *et al*., 2003).

One of the secondary defense responses during pathogen attack is the production of salicylic acid [SA] (Mohase and van der Westhuizen, 2002). Evidence that have been gathered over the years, has also implicated SA as a signal for systemic acquired resistance [SAR] (Metraux *et al*., 1990). Salicylic acid has also been implicated in hypersensitive cell death together with NO and H_2O_2 (Martinez *et al*., 2000). Thus, NO, H_2O_2 and SA might be key regulators of the defense response of plants against pathogens.

Preceding most signals, a phosphorylation cascade is thought to function (Grant *et al*., 2000). It is likely that both NADPH oxidase and nitric oxide synthase [NOS] (thought to be the major producer of reactive oxygen species [ROS] and NO during plant defense) are activated through phosphorylation (Chandra and Low, 1995; Xing *et al*.,

1996). Most defense responses are thus triggered either directly or indirectly through phosphorylation (Xing *et al*., 1996).

The improvement of our understanding of the complexity of signaling cascades leading to an effective resistance in plants will be the key in engineering durable disease resistance (Stuiver and Custers, 2001).

This study will contribute to the understanding of the early signaling events leading to the onset of defense responses in wheat infected with stripe rust, thus improving our knowledge of the complexity of signal transduction in plant-pathogen interactions.

The aim of this study was to establish the oxidative burst and the involvement of protein kinases in the early responses involved in the resistance of a resistant wheat cultivar (Yr1) to *Puccinia striiformis*, thereby establishing the earliest point of recognition and the onset of defense responses to the intruding pathogen by the plant. This time period was then used in an attempt to clone genes involved in the downstream signaling.

CHAPTER 2

Literature Review

2.1. RUSTS OF WHEAT

Wheat (*Triticum aestivum*) is one of the most important crop plants. Not only is it of economical value, but it is also a primary source of food all over the world. Wheat is produced in most countries of the world ranging from the USA, most if not all European and Asian countries, Australia, New Zealand and especially in the southern parts of Africa (Payne *et al*., 2001).

South African wheat farmers produce on average 2 million tons of grain per year (1990 – 1997) on approximately 1 million hectares of land. Of this 50% is derived from dry-land winter wheat (summer rainfall production), 30% from dry-land spring wheat (winter rainfall production) and 20% from irrigated spring wheat (Payne *et al*., 2001).

Wheat crops are subjected to a variety of pests and pathogens, including insects such as the Russian wheat aphid [RWA] (Mohase and van der Westhuizen, 2002), viruses (Truol *et al*., 2004), bacteria (Duveiller *et al*., 1992) and fungi (Pretorius *et al*., 1997). Among the fungi are the rusts, which have the potential to develop into widespread epidemics (Boshoff *et al*., 2002). Besides reducing seed yields, rusts lower the crop's forage value and winter hardiness and predispose plants to certain other plant diseases (Stubbs, 1985).

Three different rust diseases occur on wheat. They are stem rust (*Puccinia graminis* f.sp. *tritici*), leaf rust (*Puccinia triticina* f.sp. *tritici*; previously *P. recondita* f.sp. *tritici*) and stripe rust (*Puccinia striiformis* f.sp. *tritici*). They are so called because of the dry, dusty, yellow-red or black spots and stripes that erupt through the plant epidermis. The size and surrounding coloration of rust pustules determine the specific infection types, which can vary with different wheat cultivars, temperature and rust races (McIntosh *et al*., 1995).

2.1.1. Stripe Rust – Origin, History and Distribution in South Africa

Stripe rust (also called yellow rust), caused by the obligated pathogen *Puccinia striiformis* Westend. f.sp. *tritici* Eriks., is one of the most important diseases of wheat (Stubbs, 1985). Historically, stripe rust occurs more often in areas with cool and wet climates and hence found regularly in northern Europe, the Mediterranean region, Middle East, western United States, Australia, East African highlands, China, the Indian subcontinent, New Zealand and the Andean regions of South America (Danial *et al*., 1995).

In comparison with leaf rust and stem rust, the distribution of stripe rust is more restricted. Stripe rust was first reported in northern Zambia in 1958 (Angus, 1965), Australia in 1979 (O'Brain *et al*., 1980), New Zealand in 1980 (Beresford, 1982) and was only reported in South Africa in 1996 (Pretorius *et al*., 1997).

Stripe rust was first observed during 1996 on the bread wheat cultivar Palmiet, in the winter rainfall region near Moorreesburg in the Western Cape, South Africa (Pretorius *et al*., 1997). In stripe rust surveys conducted in the major wheat-producing areas in South Africa during 1996 and 1997, only one pathotype (6E16A) was detected (Pretorius *et al*., 1997). During the 1998 season stripe rust infection reached epidemic proportions when a second pathotype (6E22) occurred in the eastern Free State (Boshoff and Pretorius, 1999). The main regions that are affected by or are threatened by stripe rust in South Africa is the Western and Eastern Cape, Free State, especially the eastern parts of the Free State and KwaZulu–Natal where irrigation farming is practised (Boshoff *et al*., 2002).

2.1.2. Symptoms, Cultivar Resistance and Control

The symptoms of stripe rust vary, but usually appear earlier in spring than the symptoms for leaf or stem rust. Uredia are yellow, appear mainly on leaves and heads of wheat and are often arranged into conspicuous stripes (Fig. 2.1).

A

B

Fig. 2.1: Stripe rust infection of wheat. In (A), a close-up of linearly arranged uredinial sori of *P. striiformis* is shown (http://www.doacs.states.fl.us./pi/enpp /pathology/ images/striperustwheat4). In (B), stripe rust lesion with mature rust pustules on wheat leaf is shown (http://pdc.unl.edu/forecast).

Crop losses due to the disease and higher input costs due to repeated application of fungicides necessitated the development of more affordable control strategies against stripe rust. For instance, the outbreak of stripe rust in 1996 had producers spending R28 million on fungicides to control the epidemic (Boshoff *et al*., 2002).

Breeding of resistant cultivars represent the most cost effective means of controlling stripe rust. However, a proper knowledge of genetic variance in both pathogen and host is needed. Table 2.1 shows different infection types of stripe rust on wheat at seedling stages produced in the world (McIntosh *et al*. 1995). Certain other factors might also play a role in breeding resistance. For instance, different genes seem to be involved in conferring stripe rust resistance to both seedling and mature wheat plants (Allan *et al*., 1966). The fact that these different genes confer resistance to wheat at different growth stages, might implicate different mechanisms of resistance to stripe rust or simply different signaling pathways to similar defense mechanisms (Allan *et al*., 1966).

2.2. OVERVIEW OF PLANT DEFENSE

Plant disease is only one of various external stresses that plants are constantly subjected to. Other external stresses include, amongst others, environmental changes such as heat, cold, water stress, mechanical stress and chemical stresses (Zhang *et al*., 2005). Chemical stress can be caused by an excess of heavy metals or by high salt concentrations present in the soil (De Azevedo Neto *et al*., 2005).

Although all of the above-mentioned stresses affect the plant, attack by pathogens still account for most of the losses in overall crop yield and are thus of greater economical importance. Pathogens include viruses, bacteria, fungi, nematodes and insects (Jackson and Tayler, 1996). To survive, the plant is required to respond in an appropriate manner to these external stresses. In order to respond, the plant needs certain defense mechanisms that would help it either to prevent the stress from causing any damage or to heal the damage that has already occurred. These defense mechanisms include both structural defenses and biochemical mechanisms (Bayles *et al*., 1990; Zhang *et al*., 2005).

Table 2.1: Seedling infection types produced on World (nr. 1-9) and European (nr. 10-17) differentials and supplemental tester lines (nr. 18-44) by pathotypes 6E16A and 6E22A of *P. striiformis* f.sp. *tritici.* Seedling reaction types were determined according to McIntosh *et al*. (1995):

0 = no uredia, ; = fleck, 1 = necrotic and chlorotic areas with restricted sporulation, 2 = small to medium uredia with necrosis and chlorosis, 3 = medium – sized uredia with chlorosis, 4 = abundant sporulation without chlorosis, C and N = more than usual degrees of chlorosis or necrosis, … not tested.

Nr	Cultivar	Yr gene(s)	Low infection type	Seedling reaction pt. 6E16A	Seedling reaction pt. 6E22A
	Differentials				
1	Chinese 166	1	0;	0;	;
2	Lee	7	;N,1N	4	4
3	Heines Kolben	2,6	;,N1	4	4
4	Vilmorin 23	3a,4a	;	;N	;N, 1C
5	Moro	10, Mor	0;	;	;
6	Strubes Dickkopf	Sd,25	…	;C, 1CN	;C, 1CN
7	Suwon 92/Omar	4,Su	;	0;	0;, 1C
8	Clement	2,9,25, Cle	0;	0;	;
9	Triticum spelta album	5	0;,;	0;	;
10	Hybrid 46	4b	;	;	;
11	Reichersberg 42	7,25	;N,1N	;1CN	4
12	Heines Peko	2,6,25	;N,1N	;N	4
13	Nord Desprez	3a,4a	;	;	;,;C
14	Compare	8,19	0;,;	4	4
15	Carstens V	Cv	…	;	;C
16	Spaldings Prolifie	Sp	…	0;	0;
17	Heines VII	2,25,HVII	0;,2	;C	;C,;1C

	Supplemental set				
18	Yr1/6*AvS	1	0;	;	;
19	Kalyansona	2	0;,2	4	4
20	Yr5/6*AvS	5	0;,;	0	0;
21	Yr6/6*AvS	6	;,;N1	3	3
22	Yr7/6*AvS	7	;N,1N	3	3
23	Yr8/6*AvS	8	0;, ;	3	3
24	Federation/4*Kavkaz	9	0;	0;	0;
25	Yr9/6*AvS	9	0;	0	0;
26	Yr10/6*AvS	10	0;	;	0;
27	Yr11/3AvS	11	…	3	3
28	Wembley	14	…	3	3
29	Yr15/6*AvS	15	0;	0	;
30	Trident	17	;C, ;1	4	4
31	Yr17/3*AvS	17	;C, ;1	3	3
32	Jupateco R	18	…	4	4
33	Yr18/3*AvS	18	…	4	4
34	Yr24/3*AvS	24	…	;	;
35	TP981	25	…	;	4
36	TP1295	25	…	;	4
37	Yr26/3*AvS	26	…	;	;
38	Selkirk	27	…	1CN, 3	1CN, 3
39	Yr27/3*AvS	27	…	;, 1p=4	;
40	Avocet R	A	;CN1, 2+	;C, 1C	;C, 1C
41	YrSp/3*AvS	Sp	…	;	;
42	Avocet S	…	…	4	4
43	Federation 1221	…	…	4	4
44	Jupateco S	…	…	4	4

Of these defense mechanisms, most of the responses need to be activated through a signal (Suzuki *et al*., 2004). The signal might originate from the pathogen itself. It can however also be produced by the damaged plant after herbivore or pathogen attack (Yamaguchi *et al*., 2000).

Finally, volatile signals could also be produced by neighbouring plants whose defenses were activated (Thaler *et al*., 2002). Signals from the plant itself may include polypeptides such as systemin (Scheer and Ryan, 2002), oligosaccharides (Okada *et al*., 2002), microbial proteins (Ji *et al*., 1997; Asai *et al*., 2002) and/or lipid-based signaling molecules (Li *et al*., 2002b). These molecules, which can originate from the plant itself through the degradation of the cell wall or from the pathogen, are termed elicitors. Signals coming from a neighbouring plant might include ethylene (Hoffman *et al*., 1999), methyl jasmonate [MeJA] (Seo *et al*., 2001) and methyl salicylate [MeSA] (Shulaev *et al*., 1997), but evidence of these signals is limited and further research is needed in this area.

Elicitors that are produced must be recognized by the plant in order to respond to it. For this purpose, the plant uses receptor proteins (Martin *et al*., 2003). These receptor proteins contain various structural motifs for elicitor recognition and binding, such as leucine-rich-repeats [LRR's] (Ellis *et al*., 1999), nucleotide binding sites [NBS] (Van der Biezen *et al*., 2002), leucine-zippers [LZ] (Song *et al*., 1995) and coiled–coil [CC] sequences (Warren *et al*., 1999). Other common motifs include serine/threonine kinase domains (Salmeron *et al*., 1996) and regions with similarity to the N–terminus of Toll- and Interleukin-1-receptor proteins called TIR regions (Dinesh-Kumar *et al*., 2000).

The recognition of the elicitors by the receptor protein is the first step in responding to the external stress. Elicitors can be divided into general and specific elicitors, with the specific elicitors being subdivided into race–specific and race–nonspecific elicitors. The general elicitors may include e.g. jasmonic acid [JA], ethylene (Zhao *et al*., 2004), SA (Taguchi *et al*., 2001) and NO (Neill *et al*., 2002) and even certain ROS (Zhang *et al*., 2005).

Race–specific pathogen recognition was first proposed by Flor's gene–for–gene model for the genetic interaction between plant and pathogen (Flor, 1956). This model states that a dominant or semi–dominant resistance gene [R-gene] product from the plant interacts with the corresponding dominant avirulence [Avr] gene product from the pathogen. One of the best-studied race specific interactions is the Pto–AvrPto interaction (Salmeron *et al*., 1996). Pto is a serine/threonine protein kinase that confers race specific resistance, to strains of *Pseudomonas syringae*, in tomato that carry the corresponding *avrPto* avirulence gene. In addition to specific resistance determined by the gene–for–gene interaction, plant defenses can be activated without a matching pair of Avr and R–genes. Many fungal and bacterial proteins and glycoproteins can function as non-race-specific elicitors to induce defense responses in plants that do not carry any specific R–genes (Benhamou, 1996).

The interaction of the elicitors with the receptors are likely to activate a signal transduction cascade that may involve protein phosphorylation and dephosphorylation (Grant *et al*., 2000), ion exchange (Atkinson *et al*., 1996), reactive oxygen species production and other signaling events (Clarke *et al*., 2000a). Subsequent transcriptional and/or post-translational activation of transcription factors will eventually lead to the activation or induction of plant defense gene expression leading to, the so called hypersensitive response [HR] (Zhu *et al*., 1996).

The induction of a defense response first occurs at the site of infection through the HR, but the plant also establishes a systemic acquired resistance [SAR], which is a long lasting systemic immunity that protects the entire plant against a broad range of potential pathogens (Ryals *et al*., 1996; Sticher *et al*., 1997). Establishment of SAR is associated with the systemic expression of defense gene families encoding pathogenesis – related [PR] proteins (Van Wees *et al*., 2000). Two major role players in the establishment of SAR are SA (Yalpani *et al*., 1991) and NO (Song and Goodman, 2001). Recently additional molecules, such as lipid and lipid derivatives, have been suggested to be short and long distance mobile signals for SAR (Maldonaldo *et al*., 2002). Aspartic protease has also been suggested to play a role as a long distance signal for SAR (Xia *et al*., 2004).

2.3. DEFENSE MECHANISMS

Defense mechanisms that plants utilize can be divided into both structural and biochemical defenses. Both categories can themselves be subdivided into pre-existing defense through constitutive expression or through induction upon the elicitation event (Hammerschmidt and Schultz, 1996; Agrios, 1988).

2.3.1. Structural Defense Mechanisms

Constitutively expressed structural defense mechanisms include waxes that are deposited on the leaves (Tsuba *et al*., 2002). These waxes prevent the leaves from getting wet and thus prevent a suitable place for the germination of fungi. Hairs on the leaf will also have a similar effect. The structure of stomata also plays an important role in that any structural modification would make penetration by pathogens more difficult (Agrios, 1988). The thickness of the cuticle, as well as the thickness and strength of the epidermal cells will make penetration by pathogens more difficult (Barthlott and Neinhuis, 1997). These mechanisms are present in most plants (Hammerschmidt and Schultz, 1996).

Induced structural defense mechanisms can be divided into two classes, namely histological and cellular defense mechanisms. Histological defense mechanisms include the deposition of gum or the production of cork, which will prevent the spread of the pathogen throughout the plant (Agrios, 1988).

Cellular defense mechanisms include the fortification of cell walls (El-Gendy *et al*., 2001). One type of cell wall fortification that occurs rapidly in response to fungal invasion is the formation of papillae. They are thought to physically block fungal penetration of the host cells (Bayles *et al*., 1990). Rapid callose deposition in cell walls is also frequently associated with sites of pathogen penetration. The blockage of plasmodesmata with callose is an essential component of the defense response required to impede cell-to-cell movement of viruses (Beffa *et al*., 1996).

An additional but probably slower mechanism that renders cell walls more resistant is the localized increase of their lignin content (Mulosevic and Slusarenko, 1996). The most compelling evidence for the role of lignification in resistance has been provided by Moerschbacher *et al*., (1990). They showed that after resistant wheat was infected with an avirulent race of the stem rust fungus, the hypersensitive cell death was correlated with cellular lignification, which restricted further fungal growth.

Furthermore, the cross-linking of cell wall proteins can be induced by hydrogen peroxide [H_2O_2] by activating glutatione peroxidases as well as plant cell wall phenolics that contribute to prevent penetration of fungal hyphae (Thordal-Christensen *et al*., 1997; Grant and Loake, 2000). These induced structural mechanisms are only employed after pathogen infection and thus prevents the pathogen from spreading throughout the plant.

2.3.2. Biochemical Defense Mechanisms

Constitutively expressed biochemical defenses include the presence of phenolics in the plant, which inhibit hydrolytic enzymes from the pathogen, thus preventing the pathogen from entering the plant cell (Hammerschmidt and Schultz, 1996).

The predominant defense strategy used by plants against pathogens is the HR. The HR is an induced biochemical defense mechanism that is characterized by a rapid, localized cell death at the point of pathogen attack/recognition (Lam *et al*., 2001). Various physiological changes occur in plants after the recognition of the pathogen by the host plant. These physiological changes occur in both resistant and susceptible plants. The main difference between resistant and susceptible plants is the response time after the recognition event (Maleck *et al*., 2000). Therefore, if a plant can respond faster to an invading pathogen, it seems that resistance would be acquired, but a delay in responding to an attacking pathogen would lead to infection.

The accumulation of ROS and phenolics forms part of these changes (Schmelzer *et al*., 1993). Some of these ROS are involved in the cross-linking of cell wall polymers to strengthen it against penetration by pathogens (Thordal-Christensen *et al*., 1997).

They include amongst others super oxide anions [O_2^-], which can be converted to H_2O_2 via superoxide dismutase [SOD], singlet oxygen and the hydroxyl radical [OH^-] (Apel and Hirt, 2004). The above-mentioned leads to cell destruction. Hydrogen peroxide might also be responsible for the activation of other defense pathways in the HR (Levine *et al.*, 1994).

The accumulation of phytoalexins is also associated with the HR (Rustérucci *et al*., 1996). Phytoalexins are novel phenolics that accumulate after infection has occurred and have antimicrobial functions (Hain *et al.*, 1993). The antimicrobial activity of the phytoalexins allows the plant to defend itself against penetration by a pathogen (Schmelzer *et al*., 1993).

The production of PR-proteins that accumulate in the extracellular regions and vacuoles of plant cells is strongly related to the HR (Bertini *et al*., 2003). The degradation of fungal cell wall structural polysaccharides or the alteration of fungal cell wall architecture could arrest or severely impair fungal growth (Collinge *et al.,* 1993). Beta-1,3-glucanases and chitinases fall under the PR-2 and PR-3 classes of PR proteins respectively. They possess anti-fungal activity, which allows them to protect the plant directly against the penetration of fungal hyphae by degrading β-1,3-glucans and chitin in fungal cell walls (Sela-Buurlage *et al.,* 1993). Table 2.2 contains all the recognized families of PR-proteins as well as their specific properties.

2.4. RECEPTORS

As a first step in plant defense, the plant is required to recognize or to sense the presence of a pathogen. This is done through receptor proteins. Most known receptors are associated with the plasma membrane, although some are located either in the cell wall [WAK's] (He *et al.,* 1996) or in the cytosol of the cell (Salmeron *et al*., 1996).

The majority of receptor proteins that are activated upon elicitor recognition fall into five major classes based primarily upon their combination of a number of limited structural motifs (Martin *et al*., 2003).

Table 2.2: Recognized families of pathogenesis-related proteins.

Families	***Type member***	***Properties***	***Reference***
PR-1	Tobacco PR-1a	Antifungal	Antoniw *et al*., 1980
PR-2	Tobacco PR-2	β-1,3-glucanase	Antoniw *et al*., 1980
PR-3	Tobacco P, Q	Chitinase type I,II, IV,V,VI,VII	Van Loon, 1982
PR-4	Tobacco 'R'	Chitinase type I,II	Van Loon, 1982
PR-5	Tobacco S	Thaumatin-like	Van Loon, 1982
PR-6	Tomato Inhibitor I	Proteinase-inhibitor	Green and Ryan, 1972
PR-7	Tomato P_{69}	Endoproteinase	Vera and Conejero, 1988
PR-8	Cucumber chitinase	Chitinase type III	Métraux *et al*., 1988
PR-9	Tobacco 'lignin-forming peroxidase'	Peroxidase	Lagrimini *et al*., 1987
PR-10	Parsley 'PR1'	'Ribonuclease-like'	Somssich *et al*., 1986
PR-11	Tobacco 'class V' Chitinase	Chitinase, type I	Melchers *et al*., 1994
PR-12	Radish Rs-AFP3	Defensin	Terras *et al*., 1992
PR-13	Arabidopsis THI2.1	Thionin	Epple *et al*., 1995
PR-14	Barley LTP4	Lipid-transfer protein	García-Olmedo *et al*., 1995
PR-15	Barley OxOa (germin)	Oxalate oxidase	Zhang *et al*., 1995
PR-16	Barley OxOLP	'Oxalate oxidase-like'	Wei *et al*., 1998
PR-17	Tobacco PRp27	Unknown	Okushima *et al*., 2000

The classes are divided as follows; class 1 has a serine/threonine kinase catalytic region and a myristylation motif in the N–terminus region, while class 2 comprises of receptor proteins that has LRR, NBS and a LZ or CC motifs. Class 3 is very similar to class 2 but instead of a CC sequence the proteins have a region similar to the N–terminus of the IL 1R proteins, which are referred to as TIR regions. Class 4 lacks the NBS region but instead contains a transmembrane [TM] region with an extracellular LRR region and a cytoplasmic tail without any obvious motifs. The last identifiable class contains an extracellular LRR region, a TM region and a cytoplasmic serine/threionine kinase region. The last class of receptor proteins also contains the receptor protein kinases [RPK's] (Martin *et al*., 2003).

Various receptor proteins function as disease resistance proteins [R-proteins]. Some of the known plant disease R–proteins are listed in their various classes in table 2.3, while the specific domains that are involved in recognition are briefly discussed.

2.4.1. The LRR Domain

The LRR domain is present in a vast array of proteins of diverse functions and is implicated in protein–protein interactions (Kobe and Deisenhofer, 1994). The leucine rich repeats are tandem repeats of approximately 24 amino acids with the following consensus sequence: PXXLG-XLXXLXXLXLXXNXLXGXI (X represent non-conservative amino acids) (Torii *et al.*, 1996). Suitable support for LRR as a role player in signal recognition was presented by He *et al*. (2000). They replaced the extracellular LRR domain of Xa21 with that of BRI1, a receptor – like kinase involved in brassinosteriod perception. This yielded a brassinosteriod–inducible plant defense response in rice cells. Additionally, a point mutation in the LRR region of RPS5, an R–protein in *Arabidopsis* conferring resistance to *Pseudomonas syringae*, compromised the function of different, structurally related R–proteins. (Warren *et al*., 1998)

Table 2.3: Plant disease resistance proteins. Except for viruses, pathogen or pest type is indicated in parentheses, abbreviated as B, bacterium; F, fungus; I, insect; N, nematode and O, oomycete (Martin *et al*., 2003).

Class	***R–Protein***	***Plant***	***Pathogen***	***Reference***
1	Pto	Tomato	*Pseudomonas syringae* (B)	Kim *et al*., 2002
2	Bs2	Pepper	*Xanthomonas campestris* (B)	Minsavage *et al*., 1990
	Dm3	Lettuce	*Bremia lactucae* (F)	Meyers *et al*., 1998
	Gpa2[a]	Potato	*Globodera pallida* (N)	Van der Vossen *et al*., 2000
	Hero	Potato	*G. pallida* (N)	Ernst *et al*., 2002
	HRT[b]	*Arabidopsis*	Turnip Crinkle Virus	Cooley *et al*., 2000
	I2	Tomato	*Fusarium oxysporum* (F)	Ori *et al*., 1997
	Mi	Tomato	*Meloidogyne incognita* (N)	Milligan *et al*., 1998
	Mla	Barley	*Blumeria graminis* (F)	Zhou *et al*., 2000
	Pib	Rice	*Magnaporthe grisea* (F)	Wang *et al*., 1999
	Pi-ta	Rice	*M. grisea* (F)	Orbach *et al*., 2000
	R1	Potato	*Phytophthora infestans* (O)	Ballvora *et al*., 2002
	Rp1	Maize	*Puccinia sorghi* (F)	Collins *et al*., 1999
	RPM1	*Arabidopsis*	*P. syringae* (B)	Debener *at al*., 1991
	RPP8[b]	*Arabidopsis*	*Peronospora parasitica* (O)	McDowell *et al*., 1998
	RPP13	*Arabidopsis*	*P. parasitica* (O)	Bittner-Eddy *et al*., 2000
	RPS2	*Arabidopsis*	*P. syringae* (B)	Mindrinos *et al*., 1994
	RPS5	*Arabidopsis*	*P. syringae* (B)	Warren *et al*., 1998
	Rx1	Potato	Potato virus X	Bendahmane *et al*., 1995
	Rx2	Potato	Potato virus X	Bendahmane *et al*., 1995
	Sw – 5	Tomato	Tomato Spotted wilt Virus	Brommonschenkel *et al*., 2000
	Xa1	Rice	*X. oryzae* (B)	Yoshimura *et al*., 1998
3	L	Flax	*Melampsora lin*i (F)	Lawrence *et al*., 1995

	M	Flax	*M. Lini* (F)	Lawrence *et al*., 1995
	N	Tobacco	Tobacco Mosaic Virus	Lawrence *et al*., 1995
	P	Flax	*M. Lini* (F)	Dodds *et al*., 2001
	RPP1	*Arabidopsis*	*P. parasitica* (O)	Botella *et al*., 1998
	RPP4	*Arabidopsis*	*P. parasitica* (O)	van der Biezen *et al*., 2002
	RPP5	*Arabidopsis*	*P. parasitica* (O)	Parker *et al*., 1997
	RPS4	*Arabidopsis*	*P. syringae* (B)	Gassmann *et al*., 1999
4	Cf-2	Tomato	*Cladosporium fulvum* (F)	Dixon *et al*., 1998
	Cf-4	Tomato	*C. fulvum* (F)	Joosten *et al*., 1994
	Cf-5	Tomato	*C. fulvum* (F)	Dixon *et al*., 1998
	Cf-9	Tomato	*C. fulvum* (F)	Jones *et al*., 1994
5	Xa21	Rice	*Xanthomonas oryzae* (B)	Song *et al*., 1995

2.4.2. The NBS Region

Mutational analyses indicate a critical role for the NBS region (Tornero *et al*., 2002). It is thought that the NBS region affects R-protein function through nucleotide binding or hydrolysis, although to date these properties have not been reported. Several R-proteins align over a 320 amino acid region that include NBS, with the APAF–1 and CED–4, two proteins involved in regulating programmed cell death in animals (Van der Biezen *et al*., 1998). In addition, the alignment also contains five other short motifs of undefined function and was designated the NB–ARC [nucleotide binding in APAF–1, R–gene products, and CED–4] domain. Functional relevance of the alignment has not yet been determined, but it was suggested that R-proteins may control plant cell death by virtue of the NB–ARC domain, activated via LRR–dependent recognition of the pathogen (Van der Biezen *et al*., 1998).

Structure predictions suggest that the NB–ARC domain might be involved in ATP–dependent oligomerization (Jaroszewski *et al*., 2000) or histidine–aspartic acid phosphotransfer without nucleotide binding (Rigden *et al*., 2000).

2.4.3. The CC Motif

The CC structure is a repeated heptad sequence with interspersed hydrophobic amino acid residues of which the LZ is one example. It consists of two or more alpha helices that interact to form a supercoil. The motif is found in a variety of proteins with diverse functions and is implicated in protein–protein interactions including oligomerization and oligomerization dependent nucleic acid binding. The role of the CC domain in resistance is still to be unravelled, although it is thought to be involved in signaling rather than recognition (Aarts *et al*., 1998; Warren *et al*., 1999).

2.4.4. The TIR Domain

The TIR domain is implicated in signaling by its similarity to the cytoplasmic domain of Toll and IL–1R (Van der Biezen *et al*., 2002). It was shown by Dinesh-Kumar *et al*.

(2000) that deletions and point mutations lead to partial loss–of–function alleles or dominant negative alleles. In addition to signaling, the TIR domain can also play a part in pathogen recognition (Dinesh-Kumar *et al*., 2000). Initial search of plant EST databases suggested that monocots do not have TIR-NBS-LRR–like proteins (Pan *et al*., 2000). However a recent search has yielded a candidate TIR–domain containing protein on chromosome 1 of rice (Martin *et al*., 2003). The protein has an NB–ARC domain but lacks a typical LRR. It is located in a region containing a number of R–gene homologs and near a known R-locus, but whether it functions in disease resistance is not yet known.

2.4.5. Other Motifs and Important Structures

A myristylation motif is found in the sequence of Pto, but it is not required for AvrPto recognition when Pto is expressed from a strong promoter in transgenic plants (Loh *et al*., 1998). Covalent attachment of myristic acid to the N–terminal motifs targets a protein to the membrane. It has not been determined whether Pto is myristylated or membrane localized during recognition, but AvrPto shares and requires the myristylation motif (Shan *et al*., 2000). Several other bacterial elicitor proteins also appear to depend on myristylation in the plant cell for membrane localization and function (Nimchuk *et al*., 2000).

Another important domain is the serine/threonine kinase domain found in various R–proteins, such as Pto (Liu *et al*., 2002) and Xa21 (Song *et al*., 1995). These proteins play an important role in the plant's ability to respond to external stimuli and will be discussed as a group.

2.4.6. Receptor-Like Protein Kinases

Receptor-like protein kinases [RLK's] are a diverse group of proteins that span the plasma membrane and allow cells to recognize and respond to their extracellular environment (Becraft, 2002). A recent analysis showed that plant RLK's belong to a large monophyletic gene family that contains Pelle cytoplasmic kinases of animals

(Shiu and Bleecker, 2003). This family includes receptor kinases and non-receptor kinases (receptor-like cytoplasmic kinases) [RLCK], (Shiu and Bleecker, 2001).

The first RLK to be identified was ZmPK 1 of maize (Walker and Zhang, 1990). The *Arabidopsis* genome, which is completely sequenced, showed that *Arabidopsis* contains more that 600 genes coding for RLK's (Shiu and Bleecker, 2003). This suggests that higher plants use receptor kinase signaling commonly and broadly in response to a vast array of stimuli to modulate gene expression.

All of the plant RLK's thus far identified was shown to phosphorylate serine and threonine residues with the exception of two members that showed duel specificity (Mu *et al*., 1994; Shah *et al*., 2001). The extracellular domain of RLK's, which varies in structure, is used to classify plant RLK's into subfamilies. More than 21 different subfamilies have been identified up to date (Shiu and Bleecker, 2001). Some of the RLK's with known functions are tabulated in Table 2.4. The structures of several well-characterized RLK's are shown in Fig. 2.2.

2.5. SIGNAL TRANSDUCTION

2.5.1. Signaling in General

A variety of processes regulating growth, developmental and defense responses are triggered through phosphorylation events caused by protein kinases (Xing *et al*., 1996) and phosphatases (Braun *et al*., 1997). In addition to protein phosphorylation and dephosphorylation, other early signaling events in plant responses may involve ion channels (Hahlbrock *et al.*, 1995; Levine *et al*., 1996), GTP-binding proteins (Joo *et al*., 2005) and phospholipases (Van der Luit *et al*., 2000). Most of these signals are involved in normal plant metabolism but also play crucial roles in plant defense.

Table 2.4: Biological functions for plant receptor kinases (Haffani *et al*., 2004).

Gene Name	Biological Function	Reference
CRINKLY 4-like receptor kinases		
CRINKLY 4	Cell differentiation	Becraft *et al*., 1996
LRR receptor kinases		
CLV1	Apical meristems maintenance	Clark *et al*., 1997
ERECTA	Organ initiation and elongation	Torii *et al*., 1996
EXS	Anther and embryo development	Canales *et al*., 2002
EMS1	Microsporogenesis and tapetal development	Zhao *et al.*, 2002
HAESA	Floral organ abscission	Jinn *et al*., 2000
PRK1	Pollen development	Mu *et al*., 1994
VH1	Vascular development	Clay and Nelson, 2002
BAK1	Brassinosteroid signalling	Li *et al*., 2002a
BRI1	Brassinosteroid signalling	Li and Chory, 1997
PSK	Phytosulfokine signalling	Matsubayashi *et al*., 2002
Xa21	Race-specific resistance to bacterial blight in rice	Song *et al*., 1995
FLS2	Flagellin perception in the innate immunity response	Gómez-Gómez and Boller, 2000
HAR1/NARK	Rhizobial symbiosis and nodule proliferation	Searle *et al*., 2003
SYMRK/NORK	Rhizobial symbiosis and nodule initiation	Stracke *et al*., 2002
LRK10 – like receptor kinases		
LRK10	Resistance to wheat rust fungi	Feuillet *et al*., 1997
LysM receptor kinases		
NFR1/LYK3, NFR5	Rhizobial symbiosis and nodule initiation	Limpens *et al*., 2003
S – domain receptor kinases		
SRK	Female determination of *Brassica* self-incompatibility	Takasaki *et al*., 2000
WAK – like receptor kinases		
WAK's	Cell expansion	Anderson *et al*., 2001

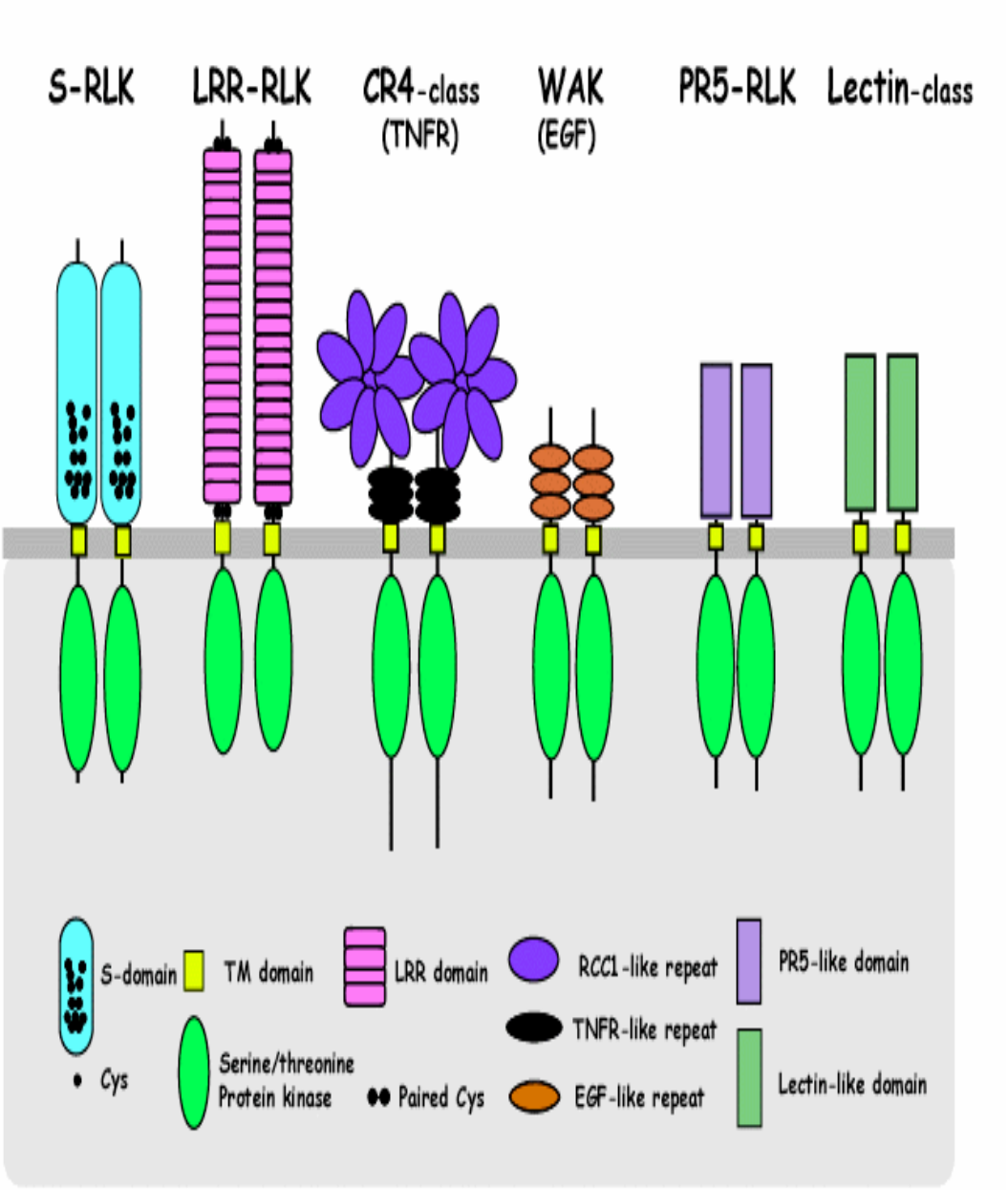


Fig. 2.2: Several extracellular domains of receptor–like kinases found in plants are indicated on which their classification is based (Torii, 2001).

2.5.1.1. Protein Kinases and Phosphatases

Protein kinases and phosphatases play a central role in signal transduction through the phosphorylation and dephosphorylation of proteins. This not only leads to the activation of defense responses, but also to the activation of developmental processes like cell growth and differentiation (Felix *et al*., 1991).

The discovery that the *Pto* resistance gene from tomato (Martin *et al*., 1993) and the *Xa21* resistance gene from rice (Song *et al*., 1995) encode serine/threonine protein kinases, strengthened the suggestion that protein phosphorylation plays a central role in signal transduction in disease resistance. In a situation where the R–gene encodes a cytosolic protein with serine/threonine kinase activity, the activated kinase may trigger a phosphorylation cascade.

The *Pto* gene is an example of this. Pto mediates resistance to bacterial speck disease caused by *P. syringae* pathovar tomato strains carrying the cognate *Avr* genes, *AvrPto* and *AvrPto*B (Martin *et al.,* 1993). It has been shown that Pto mediated resistance requires the NBS – LRR gene *Pfr* (Salmeron *et al*., 1996). It is thought that Pto and Pfr interacts in a receptor complex to bind AvrPto (Rathjen *et al*., 1999). A number of proteins were found that interact with Pto in yeast two-hybrid assays and are phosphorylated by Pto kinase activity *in vitro*. These include another kinase called Pti1 and a small family of transcription factor–like proteins called Pti4/5/6 (Zhou *et al*., 1995, 1997). Thus, a prevailing model of Pto mechanism of action states that Avr-activated Pto phosphorylates downstream targets, including the various Pti proteins, which in turn activate other downstream components of plant defense pathways (Pedley and Martin, 2003)

Other protein kinases that are also involved in signal perception and transduction are calcium-dependent protein kinases [CDPK's] and mitogen-activated protein kinases [MAPK's] (Fig. 2.3).

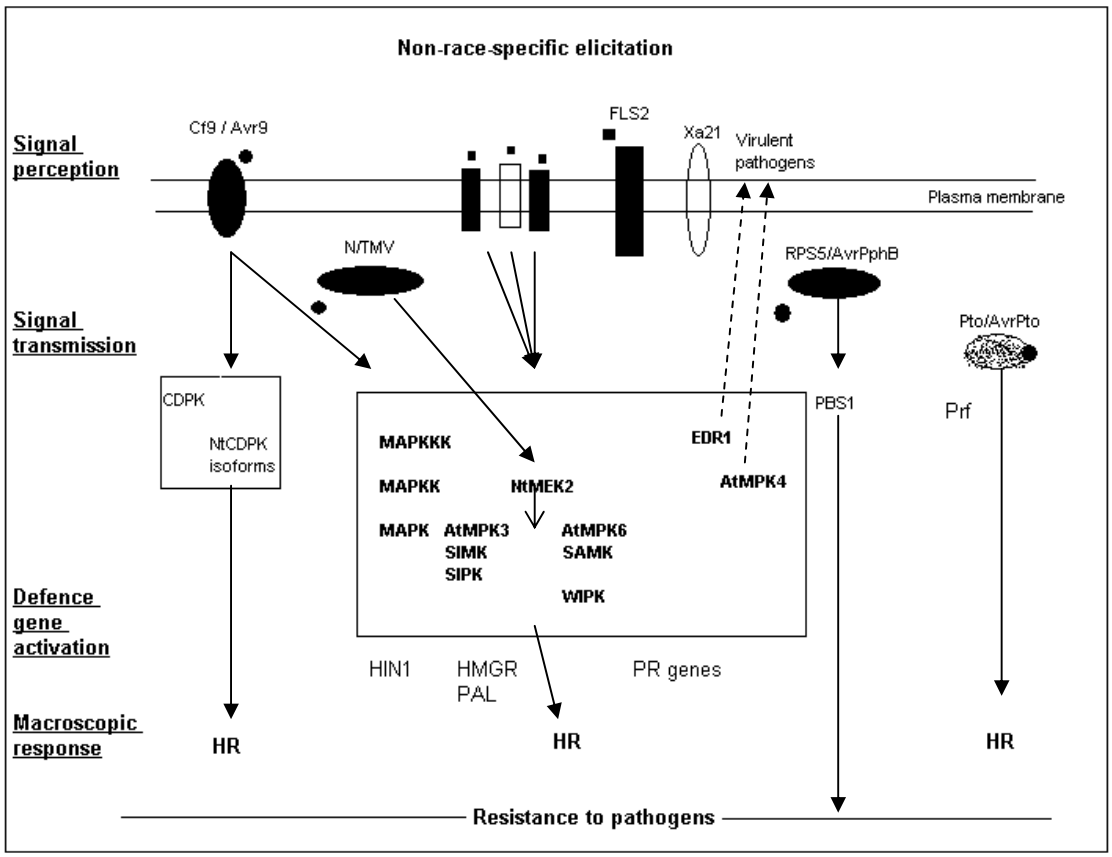


Fig. 2.3: Protein kinases involved in defense signaling (Romeis, 2001).

CDPK's comprise a family of plant-specific, multi-functional serine/threonine protein kinases in which a regulatory calcium-binding domain is directly linked to the kinase domains (Harmon *et al.*, 2000). CDPK's are ideally structured for sensing changes in intracellular calcium concentrations and translating them into kinase activity.

Since intracellular calcium levels are modulated in response to various signals such as hormones, light, abiotic stresses and pathogen elicitors, CDPK's have been implicated in general stress responses (Evans *et al*., 2001). Increasing evidence for the participation thereof in signal transduction during plant–pathogen interactions is being suggested since elevated calcium concentrations is a common consequence of pathogen perception (Gelli *et al*., 1997; Klusener *et al*., 2002).

MAPK's from several plant species were shown to be activated during plant defense response to elicitors or pathogens (Meskiene and Hirt, 2000; Ligterink *et al*., 1997). MAPK kinase signaling is complex since gene families for each of the three members of the phosphorylation cascade exists, namely mitogen-activated protein kinase, kinase, kinase [MAPKKK], mitogen-activated protein kinase, kinase [MAPKK] and MAPK. These enzymes are multi-functional and different iso-forms are activated upon pathogen-related elicitation compared to environmental stimuli (Romeis *et al*., 1999). The fact that MAP kinases were shown to be activated biochemically upon elicitation, suggests a regulatory role in defense signaling. One example of this is the *Arabidopsis edr1* mutant, isolated from a genetic screen (Frye and Innes, 1998). *EDR1* codes for a MAPKKK (Frey *et al*., 2001). The mutant line displayed enhanced resistance against the usually virulent bacterial strain *P. syringae* and the fungal powdery mildew pathogen *Erysiphe cichoracearum* (Frye and Innes, 1998). The recessive nature of the mutation suggests that EDR1 may function at the top of a MAP kinase cascade that negatively regulates defense responses (Frey *et al*., 2001).

It was found that reversible phosphorylation play an important role in the functioning of MAPK's (Asai *et al*., 2002). This implicated protein phosphatases in signaling pathways triggered by elicitors, since MAPK's can biochemically be activated by elicitors and are also implicated in defense signaling cascades (Tena *et al*., 2001).

Protein phosphatases can be divided into two groups according to their substrate specificity, namely serine/threonine protein phosphatases [PPases] and protein tyrosine phosphatases [PTPases] (Cohen, 1997). PPases specifically catalyze the dephosphorylation of phosphoserine and phosphothreonine. PPases can be subdivided into four groups according to their biochemical and pharmacological properties, namely PP1, PP2A, PP2B and PP2C (Cohen, 1989). A number of studies have implicated both PPases and PTPases in signal transduction pathways (Neel and Tonks, 1997).

One PPase that have been implicated in plant RLK signaling was KAPP [kinase associated protein phosphatase]. KAPP was isolated by Stone *et al*. (1994) after it was discovered that it interacted with a LRR-RLK, RLK5. They found that KAPP had three functional domains namely a N-terminal type 1 signal anchor, a KI [kinase interacting] domain and a type 2C protein phosphatase catalytic region.

KAPP was found to interact with various plant RLK's *in vitro* (Braun *et al*., 1997). Stone *et al*. (1998) demonstrated that KAPP bound to an autophosphorylated CLV1 recombinant protein, while it failed to do so to an inactive mutant version of CLV1. This showed that the interaction with KAPP was dependent on a functional protein kinase domain. They concluded that KAPP functions as a negative regulator of CLV1 signaling in plant development.

2.5.1.2. Ion Fluxes

Functioning immediately downstream of the initial elicitor recognition event, the activation of ion fluxes is an early response detected in plant cells (Jabs *et al*., 1997). Various bacterial and fungal elicitors have been reported to trigger fluxes of H^+, K^-, Cl^- and Ca^{2+} across the plasma membrane (Hahlbrock *et al.*, 1995).

These processes occur prior to the activation of defense related gene expression and suggest that the ion fluxes are regulated through plasma membrane bound enzymes. These enzymes include Ca^{2+}-ATPases (Pei *et al*., 2000) and H^+-ATPases (Vera-Estrella *et al*., 1994; Schaller and Oecking, 1999).

Atkinson *et al*. (1996) demonstrated that Ca^{2+} ion channel blockers not only inhibited ion fluxes but also defense responses induced by fungal and bacterial elicitors. On the other hand, an increase in extracellular Ca^{2+} concentration was shown to activate defense responses in tobacco (Suzuki *et al*., 1995; Levine *et al*., 1996). Thus, calcium seems to play an integral role throughout plant defense, since it is necessary for the activation of CDPK's and acts as a second messenger (Grant *et al*., 2000).

H^+-ATPase activity seems to be regulated by reversible phosphorylation, which appears to prevent prolonged stimulation that could otherwise result in cell death (Mittler *et al*., 1995). H^+-ATPase activity was shown to increase with the dephosphorylation of the membrane bound H^+-ATPase (Vera-Estrella *et al*., 1994). This led to the acidification of the extracellular medium. The rephosphorylation was shown to be mediated by a Ca^{2+}/calmodulin dependent protein kinase, which in turn is activated by a Ca^{2+} dependent protein C-like kinase (Xing *et al*., 1996). It has also been reported that several fungal toxins target H^+-ATPases in activating them (Wevelsiep *et al*., 1993). This leads to the prolonged stimulation of H^+-ATPases, causing acidification of the extracellular region of the cell, which will result in cell death. One example of such a toxin is NIP1 from the barley pathogen *Rhynchosporium secalis* (Wevelsiep *et al*., 1993). NIP1 function as a specific Avr elicitor on host plants carrying the *Rrs1* resistance gene (Rohe *et al*., 1995).

2.5.1.3. G-Proteins

Another player in plant signaling is the G-protein (Joo *et al*., 2005). Heterotrimeric G-proteins have been implicated in several processes during growth and development and transduce extracellular environmental signals into the cell (Ullah *et al*., 2003). A possible direct involvement of G-proteins in the stimulation of Ca^{2+} channel activity and in CDPK activation has been suggested (Gelli *et al*., 1997). This was based on the action of G-protein inhibitors and activators on elicitor activation of Ca^{2+} channels (Gelli *et al*., 1997) and on the absence of a requirement for diacylglycerol [DAG] which is needed for the activation of protein kinase C [PKC] during NADPH oxidase assembling in neutrophil cells (Suharsono *et al*., 2002). In addition, G-proteins also play a role in the regulation of stomata opening (Assmann, 1996), pollen tube

elongation in lily (Ma *et al*., 1999), and light signaling pathways in tomato cells (Neuhaus *et al*., 1993). G-proteins might also be involved in phospholipid signaling seeing that it activates phospholipase D [PLD] (Munnik *et al*., 1995).

2.5.1.4. Phospholipid Signaling

Phospholipid-derived molecules produced by the enzymes phospholipase C [PLC], PLD and phospholipase A2 [PLA2] are emerging as novel second messengers in plant defense signaling (Munnik *et al*., 1998; Wang *et al*., 2000). One of these second messengers produced, is phosphatidic acid [PA] (Lee *et al*., 2001).

PA levels have been shown to increase in plants within a few minutes after the onset of a variety of stress treatments including ethylene (Lee *et al*., 1998), wounding (Wang *et al*., 2000), pathogen elicitors (Van der Luit *et al*., 2000), osmotic and oxidative stress (Sang *et al.*, 2001) and abscisic acid [ABA] (Jacob *et al*., 1999). Various PA targets are also emerging that includes CDPK's (Farmer and Choi, 1999), MAPK's (Lee *et al*., 2001) and a K^+ channel (Hahlbrock *et al*., 1995) amongst others. This signifies the important role that PA might play in signaling and especially in defense signaling.

What is especially interesting is that apart from all the biochemical effects that PA influences, it also influences the physical properties of the membrane (Liscovitch *et al*., 2000). When PLD hydrolyses a zwitterionic phosphatidylcholine molecule to produce negatively charged PA, the surface properties change dramatically, affecting membrane curvature and the ability to form vesicles. In this way, PA could play a key role in vesicle trafficking, membrane recycling and secretion (Liscovitch *et al*., 2000).

2.5.2. Signal Molecules as a Defense Mechanism

Various signal molecules such as ROS (specifically H_2O_2), NO, SA, JA and ethylene have been documented to play a role in plant defense signaling. There is also a great deal of evidence indicating the cross talk between the above-mentioned molecules.

Evidence for the complexity of pathway interactions was provided in genetic experiments in *Arabidopsis* (Bowling *et al*., 1997; Clarke *et al*., 1998). They identified a recessive cpr5 and a dominant cpr6 mutant that constitutively produced high SA levels and expressed both SA- and JA/ethylene-dependent marker genes. The mutant plants also exhibited increased resistance to virulent *Pseudomonas syringae* and *Pseudomonas parasitica* strains. All of these phenotypes are SA–dependent, but differ in their requirement for non-expressor of PR [*NPR1*]. In additional experiments a dominant ssi1 mutant was identified, which completely bypassed NPR1 function (Shah *et al*., 1999). It was also found that ssi1 expressed the JA–dependent marker PDF1.2 in an SA – dependent manner (Shah *et al*., 1999). This suggested that the SSI1 protein together with CPR5 and CPR6 may participate in signal communication between SA-and JA/ethylene-dependent pathways (Fig 2.4).

Delledonne *et al*. (2001) reported the relationship between NO and H_2O_2. It is known that both NO and H_2O_2 induce phenylalanine ammonia lyase [PAL], which leads to an increase in SA (Delledonne *et al*., 1998; Desikan *et al*., 1998a). Navarre *et al*., (2000) as well as Zeier *et al*., (2004), showed that the application of NO to plants led to the inhibition of catalase, ascorbate peroxidase and aconitase, which are all enzymes involved in cellular protection through the scavenging of H_2O_2, thus, proposing a major role for NO in the signaling network of plant defense responses. Recently, it was shown that elevated NO concentration in tomato leaves strongly decreased H_2O_2 concentration without affecting other ROS (O_2^- and OH^-) levels (Malolepsza and Rozalska, 2005). In addition, activities of enzymes such as superoxide dismutase and catalyse was unchanged in the studied plants, indicating a direct NO–H_2O_2 interaction whereby NO modulated H_2O_2 levels (Malolepsza and Rozalska, 2005).

A large number of genes involved in plant defense are regulated positively and negatively by these signals. These defense genes include PR-1 (Zhang *et al*., 1999), PAL and glutatione S-transferase [GST] (Desikan *et al*., 1998a).

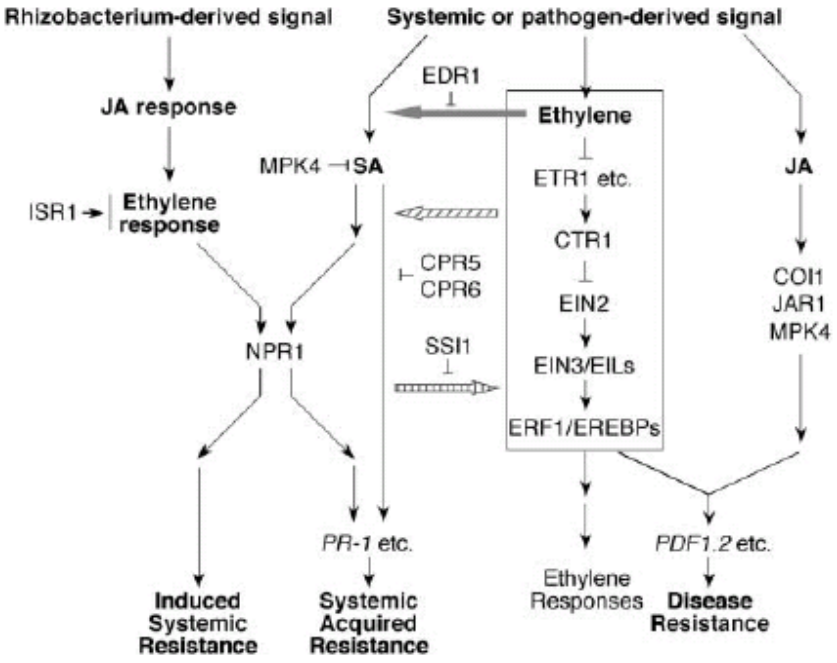


Fig. 2.4: Interactions between the ethylene signal transduction pathway and plant disease resistance. The ethylene signal transduction pathway can interact with the JA pathway to co-regulate expression of a subset of defense-related *PR* genes, for example, *PDF1.2*, involved in plant disease resistance. Meanwhile, there are considerable interactions between JA/ethylene-and SA-dependent pathways in systemic acquired resistance. In *edr1* mutant, ethylene potentiates SA-mediated *PR-1* gene expression. In the absence of CPR5 and CPR6, the ethylene pathway can also activate SA-dependent *PR-1* gene expression independent of NPR1 to promote systemic acquired resistance. In the *ssi1* mutant, the JA/ethylene-dependent *PDF1.2* gene is constitutively expressed. Moreover, the ethylene pathway is also required for the rhizobacteria-mediated induced systemic resistance, which is independent of SA and pathogenesis-related gene activation. Ethylene signaling acts downstream of the JA pathway but upstream of NPR1 in ISR activation. Plants that lack ISR1 fail to develop ISR and display ethylene insensitivity. Arrows indicate positive regulation, and open blocks indicate negative regulation (Wang *et al*., 2002).

2.5.2.1. The Oxidative Burst as a Secondary Messenger

2.5.2.1.1. Mechanisms for the Generation of ROS

One of the earliest events that occur after elicitation is the rapid production and accumulation of reactive oxygen species such as O_2^- and H_2O_2 (Doke *et al*., 1996). This is known as the oxidative burst.

Elicitation of the oxidative burst by pathogen elicitors appears to be mediated through multiple signaling cascades, which may include G – protein signaling (Diekmann *et al*., 1994), Ca^{2+} influx, H^+/K^+ exchange and protein phosphorylation (Low and Merida, 1996). The generation of ROS in an incompatible plant-pathogen interaction is thought to be mediated by a plasma membrane bound NADPH oxidase, a mechanism analogous to the mammalian NADPH oxidase system (Keller *et al*., 1998).

It is proposed that this system is activated through phosphorylation (Desikan *et al*., 1996). In addition to NADPH oxidase, pH-dependent cell wall peroxidases (Elstner and Heupel, 1976), germin-like oxalate oxidases (Hurkman and Tanaka, 1996a and b) and amine oxidases (Angelini *et al*., 1993 and 1995) have been proposed as sources of H_2O_2 in the apoplast.

2.5.2.1.2. NADPH Oxidases

The NADPH oxidase system has received the most attention of all systems involved in the oxidative burst. Chemical inhibitors of NADPH oxidases, such as diphenylene iodonium [DPI], have been shown to block or severely reduce ROS production upon biotic and abiotic stresses (Pellinen *et al*., 1999). It was also shown that the accumulation of H_2O_2 in wounded or systemin-treated tomato leaves was inhibited by DPI (Orozco-Cardenas and Ryan, 1999). In several other model systems studied, it appears that a membrane bound NADPH complex is responsible for the oxidative burst (Keller *et al*., 1998). Thus, these results suggested that NADPH oxidase is responsible for the synthesis of O_2^-, which is consistent with the hypothesis of Auh and Murphy (1995) that the synthesis of O_2^- is catalysed by a membrane bound enzyme.

The NADPH oxidase present in mammalian neutrophils, consists of five components: $p40^{phox}$ (phox for phagocyte oxidase), $p47^{phox}$, $gp91^{phox}$, $p22^{phox}$ and $p67^{phox}$ (Babior, 1999). Two of these components, $gp91^{phox}$ and $p22^{phox}$, are membrane located proteins, which together form a heterodimeric flavocytochrome b-558 (Segal and Abo, 1993). The other three components, $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$, exist in the cytosol as a complex of regulatory proteins (Babior, 1999). When resting cells are stimulated, the cytosolic component $p47^{phox}$ becomes heavily phosphorylated and the entire cytosolic complex is recruited to the membrane, where it associates with the two membrane – bound components to assemble the active oxidase. Activation requires not only the participation of the core components, but also the participation of two low molecular weight guanine – nucleotide – binding proteins, Rac2 and Rap1A (Babior, 1999). Tenhaken *et al*. (1995) showed the existence of plant homologues of $p22^{phox}$, $p47^{phox}$ and $p67^{phox}$ through the production of antibodies raised against the human proteins.

The examination of the primary structure of the plant homologue of NADPH oxidase showed important regulatory differences to the mammalian enzyme, which is activated by cytosolic polypeptides. Torres *et al*. (2002) succeeded in the cloning of a respiratory burst oxidase homolog [Rboh] in *Arabidopsis* with a protein size of 105-kD with a C-terminal region with high similarity to the 69-kD apoprotein of the $gp91^{phox}$. In the case of the plant protein, there is an additional N-terminus with EF-hands suggesting its direct activation by Ca^{+2} ions (Sagi and Fluhr, 2001). In *Arabidopsis* six genes coding for homologues of mammalian NADPH oxidases have been characterized (Torres *et al*., 1998), while one was found in rice (Groom *et al*., 1996).

2.5.2.1.3. pH Dependent Peroxidases

The production of ROS by pH-dependent cell wall peroxidases has been proposed as an alternative for the oxidative burst during biotic stress (Bolwell *et al*., 1995). It seems that pH-dependent cell wall peroxidases are activated by an alkaline pH and in the presence of a reductant, H_2O_2 is formed (Kiba *et al*., 1997). Alkalinization of the apoplast upon elicitor recognition precedes the oxidative burst and the production of H_2O_2 by pH dependent cell wall peroxidases during biotic stress (Bolwell *et al.*, 1995).

It was reported by Robertson *et al*. (1995) that in elicitor treated French bean cells, H_2O_2 appears to originate from a cell wall peroxidase in a three-component system requiring ion fluxes and leading to extracellular alkalinization and the release of a reductant. Evidence for a peroxidase-dependent oxidative burst in lettuce infected by *Pseudomonas syringae* pv *phaseolicola* was found by Bestwick *et al*. (1997) where the highly localized accumulation of H_2O_2 was detected cytochemically using $CeCl_3$. This H_2O_2 production was found to be more sensitive to inhibitors of peroxidases (KCN and NaN_3) than to inhibitors of NADPH oxidases (DPI). This peroxidase is capable of producing H_2O_2 at neutral pH (Zimmerlin *et al*., 1994). More recently a peroxidase gene designated *FBP1* from French beans were characterized (Blee *et al*., 2001). This specific peroxidase is believed to be responsible for the apoplastic oxidative burst demonstrated by suspension-cultured cells in response to fungal elicitor. They also showed that FBP1, when expressed in *Pichia pastoris*, generated H_2O_2 using cysteine at pH 7.2, a specific property of the native protein when isolated from suspension-cultured cells.

2.5.2.1.4. Germin-like Oxalate Oxidases

The specialized family of germin-like proteins include the oxalate oxidases in cereals, which can generate H_2O_2 from oxalate under acidic conditions that might arise from vascular damage caused by pathogen attack (Dumas *et al*., 1995).

Some evidence of germin-like oxalate oxidases in the production of H_2O_2 was found in barley where the activity of germin-like oxalate oxidases was induced upon infection by *Erysiphe graminis f.sp hordei* but not wounding (Dumas *et al*., 1995). Similar gene expression of germin-like oxalate oxidases was found in wheat after infection by *Erysiphe graminis f.sp tritici*, although this induction was reported in both susceptible and resistant cultivars (Hurkman *et al*., 1996b). Thompson *et al*. (1995) tried to exploit the possibility of germin-like oxalate oxidases expression in plants to increase their resistance to fungal pathogens. However, initial data indicated that germin gene expression and oxalate oxidase activity in transgenic dicotyledoneous plants were regulated more or less similarly to those observed in monocotyledonous species.

It was also found that the expression of germin-like oxalate oxidases was induced upon treatment with plant growth regulators, SA, MeSA and JA as well as in response to salt stress (Hurkman *et al*., 1996a). This evidence might then rather implicate germin-like oxalate oxidases in a general defense response, rather than in a cultivar-specific reaction.

2.5.2.1.5. Amine Oxidases

Amine oxidases catalyse the oxidation of a wide variety of biogenic amines to their corresponding aldehydes with the release of NH_3 and H_2O_2 (Bolwell and Wojtaszek, 1997). This H_2O_2 that is formed can directly be used by wall bound peroxidases for lignification and cell wall strengthening both for normal growth and in response to external stimuli such as pathogenesis (Allan and Fluhr, 1997). A previous report has implicated amine oxidases in responses to infection (Angelini *et al*., 1993). They found increased activities of both peroxidases and diamine oxidases in chickpea stems inoculated with *Ascochyta rabiei.* Using histochemical staining, it was shown that the activation was localized to the lignosuberized barriers set up by cortical and pith parenchyma cells in response to infection.

The oxidase activity of lentil seedling amine oxidases was tested with a variety of substrates. It was found that various amines as well as L-ornithine and L-lysine at high concentrations and β-alanine and γ-aminobutyric acid was utilized as substrates in the production of H_2O_2 (Medda *et al*., 1996)

2.5.2.1.6. Other Sources of ROS

The mitochondrion is a major source of ROS and it is possible that this organelle could contribute to the oxidative burst (Tiwari *et al*., 2002). Braidat *et al*. (1999) showed that oxidative stress, when applied at mild constant levels to *Arabidopsis* cells led to increased respiratory electron transport and oxygen uptake in isolated mitochondria and finally to increased H_2O_2 levels.

Potentially, chloroplasts could also generate ROS (Field *et al*., 1998). Two primary processes involved in the formation of ROS during photosynthesis are the direct photoreduction of O_2 to O_2^- by reduced electron transport components associated with photo system I [PS-I] and reactions linked to photorespiratory cycle. This includes Rubisco in the choloroplast and glycolate–oxidase and catalase [CAT]–peroxidase reactions in the peroxisomes (Asada, 1999). Superoxide radicals generated by the one–electron reduction of molecular oxygen by PS-I are rapidly converted within the chloroplast to H_2O_2 by Cu-Zn SOD (Asada, 1999).

Another potential source of ROS could be chlororespiration. Chlororespiration describes the reduction of oxygen resulting from the presence of a respiratory chain consisting of a NAD(P)H dehydrogenase and a terminal oxidase in chloroplasts that competes with the electron transport chain for reducing equivalents (Field *et al*., 1998). Only in recent years has such a system been found in higher plants (Nixon, 2000). It is however still unknown to what extent this process might contribute to ROS formation in chloroplasts of higher plants (Field *et al*., 1998).

2.5.2.1.7. ROS Scavenging Machinery in Plant Cells

In plant cells, enzymes and redox metabolites act in synergy to carry out ROS detoxification. SOD catalyses the dismutation of O^-_2 to H_2O_2, CAT dismutates H_2O_2 to oxygen and water, ascorbate peroxidases [APX] reduces H_2O_2 to water by utilising ascorbate as a specific electron donor and glutathione peroxidase [GPX] oxidises glutathione to form oxidized glutathione (Noctor and Foyer, 1998).

Of all these mentioned scavenging mechanisms, SOD contributes to resistance in producing H_2O_2 from O_2^-, thus playing a major role in plant defense (Gupta *et al*., 1993).

2.5.2.1.8. Superoxide Dismutases

ROS are continuously produced in both stressed and unstressed plants (Apel and Hirt, 2004). Therefore plants have a well-developed defense system against ROS, involving limiting the formation of ROS as well as instituting its removal. Within a cell, the SOD's constitute the first line of defense against ROS (Ogawa *et al*., 1997). O_2^- is produced at any location where an electron transport system is present and hence O_2^- activation may occur in different compartments of the cell (Alscher *et al*., 1997). This being the case, it is not surprising that SOD's are found throughout all subcellular locations (Fridovich, 1986; Kliebenstein *et al*., 1998).

Based on the metal cofactor used by the enzyme, SOD's are classified into three groups: iron SOD [Fe SOD], manganese SOD [Mn SOD] and copper-zink SOD [Cu-Zn SOD] (Alscher *et al*., 2002). The three groups have different cellular localizations. Fe SOD's are predominantly found in the chloroplasts (Salin, 1988), Mn SOD has been located in the peroxisomes and mitochondria (Del Rio *et al*., 1992) and Cu-Zn SOD's are found throughout the plant cell (Alscher *et al*., 2002).

There are two different groups of Cu-Zn SOD's. The first group consists of cytoplasmic and periplasmic forms, which are homodimeric (Kliebenstein *et al*., 1998). The second group comprises the chloroplasmic and extracellular Cu-Zn SOD's, which are homotetradimeric (Bordo *et al*., 1994). Ogawa *et al*. (1997) have proposed that Cu-Zn SOD's in the apoplast functions in the lignification of the cell wall and that it protects the nucleus against fatal mutations caused by O_2^- molecules. Recently Ivanov *et al*., (2005) established that after infecting susceptible and resistant (expressing HR) wheat plants with leaf rust (*P. recondita* f.sp. *tritici*), the levels of hydrogen peroxide and superoxide dismutase activity in the resistant plants were constitutively higher than in the susceptible plants. Also in the susceptible plants, an inhibition of activity of catalase and GST was found.

2.5.2.1.9. Plant Defense Signaling Through ROS.

A well-established role for H_2O_2 is to function as a signal molecule during HR (Clarke *et al*., 2000b). When H_2O_2 is produced in response to pathogen infection, it mediates the cross-linking of cell wall proteins (Bradley *et al*., 1992) and plant cell wall phenolics (Grant and Loake, 2000). Although this is still somewhat controversial, H_2O_2 may also have some antimicrobial function (Wu *et al*., 1995). However, the most important function of H_2O_2 and O_2^- is thought to act as second messengers, not only to induce plant defense related genes, but also the hypersensitive host cell death (Desikan *et al*., 2000).

The expression of defense related genes such as GST and glutatione peroxidase has been shown to be induced by H_2O_2 in soybean (Levine *et al*., 1994), while H_2O_2 induced the expression of GST and PAL in *Arabidopsis* suspension cultures (Desikan *et al*., 1998a). Recently, a tobacco gene encoding a proteasome subunit was identified after being induced by H_2O_2 (Etienne *et al*., 2000). All 4 these genes are implicated to function during plant defense. GST comprises a family of enzymes involved in cellular detoxification processes following various stresses (Foyer *et al*., 1997), GPX scavenge H_2O_2 in the ascorbate-glutatione cycle (Foyer *et al*., 1997), PAL is an enzyme involved in the synthesis of defense-related compounds (Mauch-Mani and Slusarenko, 1996), while proteasomes are involved in protein degradation, a common feature of the HR cell death response (Etienne *et al*., 2000).

Micro-array analysis identified a large number of up-regulated genes after H_2O_2 treatments (Desikan *et al*., 2001a). The genes found to be up regulated coded for antioxidant enzymes, defense and stress related proteins, transcription factors, protein phosphatases and protein kinases (Desikan *et al*., 2001b). This demonstrated that H_2O_2 can modulate the expression of a subset of genes within the *Arabidopsis* genome and also generates the possibility that H_2O_2 can act as a modulator of gene expression in other plants. Thus, H_2O_2 is being proposed as a major player in cell signaling.

A further role for H_2O_2 in signaling events was demonstrated by Pei *et al*. (2000). They demonstrated that calcium channels were activated by H_2O_2 in intact *Arabidopsis*

guard cells. Thus, if calcium elevation is an early response to H_2O_2, it is likely that the activation of calcium dependent protein kinases may mediate downstream signaling together with protein kinases/phosphatases and other effector proteins. Researchers have suggested that reversible protein phosphorylation is a key-regulating event in the oxidative burst in response to plant-pathogen interaction (Levine *et al*., 1994; Desikan *et al*., 1996). Pharmacological data has shown that reversible protein phosphorylation is indeed involved in downstream signaling following H_2O_2 generation and/or perception (Grant *et al*., 2000).

A protein phosphorylation cascade that has been shown to be activated by H_2O_2 is a MAPK cascade in *Arabidopsis* suspension cultures (Desikan *et al*., 1999). Other MAPK's that are known to be activated in *Arabidopsis* are MPK3 and MPK6 via MAPKKK ANP1 (Kovtun *et al*., 2000). Overexpression of ANP1 in transgenic *Arabidopsis* plants resulted in increased tolerance to heat shock, freezing and salt stress (Kovtun *et al*., 2000). H_2O_2 also increases expression of the *Arabidopsis* nucleotide diphosphate [NDP] kinase 2 (Moon *et al*., 2003). Overexpression of AtNDPK2 reduced accumulation of H_2O_2 and enhanced tolerance to multiple stresses including cold, salt and oxidative stress. The effect of AtNDPK2 might be mediated by the MAPK's, MPK3 and MPK6 because NDPK2 can activate the MAPK's (Moon *et al*., 2003). This suggests a scenario in which various stresses induce ROS generation that in turn activate MAPK signaling cascades. In addition to MAPK's, a receptor–like protein kinase gene that is transcriptionally activated by ROS has been identified in *Arabidopsis* (Czérnic *et al*., 1999).

It has been speculated that ROS would have the ability to inhibit protein phosphatases. Recent work by Van Montfort *et al*. (2003) has confirmed these speculations since they showed that the human protein tyrosine phosphatase PTPB1 was modified by H_2O_2 at the active cysteine site. The inactivation of PTPB1 was found to be reversible and could be brought about by incubation with glutathione. A similar regulation likely occurs in plants because PTP1, an *Arabidopsis* PTP that can inactivate the *Arabidopsis* MPK6, can be inactivated by H_2O_2 (Gupta and Luan, 2003). Also, phosphatases involved in ABA signaling within guard cells have been identified whose *in vitro* activity was modulated reversibly by H_2O_2 (Meinhard *et al*., 2002)

2.5.2.2. Nitric Oxide

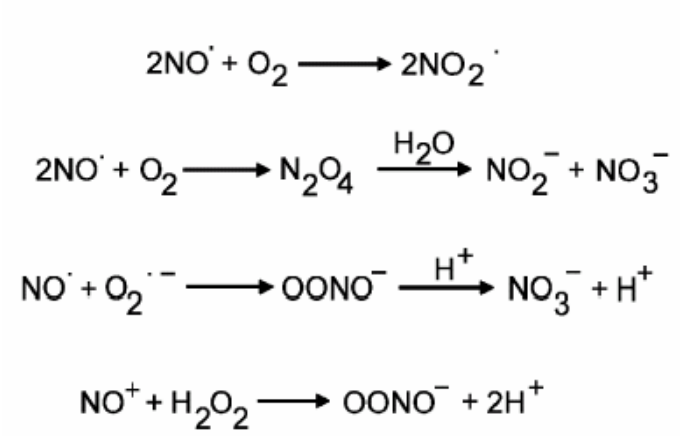
2.5.2.2.1. Signaling in Defense

In recent years NO, which is a water- and lipid-soluble gas, has emerged as a major signal molecule of ancient origin and ubiquitous importance (Durner *et al*., 1999). While it is known that NO functions as an important redox-active signal in the induction of innate immunity in animals, it also has a broad spectrum of other regulatory functions in the central nervous and cardiovascular systems including programmed cell death (PCD; Moncada *et al*., 1991). In 1998 NO synthase [NOS] activity was monitored in Tobacco mosaic virus [TMV] infected resistant and susceptible tobacco plants (Durner *et al*. 1998). It was observed that NOS activity increased dramatically in resistant plants but not in susceptible plants. In another publication by Delledonne *et al*. (1998), NO was described as a defense signal in plants. These were the first two articles to implicate NO in plant defense response.

NO is a gaseous free radical which can react rapidly with oxygen to form nitrogen dioxide [NO_2], which in turn is rapidly degraded to nitrate and nitrite in an aqueous solution (Fig. 2.5a) (De Gara *et al.,* 2003). NO can also react with O_2^- and H_2O_2 to form the ion peroxynitrite [$OONO^-$], which is a reactive as well as a destructive compound. Such interaction between NO and ROS has been reported during plant-pathogen interactions (Delledonne *et al*., 2001). It can also be noted that both NO and H_2O_2 are responsible for the regulation of various genes implicated in plant defense (Fig. 2.6).

The application of exogenous NO to plants has provided some insights into the role that NO plays in the physiological functions of plants (Huang *et al*., 2002a). For example, the application of NO to plants leads to the inhibition of catalase, ascorbate peroxidase and aconitase, which are all enzymes involved in cellular protection through the scavenging of H_2O_2 (Navarre *et al*., 2000). Nitric oxide has also been reported to play a mediating role in cell wall lignification (Ferrer and Ross-Barcelo, 1999), the regulation of ion channels of guard cells (Garcia-Mata *et al*., 2003), mitochondrial and chloroplastic functionality (Zottini *et al*., 2002; Takahashi and Yamasaki, 2002), cell death (Hung and Kao, 2003), senescence, accumulation of ferritin (Murgia *et al*., 2002) and wound signaling (Orozco-Cardenas and Ryan, 2002).

A



B

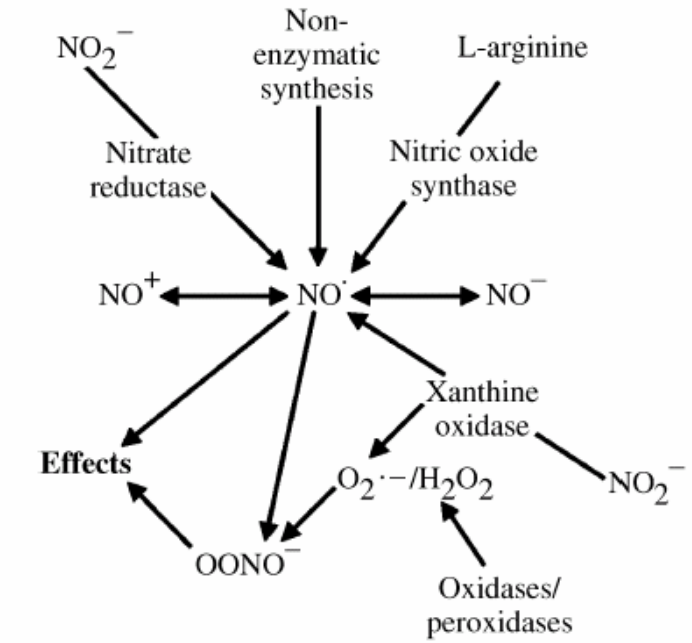


Fig. 2.5: Reactions and production of nitric oxide. **(A)** Some of the reactions of nitric oxide (NO). NO can react with oxygen, and in aqueous solution such reaction will lead to the generation of nitrite and nitrate. NO, either as the radical or NO+ ion, can react with superoxide and hydrogen peroxide, respectively, to produce peroxynitrite. NO can also undergo many other reactions with bio-molecules, not shown here. **(B)** The production of nitric oxide (NO). There is evidence for several potential sources of NO in plants, including nitric oxide synthase (NOS), nitrate reductase (NR), xanthine oxidoreductase or nonenzymatic sources. Once generated, NO can induce various effects, or react with reactive oxygen species to generate peroxynitrite. It should be noted that NO can exist in three forms, and although it is implied here that only the radical is biologically active, both the NO^+ and NO^- may have biological effects (Neill *et al*., 2003).

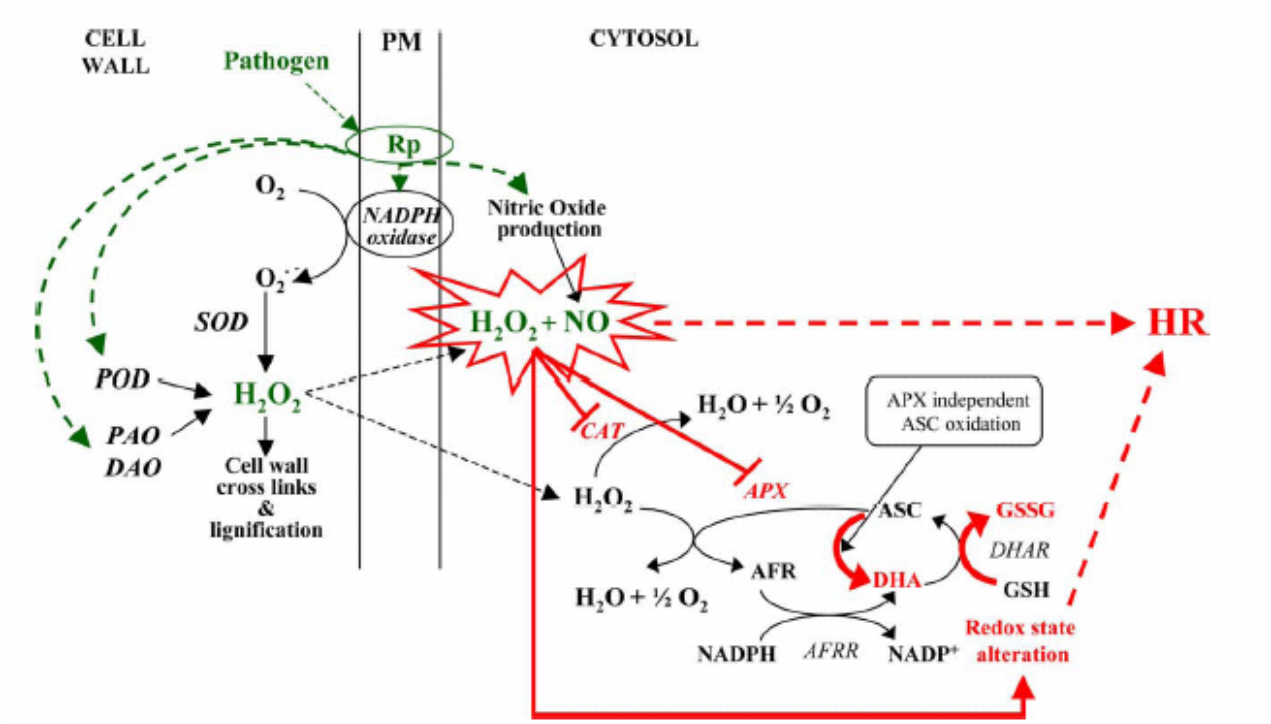


Fig. 2.6: Changes in the antioxidant system induced during HR. An oxidative burst producing reactive oxygen and/or nitrogen species is a precocious response activated by plant cells against pathogens. These reactive species, in spite of hindering pathogen penetration, also act as signal molecules for the activation of the defense responses leading to HR. The suppression of ROS scavenging enzymes (APX and CAT) contributes to the oxidative burst with ROS producing enzymes activated by pathogen attack. The imbalance of ASC/DHA, GSH/GSSG redox pairs is also part of the transduction pathway triggering HR. APX, ascorbate peroxidase; ASC, ascorbate; AFR, ascorbate free radical; AFRR, ascorbate free radical reductase; CAT, catalase; DAO, diamine oxidase; DHA, dehydroascorbic acid; DHAR, dehydroascorbic acid reductase; GSH glutathione; GSSG, glutathione disulfide; HR, hypersensitive response; NO, nitric oxide; PAO, polyamine oxidase; PM, plasma membrane; POD, secretory peroxidase; RP, receptor proteins; SOD, superoxide dismutase (De Gara *et al.,* 2003)

.

Together with the above mentioned, it has been demonstrated that NO mediates ABA-induced stomatal closure and that ABA in turn induces rapid NO production in guard cells (Neill *et al*., 2002). Some studies has also implicated NO in the role of inducing gene expression of certain defense related genes such as *AOX1a* (Huang *et al*., 2002a), *PR-1* (Durner *et al*., 1998), *CHS* (Guo *et al*., 2003), *AtNOS1* (Guo *et al*., 2003), several peroxidases (Huang *et al*., 2002b), GST (Delledonne *et al*., 1998), *PAL* (Delledonne *et al*., 1998) and key enzymes in the JA biosynthesis pathway (Huang *et al*., 2004).

There are several potential sources of NO in plants (Fig. 2.5b) and it seems likely that the importance of each of these sources of NO will depend on the plant itself, the specific species, environmental conditions and the signals involved in activating the specific source of NO. Evidence suggests that some or all of these sources might be involved simultaneously in producing NO (De Gara *et al*., 2003).

There is evidence to suggest some NOS-like enzymes and nitrate reductase are the main sources of NO in plants (Corpas *et al*., 2002; Rockel *et al*., 2002). However, it is likely that other sources like xanthine oxidoreductase (Godber *et al*., 2000), peroxidase (Huang *et al*., 2002b), cytochrome P450, some heme-proteins (Boucher *et al*., 1992a and b) as well as other non-enzymatic sources might also play a role (Cooney *et al*., 1994).

2.5.2.2.2. Nitric Oxide Synthase [NOS]

NOS is the main NO producer in animal systems (Moncada *et al*., 1991). The enzyme catalyses the oxygen- and NADPH–dependent oxidation of L-arginine to NO and citrulline in a complex reaction requiring flavin adenine dinucleotide [FAD], flavin mono nucleotide [FMN], Tetrahydrobioterpin [BH_4], calcium and calmodulin (Alderton *et al*., 2001). In mammals there are three characterized genes that code for NOS. Two of these genes are constitutively expressed, namely endothelial NOS [eNOS] and neuronal NOS [nNOS], while another form, inducible NOS [iNOS] can be induced in the presence of lipopolysaccharide or interferon (Neill *et al*., 2003).

NOS activity has been detected in some plant species (Table 2.5). The presence of NOS has also been detected immunologically in wheat (Kuo *et al*., 1995), pea (Sen and Cheema, 1995) and maize (Ribeiro *et al*., 1999). However, Butt *et al*. (2003) showed in a proteomic study from maize embryonic axis with polyclonal rabbit antibodies against human nNOS and mouse iNOS, that the antibodies recognized many unrelated NOS proteins.

Recently, in tobacco and *Arabidopsis* plants, a variant of the P protein of the mitochondrial glycine decarboxylase was found to be a pathogen inducible plant NOS (Chandok *et al*., 2003). This variant was designated "plant iNOS" and was cloned and purified by Chandok *et al*. (2003). iNOS was dependent on the same cofactors as it's animal counterpart but its specific activity was about 30 times higher (20 – 47 umol/min/mg) than animal NOS. At the same time a NOS gene (*AtNOS1*) was identified and described in *Arabidopsis,* which regulates growth and hormone signaling (Guo *et al*., 2003).

AtNOS1 was found to work independent of cofactors that are needed by animal NOS and plant iNOS and its specific activity was also much lower (5 umol/min/mg). The amino acid sequence showed that AtNOS1 was very similar to a group of bacterial proteins with putative GTP-binding or GTPase domains. Interestingly enough neither plant iNOS nor AtNOS1 has sequence similarities to any mammalian NOS (Guo *et al*., 2003).

2.5.2.2.3. Nitrate Reductase [NR]

Dean and Harper (1986) was first to discover that NR might by involved in NO production. They used nitrate reductase-deficient mutants of soybean and discovered that they produced NO differently than wild type plants. When soybean NR activity was characterised, it was shown to be NADPH dependent, had a pH optimum of 6.75 and was cyanide sensitive (Dean and Harper, 1988).

Table 2.5: Nitric oxide synthase activity reported in some plant species.

Species	Tissue	Reference
Pisum sativum	Leaves	Leshem and Haramaty, 1996
Pisum sativum	Leaf peroxisome	Barroso *et al*., 1999
Lupinus albus	Roots and nodules	Cueto *et al*., 1996
Mucuna hassjoo		Ninnemann and Maier, 1996
Glycine max	Cell suspensions	Delledone *et al*., 1998
Nicotiana tabacum	Leaves	Durner *et al*., 1998
Glycine max	Embryonic axis	Caro and Puntarulo, 1999
Zea mays	Root tips and young leaves	Ribeiro *et al*., 1999
Taxus brevifolia	Callus	Pedroso *et al*., 2000
Nicotiana tabacum	Leaf epidermal cells	Foissner *et al*., 2000
Nicotiana tabacum	Cell cultures	Tun *et al*., 2001
Arabidopsis thaliana	Cell cultures	Guo *et al*., 2003
Glycine max	Cotyledons	Modolo *et al*., 2002
Sorghum bicolour	Seeds	Simontacchi *et al*., 2004
Petrosilenum crispum	Cell cultures	Modolo *et al*., 2002

Nitrate reductase is a key enzyme of nitrate assimilation in higher plants (Pattanayak and Chatterjee, 1998), where it uses NADPH as an electron source for the conversion of nitrate to nitrite (Lea, 1999). NR can also generate NO by using NADPH as an electron source (Yamasaki and Sakihama, 2000; Rockel *et al*., 2002).

However, *in vitro*, the NO generating capacity of total NR activity was less than 1% (Rockel *et al*., 2002). It was also discovered by Yamasaki and Sakihama (2000) that NR produces peroxinitrate simultaneously with NO.

It is possible that NR might be involved in the generation of NO during pathogen infection as a defense response, since it was recently found that NR induction took place in potato tubers after infection with *Phytophthora infestans* (Yamamoto *et al*., 2003). Some evidence also indicate that NR may also play a role in hormone signaling since NR-mediated NO synthesis was found to be necessary for ABA signaling in *Arabidopsis* (Desikan *et al*., 2002) a role that has also been assigned to AtNOS1 (Guo *et al*., 2003).

2.5.2.2.4. Other Sources of NO in Plants

Xanthine oxidoreductase [XOR] is a Moco-containing enzyme that has recently been demonstrated to produce NO in animal systems (Harrison, 2002). This enzyme also has the capability to produce O_2^- in animal systems (Godber *et al*., 2000). Corpas *et al*. (1997) found XOR activity in pea peroxisomes, although no evidence of XOR producing NO in plants has been reported till date.

Another enzyme that has been reported to produce NO in plants is horseradish peroxidase. In 1992, Boucher *et al*. (a) reported that NO and citrulline was produced by horseradish peroxidase using N-hydroxy-arginine and H_2O_2 as substrates. More recently it was demonstrated that NO can be generated from hydroxyurea and H_2O_2 (Huang *et al*., 2002b).

Recently a plasma membrane bound enzyme was found in tobacco roots that could generate NO from nitrate. This enzyme had a higher molecular weight than NR and has still to be characterized (Stohr *et al*., 2001).

Hemeproteins such as cytochrome P450 (Boucher *et al*., 1992b), hemoglobin and catalase (Boucher *et al*., 1992a) also have the capability to produce NO when supplied with the right substrate. Nitric oxide can also be produced non-enzymatically in plants by nitrification/denitrification cycles (Wojtaszek, 2000). Another non-enzymatic mechanism proposed for NO formation is the light mediated reduction of NO_2 by carotenoids (Cooney *et al*., 1994).

2.5.2.3. Salicylic Acid

Local resistance [LR] and SAR are generally accompanied by elevated levels of endogenous SA (Dorey *et al*., 1997; Metraux *et al*., 1990). There is strong evidence that SA plays a central role in LR and SAR signaling (Dorey *et al*., 1997; Durner *et al*., 1997; Martinez *et al*., 2000), since it has been demonstrated that infiltration of *Arabidopsis* with SA induced the same set of SAR genes as pathogen infection (Ward *et al*., 1991; Uknes *et al*., 1992).

SA can be synthesized via two routes, firstly through the phenylalanine biosynthetic pathway by PAL from phenylalanine (Mauch-Mani and Slusarenko, 1996). Alternatively, it can be synthesized through isochorismate synthase [ICS] and isochorismate pyrovate lyase [IPL] from chorismate (Wildermuth *et al.*, 2001).

Evidence confirming the key role of SA in defense came from analyzing transgenic plants expressing the *nahG* gene, which encodes for an enzyme salicylic acid hydroxylase that inactivates SA by converting it to catechol (Delaney *et al*., 1994). Plants with the NahG phenotype showed to be more susceptible to normally avirulent pathogens (Van Wees and Glazebrook, 2003). The NahG phenotype was found to be a direct result of reduced SA levels, since functional analogs of SA, namely INA [2, 6-dichloroisonicotinic acid] and BTH [benzothiadiazole] was able to restore resistance in the plants (Lawton *et al*., 1996).

PAL–inhibited plants also showed to be more susceptible to avirulent strains of pathogens, while resistance was restored by treating plants with SA or INA (Mauch-Mani and Slusarenko, 1996).

Salicylic acid signaling seems to be mediated through at least two mechanisms, one dependent on NPR1 (Cao *et al*., 1998) and one independent of NPR1 (Kachroo *et al*., 2000). Signaling through NPR1 has been the aim of various studies. It was found that NPR1 contains at least four ankyrin repeats, which are also found on proteins that are involved in very diverse biological functions and also in protein-protein interactions (Bork, 1993). In other studies on NPR1 interaction with proteins by Zhang *et al*. (1999), it was found that NPR1 interacted with a subclass of transcription factors in the basic LZ protein family namely AHBP-1b and TGA6. Together with this information, AHBP-1b was shown to specifically bind to an SA-responsive promoter element of the *A. thaliana PR-1* gene. Thus, Zhang *et al*. (1999) concluded that after SA activated NPR1, it is mobilised to the nucleus where it interacts with a subclass of transcription factors in the basic leucine zipper protein family to regulate *PR-1* expression.

A second mechanism where SA signaling functions without NPR1 was also described by Kachroo *et al*. (2000) as well as Takahashi *et al*. (2002). Kachroo *et al*. (2000) found that the gene-for-gene resistance of *Arabidopsis* to Turnip crinkle virus and cucumber mosaic virus Y, which are conferred by the *HRT* [hypersensitive response to turnip crinkle virus] and *RCY1* [resistance gene cucumber mosaic virus Y] resistance genes respectively, are compromised in *NahG* mutants, but not *npr1* mutants. Van Wees and Glazebrook (2003) showed that catechol, which is produced from SA by the NahG-encoded salicylate hydroxylase, is responsible for some plant defenses. Thus, other products of the phenylalanine biosynthetic pathway might be involved in defense and not only SA.

Some of the effects of SA are that it binds to certain effector proteins. Four of these effector proteins have been identified and includes a salicylic acid binding protein [SABP] catalase (Chen *et al*., 1993), ascorbate peroxidase (Durner and Klessig, 1995), carbonic anhydrase (SABP3; Slaymaker *et al*., 2002) and SABP2 (Kumar and Klessig, 2003).

All of the above mentioned proteins, except SABP2, appear to have antioxidant activity, while SABP2 has SA-stimulated lipase activity. These effector proteins might play a very important role in stress hormone mediated signal transduction.

Chen *et al*. (1993) first discovered that SA inhibited catalase H_2O_2 degrading activity. Furthermore it was found that H_2O_2 could activate *PR1* gene expression (Chen *et al*., 1993). From this it was shown that SA mediated H_2O_2-activated expression of PR1, since the application of H_2O_2 induced SA synthesis in tobacco (Leon *et al*., 1995) as well as in *Arabidopsis* (Summermatter *et al*., 1995). Thus, this shows the effect which effector proteins have in SA signaling.

Additionally, it was shown that not all plants require SA accumulation to mediate resistance, since *Cf2* and *Cf9* gene mediated resistance to *Cladosporium fulvum* in tomato was unaffected by the presence of the *nahG* gene (Hammond-Kosack and Jones, 1996).

2.5.2.4. Jasmonic Acid

Besides SA, JA has also been implicated as a signal in plant defense response (Hammond-Kosack and Jones, 1996). Jasmonic acid is derived from linolenic acid by a lipoxygenase [LOX] – mediated oxygenated process (Hamberg and Gardner, 1992).

Exogenous application of JA was found to induce expression of several defense related genes, including osmotin [PR-5] (Xu *et al*., 1994) as well as different enzymes involved in defense reactions such as PAL (Gundlach *et al*., 1992) and LOX (Bell *et al*., 1995). The fact that PAL is a key enzyme in the biosynthesis of SA demonstrates the interaction between these two signals in plant defense. One example of this antagonistic relationship between SA and JA was demonstrated by Rao *et al*. (2000) in showing how these two signaling pathways interacted in controlling the magnitude of ozone-induced HR-like cell death.

The role that JA plays in protecting plants from pathogens and especially insects are deemed critical (Huang *et al*., 2004). Previous studies on cell cultures that were

treated with fungal elicitors, showed an increase in JA biosynthesis, demonstrating the role of JA in plant–fungal interactions (Gundlach *et al*., 1992). More recently, the JA pathway was shown to be important for the resistance to the biotrophic powdery mildew fungus *Erysiphe cichoracearum* in *Arabidopsis* (Ellis *et al*., 2002). Cohen *et al*., (1993) showed that potato and tomato had an increased resistance to *Phytophthora infestans* after treatment with JA as well as with its methyl ester, MeJA.

In order to better understand the working of JA in plants, mutants in JA response have been used. Some of these mutants include *jar1* [jasmonic acid resistant 1] (Staswick *et al*., 1992), *coi1* [coronatine insensitive 1] (Feys *et al*., 1994), *jin1* [jasmonate insensitive 1] (Berger *et al*., 1996) and *opr3* [oxophytodienoic acid reductase 3] (Stintzi and Browse, 2000). The opr3 mutant carries a mutation that blocks the JA biosynthesis beyond the JA precursor 12-oxo-phytodienoic acid [OPDA]. This mutant however still shows resistance to *Bradysia* larvae and the fungal pathogen *A. brassicicola* (Stintzi *et al*., 2001). Thus, resistance was still present in the absence of JA. This suggested that JA and MeJA might not always be necessary for resistance, but that other intermediates such as OPDA might play a signaling role in defense response. Furthermore, the intermediate dinor oxo-phytodienoic acid [dnOPDA], which is synthesized from hexadecatrienoic acid rather than linolenic acid (Weber *et al*., 1997), and the two JA conjugates, JA-amino acid and JA-glycosyl might also play an important role in as signaling molecules (Staswick *et al*., 2002).

2.5.2.5. Ethylene

The plant hormone, ethylene, is involved in many aspects of the plant life cycle, including germination (Goeschl *et al*., 1966), leaf senescence (Grbic and Bleecker, 1995), nodule development (Oldroyd *et al*., 2001) and plant defense (Hoffman *et al*., 1999).

Ethylene is formed from methionine via S-adenosyl-L-methionine and the cyclic, nonprotein amino acid 1-aminocyclopropane-1-carboxylic acid (Yu *et al*., 1979; Miyazaki and Yang, 1987).

The perception of ethylene occurs through a family of five membrane–localized receptors that are homologous to bacterial two–component histidine kinases involved in sensing environmental changes (Wang *et al*., 2002). In *Arabidopsis*, all five ethylene receptors namely, ETR1, ETR2, EIN4, ERS1 and ERS2 are present (Chang *et al*., 1993; Hua *et al*., 1995; Hua and Meyerowitz, 1998; Sakai *et al*., 1998). Of these, ETR1 and ERS1 contains three transmembrane domains and a conserved histidine kinase domain and have been shown to function as homodimers (Hall *et al*., 2000), while ETR2, EIN4 and ERS2 have four membrane-spanning regions and a degenerate histidine kinase domain (Hall *et al*., 2000). Amongst these receptors, only ETR1, ETR2 and EIN4 have receiver domains at their C termini. Ethylene binding occurs at the N-terminal transmembrane domain of the receptors and a copper cofactor is required for the binding (Hua *et al*., 1995).

The *Arabidopsis* RAN1 [Responsive–to–antagonist], a copper transporter, is involved in delivery of copper to the ethylene receptor (Hirayama *et al*., 1999). In the absence of an ethylene signal, ethylene receptors activate a Raf-like kinase, CTR1 (Kyriakis *et al*., 1992) and CTR1 in turn negatively regulates the downstream ethylene response pathway, possibly through a MAP-kinase cascade (Kieber *et al*., 1993). Binding of ethylene inactivates the receptors, resulting in the deactivation of CTR1, which allows EIN2 to function as a positive regulator of the ethylene pathway (Alonso *et al*., 1999). EIN2 contains the N-terminal hydrophobic domain similar to the N-ramp metal transporter proteins and the novel hydrophilic C terminus (Alonso *et al*., 1999). EIN2 positively signals downstream to the EIN3 family of transcription factors located in the nucleus (Chao *et al*., 1997). EIN3 binds to the promoter of the *ERF1* gene and activates its transcription in an ethylene-dependent manner (Solano *et al*., 1998). Transcription factors ERF1 and other EREBPs can interact with the GCC-box in the promoter of target genes and activate downstream ethylene responses (Yamamoto *et al*., 1999).

As mentioned, ethylene seems to play an important role in various plant disease resistance pathways. However, depending on the type of pathogen and plant species, the role of ethylene can be different.

Plants deficient in ethylene signaling may show either increased susceptibility or increased resistance. For example, in soybean, mutants with reduced ethylene sensitivity produce less severe chlorotic symptoms when challenged with the virulent strains *Pseudomonas syringae* pv. *glycinea* and *Phytophthora sojae*, whereas virulent strains of the fungi *Septoria glycines* and *Rhizoctonia solani* cause more severe symptoms (Hoffman *et al*., 1999). Similarly, in *Arabidopsis*, the ethylene-insensitive mutant *ein2* develops only minimal disease symptoms as the result of enhanced disease tolerance when infected by virulent *P. syringae* pv *tomato* or *Xanthomonas campestris* pv *campestris* (Bent *et al*., 1992).

However, the *ein2* mutant also displays enhanced susceptibility to the necrotrophic fungus *Botrytis cinerea* (Thomma *et al*., 1999). On the basis of these observations, ethylene seems to inhibit symptom development in necrotrophic pathogen infection but enhances the cell death caused by other types of pathogen infection.

Interestingly, ethylene response can also be linked to a gene–for–gene resistance in tomato. It was established that the expression of *Pti4* (functioning downstream of Pto) was rapidly induced by ethylene, which then induces the expression of GCC–box–containing *PR*–genes (Gu *et al*., 2000).

Thus, it can be seen that various signals are involved in the onset of a plants responses to an intruding pathogen. Also the participation of signals varies between different plant – pathogen interactions as well as the synergy between these signals. However, the signals involved in the activation of the plant's defense responses are not solely responsible for effective responses. The time necessary for these signals to activate the plant's defense responses also play a important role in effective resistance.

2.6. Delayed Responses in Susceptible Plants

The defense response of a resistant plant can be directly linked to signaling events leading to the specific defense response or responses. This can be said since it is known that one of the major differences between susceptible and resistant plants is

the response time of the plants to external stresses (Maleck *et al*., 2000). For example, in a study on various resistant and susceptible cabbage varieties infected with *Xanthomonas campestris* pv*. campestris*, it was found that in general increases in enzyme activities were greater and occurred earlier in the resistant plants compared to the susceptible plants (Gay and Tuzun, 2000). Enzyme activities tested included SOD and total peroxidase activity while H_2O_2 levels were also assessed.

In some instances plants do not respond at all, possibly due to suppressors produced by the pathogen, thereby becoming susceptible to the intruding pathogen (Moerschbacher *et al*., 1999). The latter was made evident when it was discovered that in a *T. aestivum P. graminis* interaction, fragments from the host cell wall, possibly produced enzymatically during fungal penetration, could have acted as endogenous suppressors of resistance in the resistant wheat. Further evidence was also provided when it was found that a *Pseudomonas syringae* type III secretion system downregulated the expression of a set of cell wall and defense proteins in *Arabidopsis* (Hauck *et al*., 2003). Thus, it becomes apparent that the earlier a plant perceives and responds to an intruding pathogen the better its chance on survival. This makes early signaling events in response to pathogens a major contributing factor to effective resistance in plants

CHAPTER 3

Materials and Methods

3.1. MATERIALS

3.1.1. Biological Material

For the infection studies, the resistant Yr1 and susceptible Avoset-S wheat cultivars were used. The 6E22 pathotype of *P. striiformis* was used for infections. The wheat seeds and fungal spores were obtained from the Plant Pathology group of the Department of Plant Science, University of the Free State.

3.1.2. Other Materials

Protein concentration was determined using Biorad reagent (BioRad Laboratories GmbH) and gamma globulins (Sigma) as protein standards. The phosphatase inhibitors antipain, chymostatin, leupetin, pepstatin and aprotinin were obtained from Roche. The [α-^{32}P]-dATP radioisotope, GFX^{TM} Micro plasmid Prep Kit, a GFX^{TM} PCR DNA and Gel Band Purification Kit and Rediprime II – Random Prime Labelling System were obtained from Amersham Biosciences. TRIzol was obtained from Life Sciences. *Escherichia coli* JM 109 competent cells and the pGEM-T Easy vector were obtained from Promega. The Titan One Tube RT-PCR System, all restriction enzymes and the mRNA Capturing Kit were obtained from Roche. *Taq* DNA polymerase SuperPak^{TM} was obtained from Sigma. All other chemicals and reagents were of the highest quality and purity.

3.2. METHODS

All biochemical assays were done in triplicate on two separate, but identically layed-out experiments.

3.2.1. Sterilization and Planting of *T. aestivum*

T. aestivum seed was sterilized by suspending it in a 2.25% (w/v) sodium hypochloride, 0.05% (v/v) polyoxyethylensorbitanmonolaurat [Tween 20] solution for 10 min. The solution was discarded and the seeds were washed 6 – 8 times

with sterile distilled water. The seeds were then planted in small pots and grown under greenhouse conditions at 24^{o}C with a 16 h day and 8 h night cycle. To provide a constant 16 h day period, cool–white fluorescent tubes were used. Fourteen day old seedlings, which were at a three leaf stage, were used for infection with *P. striiformis*. Fertilizer with a N:P:K ratio of 3:2:1 was applied to seedlings at a final concentration of 10 g.l^{-1} 3 days before infection with *P. striiformis*. The seedlings were also treated with a 24 h light period at 24^{o}C before infection.

3.2.2. Infection of *T. aestivum* with *P. striiformis*

Fresh rust spores were suspended in carosene oil to a final concentration of 22 X 10^{4} spores.ml^{-1}. After the light treatment, the plants were inoculated with the spore suspension by spraying it on the leaves with a spray gun. Control plants were sprayed with carosene oil only. Plants were left to dry off for approximately 2 h at room temperature. Cool sterile water was then sprayed on the plants to simulate high humidity conditions. Finally, the plants were incubated for 48 h in the dark at 8^{o}C with high humidity to allow infection to occur. Plant tissue was collected at different time points, quickly frozen in liquid nitrogen and stored at –80^{o}C.

3.2.3. Protein Extraction

Plant material harvested at the different time points was ground to a fine powder in liquid nitrogen. Total protein was then extracted from the frozen tissue as described for each individual enzyme activity.

3.2.4. Protein Concentration Determination

The protein concentration of all extracts was determined according to Bradford (1976). A standard curve was constructed by preparing serial dilutions of gamma globulins in distilled water to a final volume of 400 µl. To this, 100 µl Biorad reagent was added and mixed. The absorbance value at 595 nm (A_{595}) was

determined for each dilution. A standard curve was drawn of A_{595} versus total µg proteins. The best linear region of the standard curve showed that 10 µg protein gave an A_{595} of 0.4. This value was used as an internal standard for all protein concentration determinations.

All protein samples were diluted in a final volume of 400 µl to which 100 µl Biorad reagent was added. The OD_{595} value was determined and the protein concentration expressed as $\mu g.ml^{-1}$.

3.2.5. NADPH Oxidase Activity

NADPH oxidase was extracted using a modified method of Mulosevic and Slusarenko (1996). Extraction buffer (50 mM potassium phosphate buffer, pH 7.0, 0.1% (v/v) polyoxyethylene octyl phenyl ether [Triton X-100], 1.0% (w/v) polyvinylpyrolidone [PVP], 0.04% (w/v) $Na_2S_2O_5$, 10 mM ethylenediamine tetra-acetic acid [EDTA]) was added to the frozen tissue in a 3:1 ratio. The samples were incubated on ice for 30 min and then centrifuged at 12000 *g* for 20 min at 4^{o}C. The clear supernatant was transferred to a clean eppendorf tube.

NADPH oxidase activity was measured according to Askerlund *et al*. (1987) as modified by Rao *et al*. (1996). The assay mixture consisted of 50 mM potassium phosphate buffer, pH 7.0 containing 45 µM NADPH and 5 µM KCN. The reaction was initiated by the addition of 100 µl protein extract. The decrease in A_{340} was followed for 5 min at 25^{o}C. NADPH oxidase activity was expressed as $A_{340nm}.mg^{-1}$ protein.min^{-1}.

3.2.6. SOD Activity

SOD was extracted as described previously (3.2.5). SOD activity was determined according to Keppler and Novacky (1987). The reaction mixture consisted of 50 mM potassium phosphate buffer, pH 7.8, 2 µM riboflavin, 0.1 mM EDTA, 75 µM 4-nitro-blue tetrazolium chloride [NBT], 13 mM methionine and 20 µl enzyme extract. The enzymatic reaction was started by illuminating the samples for 20 min with cool–white fluorescent tubes. The A_{560} value for each sample was then determined. A non-irradiated duplicate sample was used as a control. A sample

lacking enzyme extract was used to determine the maximal attainable A_{560} value. SOD activity was expressed as $A_{560}.mg^{-1}$ protein.min^{-1}.

3.2.7. Determination of H_2O_2 Levels

Hydrogen peroxide levels were assayed according to Brennan and Frenkel (1977) as revised by Patterson *et al*. (1983). A standard curve was constructed by preparing serial dilutions of H_2O_2 in distilled water to a final volume of 1 ml. To this, $TiCl_4$ (20% (v/v) $TiCl_4$ dissolved in concentrated HCl) to a final concentration of 2% (v/v) and 0.2 ml of a 13 M NH_4OH solution was added. The solution was shaken to precipitate the Ti-H_2O_2 complex. After centrifugation at 5520 *g,* the precipitates were dissolved in 2 N H_2SO_4 and the A_{410} values determined. A standard curve was then drawn of A_{410} versus H_20_2 concentration.

Frozen plant material was homogenized in acetone in a 1:5 ratio for 1 min. The extract was centrifuged at 5520 *g* for 20 min at 4°C. The supernatant was recovered and $TiCl_4$ (20% (v/v) $TiCl_4$ dissolved in concentrated HCl) was added to a final concentration of 2% (v/v). To this, 0.2 ml of a 13 M NH_4OH solution was added for each 1 ml of extract. The solution was shaken to precipitate the Ti-H_2O_2 complex. After centrifugation at 5520 *g*, the precipitate was washed repeatedly with acetone until the supernatant was colorless. The precipitates were finally dissolved in 2 N H_2SO_4 and the A_{410} values determined. The H_2O_2 concentrations were calculated using the standard curve. The hydrogen peroxide levels were expressed as mM $H_2O_2.g^{-1}$ fresh mass.ml^{-1}.

3.2.8. Glutatione Peroxidase Activity

A modified method from Zieslin and Ben-Zaken (1991) was used for the assay of glutatione peroxidase activity. Total protein was extracted by adding extraction buffer (50 mM Tris(hydroxymethyl)amino methane [Tris] pH 7.5, 2 mM EDTA, 10 mM β-mercapto-ethanol, 2 mM phenylmethylsulfonylfluoride [PMSF]) to the frozen tissue in a 3:1 ratio. The samples were incubated on ice for 30 min and then centrifuged at 12000 *g* for 20 min at 4°C. The supernatant was transferred to a clean eppendorf tube.

The assay mixture consisted of 80 mM potassium phosphate pH 5.5, 8.2 mM H_2O_2, 5 mM guaiacol. The increase in A_{470} was measured for 3 min at 30°C after adding 10 µl enzyme extract. Peroxidase activity was expressed as nmol tetraguaiacol.mg^{-1} protein.min^{-1}

3.2.9. Total Protein Kinase Activity

Total protein was extracted by resuspending the frozen tissue in a 3:1 ratio in extraction buffer (25 mM Tris pH 7.5, 1 mM ethylenebis(oxyethylenenitrilo) tertaacetic acid [EGTA], 1 mM EDTA, 5 mM dithiotreitol [DTT], 0.1 mM Na_3VO_4, 10 mM NaF, 50 mM β-glycerophosphate, 1 mM PMSF, 4 µg.ml^{-1} phosphatase inhibitors (antipain, chymostatin, leupetin, pepstatin, and aprotinin)). After incubation of 30 min on ice, the samples were centrifuged at 12000 *g* for 20 min at 4°C. The supernatant was transferred to a clean eppendorf tube.

Total kinase activity was determined by adding 40 µg total protein to the reaction buffer (25 mM β-glycerophosphate pH 7.5, 1.25 mM EGTA, 1 mM DTT, 10 mM $MgCl_2$, 10 mM $CaCl_2$, 10 mM $MnCl_2$, 150 µM Na_3VO_4, 20 µM adenine triphosphate [ATP], 0.5 µCi [γ-^{32}P]-dATP, 0.3 mg.ml^{-1} myelin basic protein [MBP]). The reactions were incubated at 30°C for 20 minutes thereafter, 10% (v/v) trichloroacetic acid [TCA] was added to a 1.8:1 ratio. Half of each reaction was spotted on Whatman P81 cellulose phosphate filter paper squares. The P81 filter paper was washed three times for 10 min each in 1.2% (v/v) orto-phosphoric acid followed by 1 wash with 100% (v/v) acetone for 5 min. The paper was allowed to dry whereafter the labeled proteins were determined using the Cherenkov channel of a liquid scintillation counter. The amount of incorporated ^{32}P-ATP was expressed relatively to time zero after a blank was subtracted.

3.2.10. RNA Extraction

Distilled water was treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC) for 1 h thereafter the solution was autoclaved. DEPC water was used for all RNA related work. Total RNA was isolated according to Chomczynski (1993). Approximately 0.1 g frozen ground leaf material (3.2.3) was resuspended in TRIzol in a 5:1 ratio. After incubation at room temperature for 5 min, chloroform was added in a 5:1

ratio. The material was vortexed for 15 s whereafter it was incubated at room temperature for 15 min. After centrifugation at 12000 *g* for 15 min at 4°C, the RNA suspension was precipitated from the clear supernatant using isopropanol. The RNA was centrifuged at 12000 *g* for 10 minutes at 4°C, the pellet washed with 75% (v/v) ethanol whereafter the RNA was dissolved in DEPC treated water.

The RNA concentration was calculated by determining the A_{260} value of a diluted RNA sample (Sambrook *et al*., 1989). The RNA concentration was finally expressed as $\mu g.ml^{-1}$. To confirm the quality and quantity of the extracted RNA, 200 ng RNA of each time point was separated on a 1% (w/v) agarose gel (3.2.11).

3.2.11. Agarose Gel Electrophoresis

A 1% (w/v) agarose gel was prepared in 0.5X TAE buffer (0.4 M Tris pH 8.0, 0.018 M acetic acid, 0.001 M EDTA pH 8.0) containing ethidium bromide to a final concentration of 0.2 $\mu g.ml^{-1}$. Loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) orange G, 0.375 M ficoll) was added to all samples in a 1:3 ratio. The gel was run at 12 $V.cm^{-1}$ for 1 h using 0.5X TAE as running buffer. The separated DNA or RNA was visualized by exposing the gel to ultraviolet light with a 305 nm wavelength.

3.2.12. Differential Display RT-PCR (DDRT-PCR)

The DDRT-PCR was performed using the Titan one tube RT–PCR system. Each reaction contained 50 ng total RNA, 0.25 mM deoxy nucleotide triphosphate [dNTP's], 10µCi [α-^{32}P]-dCTP, 5 mM DTT, 1.5 mM $MgCl_2$, 5% (v/v) dimethylsulfoxide [DMSO], 8 pmol of each primer and 1 µl of the enzyme mix. Two sets of reactions were done. For the first, the primer combination of subdomain-VIII (Bovis 44) and monocot (Bovis 22) was used, while in the second Bovis 22 was substituted with the dicot (Bovis 23) primer (Table 3.1).

Table 3.1: DNA oligo nucleotides used in this study for DDRT-PCR, amplification of cDNA fragments as well as RT-PCR.

NAME		**TM**	**SEQUENCE**
Monocot	Bovis 22	46.4^{o}C	5`- GAYATHAARCCNCAYAAY - 3`
Dicot	Bovis 23	49.7^{o}C	5' – GAYGTNAARCCNGARAAY – 3'
Sudomain-VIII	Bovis 44	67.8^{o}C	5' - TCYGGYGCRATRTANCCNGGITGICC - 3'
05WVZ03 (forward)	Bovis 96	56.2^{o}C	5' – CCCTCTCGACGATGAAGCTTA – 3'
05WVZ03 (reverse)	Bovis 97	55.1^{o}C	5' – CCGAACCGACTGATGTTGAA – 3'
18S rRNA (forward)	Bovis 26	51.3^{o}C	5`- CAACTTTCGATGGTAGGATAG – 3`
18S rRNA (reverse)	Bovis 27	49.4^{o}C	5`- CTCGTTAAGGGATTTAGATTG - 3`

* H = A, T, C;
* Y = C & T;
* R = A & G;
* N = A, G, C, T

The amplification regime was as follows; one cycle at 60°C for 30 min, one cycle at 94°C for 1 min, ten cycles at 94°C for 30 s, 45°C for 1 min and 68°C for 1 min and another 20 cycles where the 68°C extension step is increased by 5 s at each cycle.

A final cycle of 68°C for 7 min was included. Following the RT-PCR, equal volumes loading buffer (95% (v/v) formamide, 0.09% (w/v) bromophenol blue, 0.09% (w/v) xylene cyanol FF) was added to each reaction. The cDNA was denatured at 94°C for 10 min, whereafter it was incubated on ice for 5 min before separation on a denaturing polyacrylamide gel.

3.2.13. Denaturing Polyacrylamide Gel Electrophoresis

The denatured cDNA samples were separated on a denaturing polyacrylamide gel consisting of 7.0 M urea, 6% (w/v) Long Ranger Gel solution 0.09 M Tris buffer pH 8.0, 0.09 M boric acid, 2.0 mM EDTA, 0.1% (v/v) N, N, N`, N`-tetramethylendiamine [TEMED], 0.1% (w/v) ammoniumperoxodisulfate [APS]. The gel was pre-run at 70 W for 30 min whereafter the samples were loaded and separated at 70 W for approximately two hours. The running buffer consisted of 0.09 M Tris buffer pH 8.0, 0.09 M boric acid, 2.0 mM EDTA, pH 8.0. The gel was then dried for 1 h at 80°C and finally exposed to X-ray film for two weeks.

3.2.14. Reamplification of cDNA Fragments

DNA fragments showing differential expression were cut from the gel and resuspended in TE (10 mM Tris buffer pH 7.5, 1 mM EDTA) and incubated it for 5 min at 100°C before grinding it to a fine pulp. The cDNA fragments were re-amplified using 5 µl of the extracted DNA, 5% (v/v) DMSO, 3.0 mM $MgCl_2$, 0.25 mM dNTP's, 2 units *Taq*-pol, 10 mM Tris buffer pH 8.3, 50 mM KCl, 0.001% (v/v) gelatine and 25 pmoles of each primer. The primer combinations used, were as described for the DDRT–PCR (4.2.2.3). The conditions for the PCR were as follows: 1 cycle of 94°C for 2 min, 35 cycles of 94°C for 30 s, 48°C for 1 min and 72°C for 1 min. A final cycle of 72°C for 5 min was included. The amplified products were separated on an 1% (w/v) agarose gel (3.2.11).

3.2.15. Cloning of Differentially Expressed cDNA Fragments

The amplified cDNA fragments were cloned into pGem-T easy vector according to the manufacturer's instructions. Ligation was performed at room temperature for 1 h.

The ligation reaction was mixed with 100 µl ultra competent *E. coli* cells and incubated on ice for 30 min. After giving the cells a heat shock at 42^{o}C for 30 s, 900 µl of Luria-Bertani medium [LB] (1% (w/v) tryptone, 0.5% (w/v) yeast – extract, 1% (w/v) NaCl) was added and the cells were allowed to recover at 37^{o}C for 1 h. The transformed cells were plated on LB plates containing 50 µg.ml^{-1} ampicillin. An hour prior to plating the transformed cells, the LB plates were covered with 40 µl (20 mg.ml^{-1}) isopropylthio β-D galactoside [IPTG] and 40 µl (20 mg.ml^{-1}) 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-gal]. The plates were incubated overnight at 37^{o}C.

White colonies containing recombinant plasmids were identified and transferred to fresh LB ampicillin plates. These colonies were inoculated into 5 ml LB containing 50 µg.ml^{-1} ampicillin and grown at 37^{o}C overnight with vigorous shaking. The cells in 1 ml of the overnight culture were pelleted in a microcentrifuge at 12000 *g* for 5 min. The cells were resuspended in 200 µl cell resuspension buffer (50 mM Tris - HCl pH 7.5, 10 mM EDTA, 0.7 mg.ml^{-1} RNase A) whereafter the cells were lysed by adding equal volumes of cell lysis solution (0.2 M NaOH, 1% (w/v) sodium dodecyl sulfate [SDS]). The solution was neutralized with 200 µl neutralization solution (2.55 M KOAc, pH 4.8) and mixed through inversion. After centrifugation for 5 min, the plasmid DNA was precipitated from the clear supernatant using 100% ethanol. The precipitated plasmid DNA was washed with 70% (v/v) ethanol whereafter the pellet was air dried and resuspended in water. To confirm the presence of an insert in the plasmid, a restriction digest was performed (3.2.16).

3.2.16. Restriction Digestion of DNA

A restriction digest was performed using 500 ng plasmid DNA of each clone. The DNA was digested with 1 U *Eco*RI in the presence of 50 mM Tris buffer pH 7.5,

100 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT. The DNA was digested at 37^{o}C for 2 h. The reaction was then separated on a 1% (w/v) agarose gel (3.2.11).

3.2.17. Reverse Northern Blot

Of each recombinant plasmid, 500 ng DNA was dissolved in 200 µl SSC (150 mM NaCl, 15 mM Na–citrate, pH 7.0). A cloned actin gene was included as control. The DNA was denatured by boiling it for 5 min before transferring to Hybond N^+ membranes using a Hoefer slot blot apparatus. Each well was washed with 6X SSC (0.9 M NaCl, 0.09 M Na–citrate, pH 7.0) and the membrane was finally dried at 80^{o}C for 15 min.

Individual cDNA probes were prepared using purified mRNA isolated from tissue harvested at 0, 24, 30, 36, 42 and 48 h.p.i. This was done for both IR and IS plants. A total of 15 µg total RNA was used to purify mRNA with a Roche mRNA capture kit according to the manufacturer's instructions. The reverse transcription of the mRNA to cDNA was done at 42^{o}C for 60 min using 2 mM $MgCl_2$, 0.5 mM dNTP's, 50 µCi [α-^{32}P]-dCTP, 1 µl ImProm-II reverse transcriptase in 1X ImProm-II reaction buffer. Before adding the labelled probe to the hybridization solution, it was denatured by boiling it for 5 min.

The membranes were first prehybridized for 2 h in 50% (v/v) formamide, 5X SSC (750 mM NaCl, 75 mM Na–citrate, pH 7.0), 0.5% (w/v) SDS, 5X Denhardts (0.1% (w/v) PVP, 0.1% (w/v) ficoll, 0.1% (w/v) bovine serum albumin [BSA]), 250 µg/ml salmon sperm DNA, 10% (w/v) dextrane sulphate. The denatured probes were then added and hybridized overnight at 42^{o}C. The membranes were finally washed twice with 2X SSC (300 mM NaCl, 30 mM Na–citrate, pH 7.0), 1% (w/v) SDS for 5 min at room temperature and two times with 0.5X SSC (75 mM NaCl, 7.5 mM Na–citrate, pH 7.0), 1% (w/v) SDS for 15 min at 42^{o}C.

The slots of the individual hybridized clones were then cut from the membrane and counted on a Beckman scintillation counter using the Cherenkov channel.

3.2.18. Sequencing

Selected recombinant plasmids were purified with the GFX^{TM} Micro plasmid Prep Kit according to manufacturer's instructions. The respective inserts were sequenced at the Central DNA Sequencer facility (University of Stellenbosch).

3.2.19. Analysis of Sequence Data

Analysis of sequencing data was done using different web based analysis tools. The BLAST similarity search was done on the NCBI website http://www.ncbi.nlm.nih.gov/Genbank/Genbank Search.html (Altschul *et al*., 1997). The translation of the sequences was done at http://us.expasy.org/tools/#ptm. Sequence alignment was done with CLUSTALW on http://www.ch.embnet.org/software/ClustalW.html and ALIGN on the Expasy website according to Pearson *et al*. (1997). A motif and pattern scan was performed on the amino acid sequences according to Falquet *et al*. (2002) on the PROSITE database.

3.2.20. Genomic DNA Extraction

Genomic DNA was isolated from various resistant near-isogenic wheat lines. Seeds were sown (3.2.1) and fourteen days later the seedlings were harvested and ground to a fine powder in liquid nitrogen.

Extraction buffer (25 mM NaCl, 5 mM Tris, 2 mM EDTA, 1 mM sodium bisulphite, 2 mM SDS, 0.7 mg.ml^{-1} RNase, pH 8.0) was added to the frozen powder in a ratio of 10:1, whereafter the mixture was incubated at 65^{o}C for 30 min. One volume of chloroform:isoamyl alcohol (24:1) was added and the suspension was centrifuged at 12000 *g* for 15 min. The genomic DNA was precipitated from the cleared top phase with 2 volumes 100% ethanol. After centrifugation at 7500 *g* for 10 min, the precipitated DNA was washed with 70% (v/v) ethanol, air dried and dissolved in 200 µl of sterile TE (10 mM Tris buffer, 1 mM EDTA, pH 7.0). The DNA concentration was then calculated (3.2.10).

3.2.21. Southern Blot

Two 25 µg aliquots of genomic DNA of each extracted sample were digested with *Eco*RI and *Xho*I respectively (3.2.16). The digested DNA was run on a 0.8% (w/v) agarose gel (3.2.11) at 2 $V.cm^{-1}$ for 12 h. The DNA was then transferred to a Hybond N^+ nylon membrane according to Chomczynski *et al*., (1992). The genomic DNA was denatured for 30 min in a 3 M NaCl, 0.4 M NaOH solution, whereafter the genomic DNA was transferred to the membrane for 2 h using 1.5 M NaCl, 0.4 M NaOH as transfer buffer. After transfer, the membrane was neutralized for 10 min in 0.2 M phosphate buffer, pH 6.8 and dried at 80^oC for 15 min. Probe preparation and hybridization was done as described (3.2.21).

3.2.22. Expression Analysis Using Northern Blot

After total RNA was extracted (3.2.10), 20 µg of each sample was freeze dried. The freeze dried RNA samples were dissolved in a RNA buffer (1X MOPS buffer [20 mM 3 – (N–morpholino)propanesulfonic acid, 5 mM sodium acetate, 10 mM EDTA pH 8.0], 50% (v/v) formamide, 6.5% (v/v) formaldehyde, 0.05 $mg.ml^{-1}$ ethidium bromide) containing 1X RNA loading buffer (50% (v/v) glycerol, 1 mM EDTA pH 8.0, 0.25% (w/v) bromophenol blue) The RNA was denatured at 65^oC for 15 min, whereafter it was incubated on ice before separation. The samples were separated on a 1% (w/v) agarose gel containing 0.41 M formaldehyde, 1X MOPS in a 1X MOPS buffer. The denatured RNA was separated at 5 $V.cm^{-1}$ for 2 h.

RNA was transferred to a nylon membrane for 2 h using 3 M NaCl, 8 mM NaOH and 2 mM N–lauroylsarcosine as transfer buffer. After transfer the membrane was neutralized for 10 min in 0.2 M phosphate buffer, pH 6.8 and dried at 80^oC for 15 min.

The respective cDNA inserts that were used as probes, were labelled radioactively with 50 µCi [α-^{32}P]-dCTP using a Rediprime II Random Prime Labelling System according to the manufacturer's instructions. After labelling the probe, it was purified by separation through a Sephadex G-75, superfine column

using a TE buffer to elute the probe in different fractions (Sambrook *et al*., 1989). The fraction with the highest emission of radioactivity was used for hybridization.

After prehybridizing the membranes in Ultrahyb for 30 min at 42^{o}C, the denatured radioactive labeled probe was added. The membranes were hybridized at 42^{o}C for 16 h and then washed two times with 2X SSC (300 mM NaCl, 30 mM Na–citrate, pH 7.0), 1% (w/v) SDS for 5 min at room temperature and two times with 0.1X SSC (15 mM NaCl, 1.5 mM Na–citrate, pH 7.0) containing 1% (w/v) SDS for 30 min at 68^{o}C. The membranes were analyzed by exposure to X–ray film for a specific time period.

3.2.23. Expression Analysis Using RT-PCR

In order to confirm the induced expression of the identified clones, RT–PCR was done. Each reaction contained 10 ng total RNA, 0.5 mM dNTP's, 5 mM DTT, 5% (v/v) DMSO, 1.5 mM $MgCl_2$, 1 µl enzyme mix and 10 pmol primer. A gene specific primer set was synthesized for each of the cloned cDNA fragments (Table 3.1).

The amplification regime for all primer sets was as follows: one cycle at 60^{o}C for 30 min, one cycle at 94^{o}C for 2 min, 30 cycles at 94^{o}C for 15 s, X^{o}C for 30 s, and 68^{o}C for 45 s, with an additional 5 s that was added during the extension reaction for the last twenty cycles. A final cycle of 68^{o}C for 7 min was included. The primer annealing temperature (X) was set individually for each clone (Table 3.1). The RT–PCR's were done on RNA isolated from IR and IS plants for all the time points. As a control the 18S rRNA was also amplified from each time point. The amplified fragments were separated on a 1% (w/v) agarose gel (3.2.11) and the yield for each fragment determined. The 18S rRNA yield was used to normalize the expression of the individual fragments. Each time point was then finally expressed relative to time 0.

CHAPTER 4

Results

4.1. Infection of Yr1 and Avoset-S Cultivars

Resistant (Yr1) and susceptible (Avoset–S) wheat cultivars were infected with *P. striiformis* (stripe rust) (Fig. 4.1). The Yr1 cultivar was extremely resistant showing only necrotic lesions at 15 days post inoculation (d.p.i.). In contrast, the Avoset-S cultivar was very susceptible for infection showing the formation of rust pustules and chlorosis at the same time interval. Of the two experimental setups, the second set of plants (Fig. 4.1 B) showed a slightly better infection efficiency compared to the first set (Fig. 4.1 A). This was concluded since the lesion formation on the Yr1 cultivar as well as chlorosis on the Avoset-S plants were higher in the second set.

4.2. Biochemical Activation of the Defense Reactions

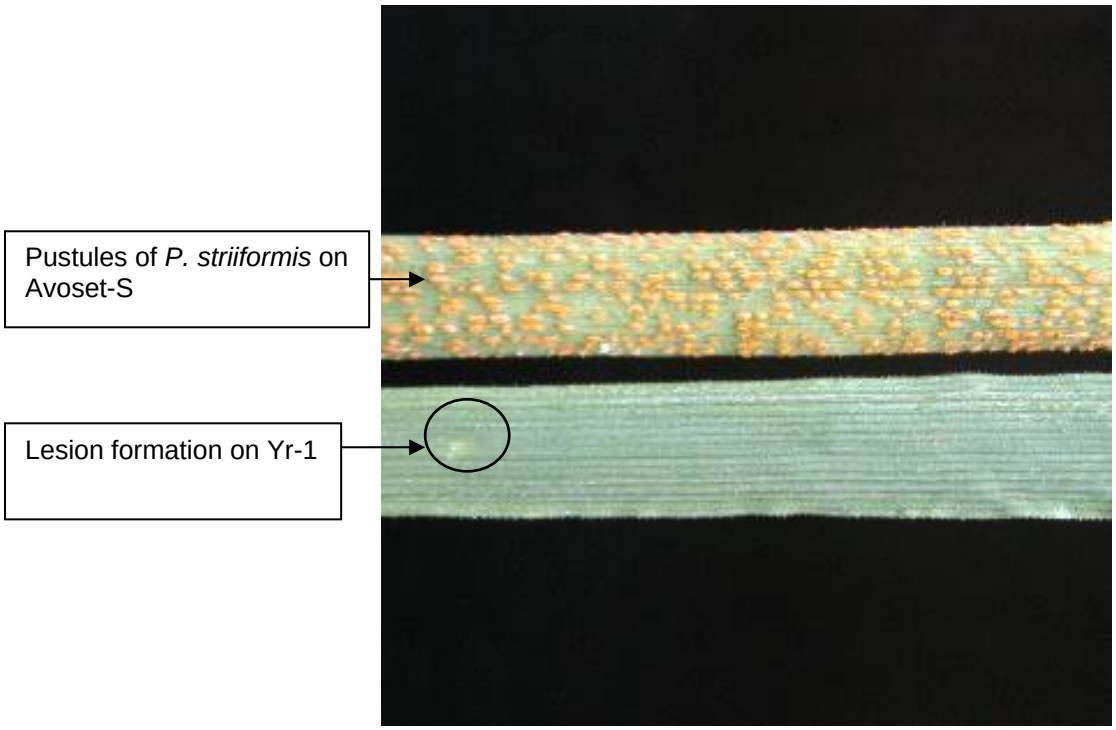
In order to determine the earliest defense response of wheat towards infection by stripe rust, a time trail over 72 h was conducted. Time zero tissue was harvested 2 h after inoculation as soon as the plants dried off. From then on, tissue was harvested with 6 h intervals for the duration of the experiment. To establish the earliest response time of the plants towards infection, several defense related enzyme activities were determined.

4.2.1. Involvement of the Oxidative Burst

A standard curve was constructed showing the linear relationship between protein levels and A_{595} values (Fig. 4.2 A). This curve was used to calculate total protein concentrations. From the graph, 10 µg standard protein gives an A_{595} value of 0.4 that falls within the linear region of the graph. For each protein concentration determination, this standard was used as an internal control.

The oxidative burst was firstly examined by assaying NADPH oxidase activity (Fig. 4.3). This was done for two reasons: firstly to determine the level of involvement of NADPH oxidase in the oxidative burst and secondly to determine how soon after infection the oxidative burst takes place.

A



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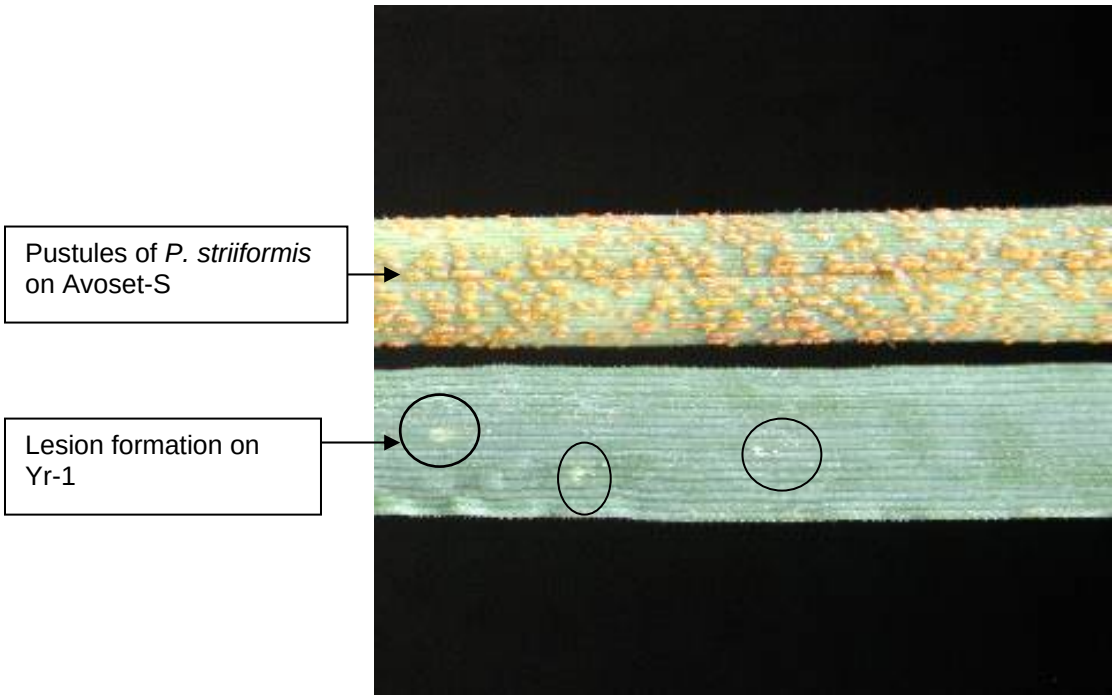
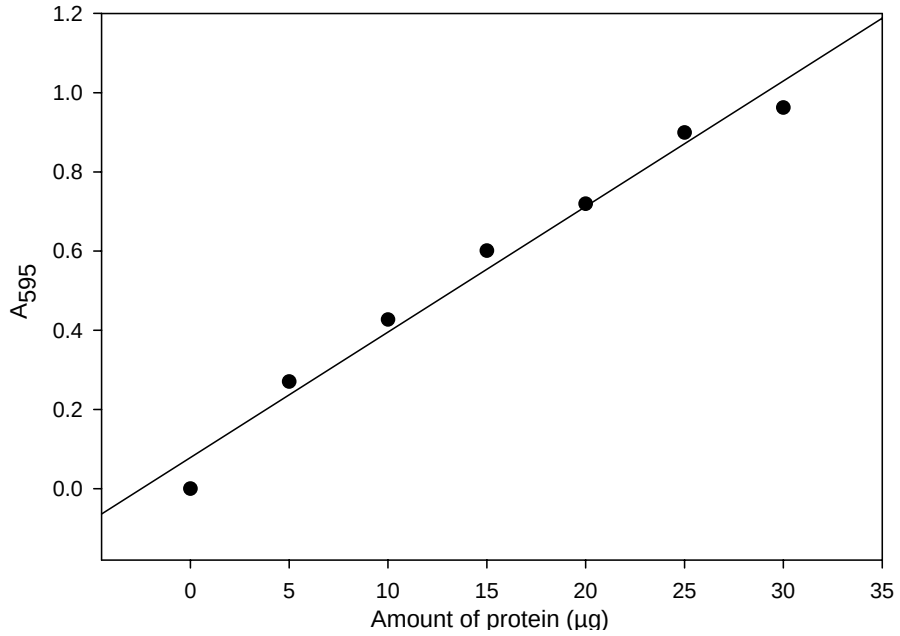


Fig. 4.1: Infection of Avoset-S (top leaf) and Yr-1 (bottom leaf) wheat cultivars with *P. striiformis.* A and B represent two separate sets of infected plants.

A



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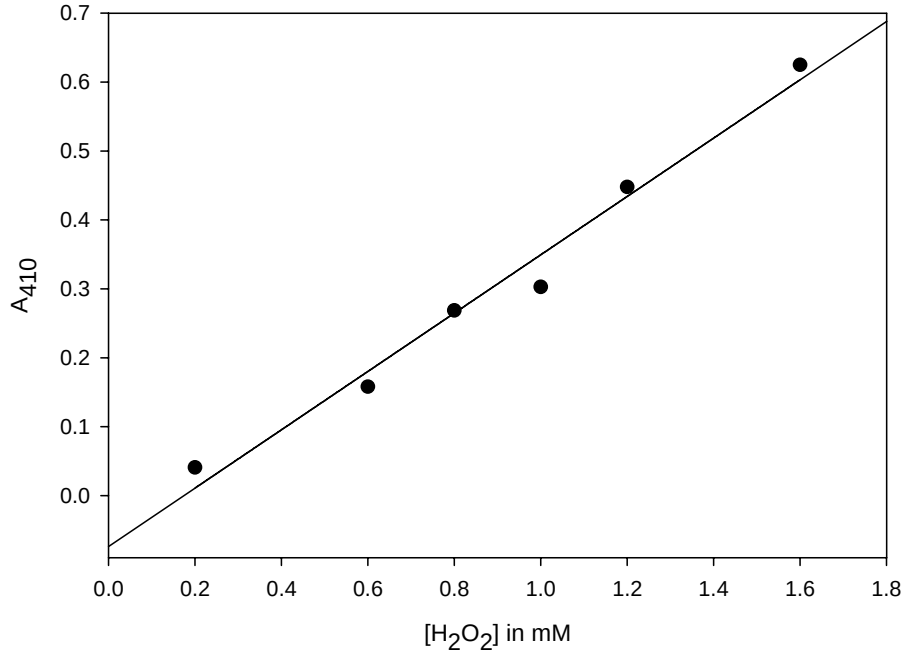
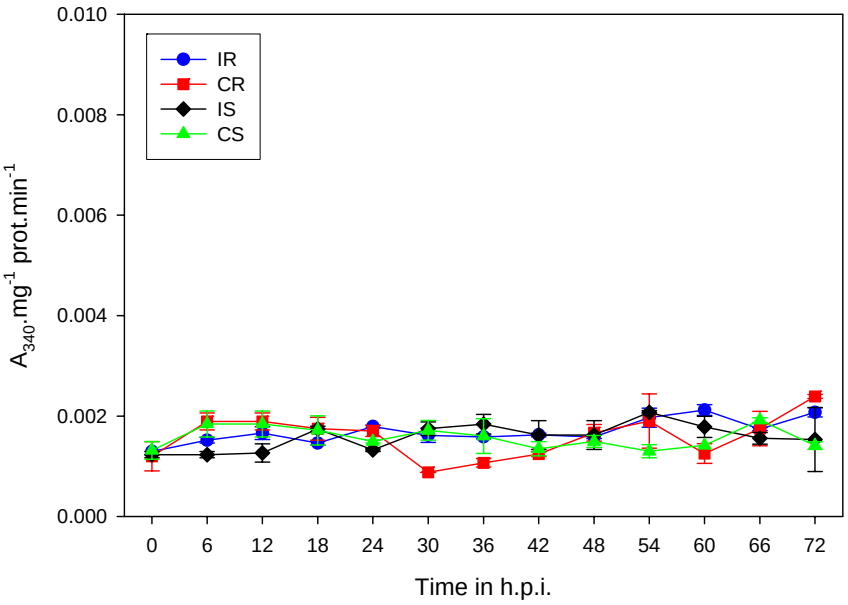


Fig. 4.2: Standard curves used for protein concentration and H_2O_2 level determination in this study. (**A**) Protein standard curve used for the determination of protein concentration of extracted protein samples, (R^2 = 0.977). (**B**) Standard curve for H_2O_2 used to determine the amount of H_2O_2 present in different samples of infected resistant and susceptible cultivars, (R^2 = 0.980).

A



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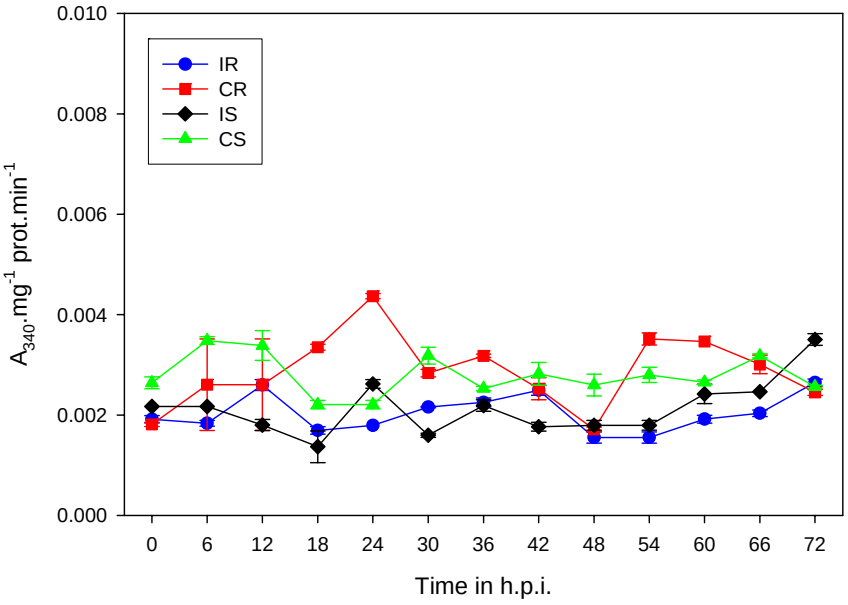


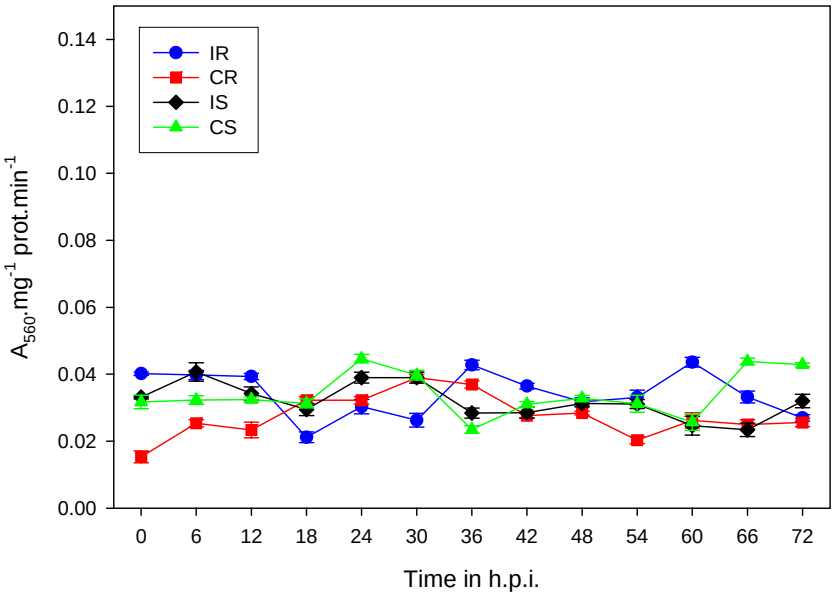
Fig. 4.3: NADPH oxidase activity of stripe rust infected wheat plants. A and B represent the two sets of experiments conducted. Non - specific enzyme activity is indicated as A_{340}.mg^{-1}prot.min^{-1} for each time interval given in hours post inoculation [h.p.i.]. Abbreviations are IR, infected resistant; IS, infected susceptible; CR, control resistant; CS, control susceptible.

NADPH oxidase activity showed no significant induction at any stage of the trial period of 72 h (Fig. 4.3). Both sets of plants had a relative constant low level of activity, although the second set of plants overall seemed to have a slightly higher level of activity. This could possibly be related to a better infection that occurred in the specific set of plants. The second experiment (Fig. 4.3 B) also showed much more variation in activity levels. However, this was present in both infected and control plants. A transient increase in activity in the control resistant [CR] plants can be seen at 24 hours post inoculation [h.p.i.] (Fig. 4.3 B).

The second enzyme that was assayed for its involvement in the oxidative burst was SOD. As with NADPH oxidase, only low and constant SOD activities were measured in both experimental sets (Fig. 4.4). It was again observed that in the second experiment, SOD activity was slightly higher with greater variation than the first experiment. A temporary increase of SOD activity was seen from 36 h.p.i. in both infected resistant [IR] and infected susceptible [IS] plants, with IR then returning to its original level of activity, while IS still increased until 48 h.p.i. before returning to its original level of activity. The increase of SOD activity was however small, not exceeding one and a half times the original level of activity of both IR and IS plants.

The internal H_2O_2 levels (Fig. 4.5) were consistent with these findings. Much variation occurred during the trial period of 72 h. This variation however was constant throughout infected and control plants. In both experiments the level of H_2O_2 was higher at time zero compared to the rest of the trial period. In the first experiment, the H_2O_2 levels when compared to time zero, remained relative constant without any significant increase in H_2O_2 levels. However, two exceptions occurred in the second experiment where two transient peaks of increased H_2O_2 levels were visible in the IR plants (Fig. 4.5 B). For the IR plants, peaks in H_2O_2 level occurred at 24 h.p.i. and 48 h.p.i. and for the IS plants at 36 h.p.i. In the case of the IR plants, the H_2O_2 levels were 2 and 4 times higher than at time zero respectively, while the IS plant levels were only slightly higher than that of time zero, almost at comparable levels.

A



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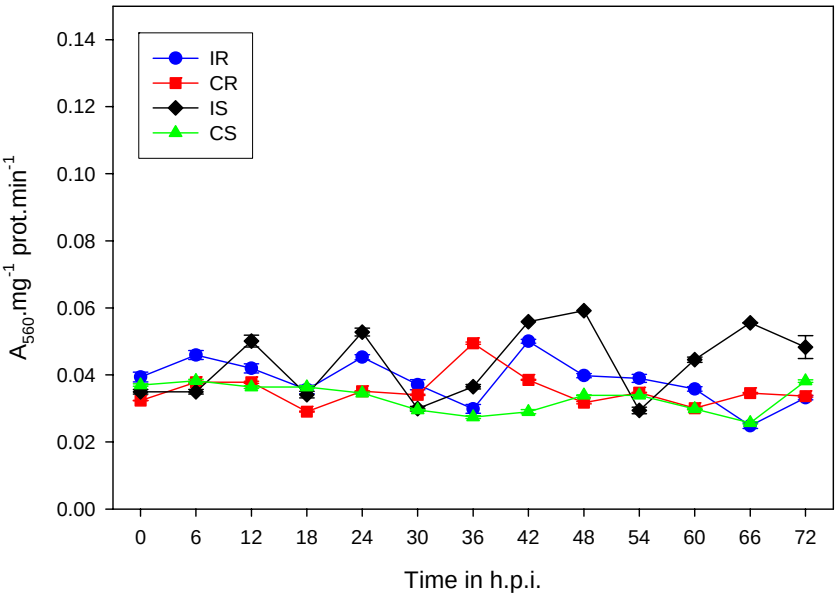
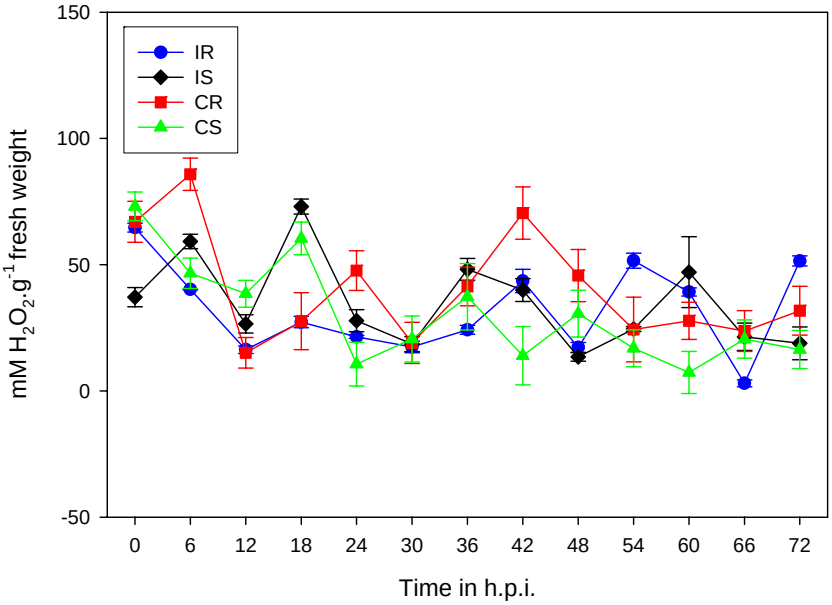


Fig. 4.4: Superoxide dismutase activity of stripe rust infected wheat plants. A and B represent the two sets of experiments conducted. Non - specific enzyme activity is indicated as $A_{560}.mg^{-1}prot.min^{-1}$ for each time interval given in hours post inoculation [h.p.i.]. Abbreviations are IR, infected resistant; IS, infected susceptible; CR, control resistant; CS, control susceptible.

A



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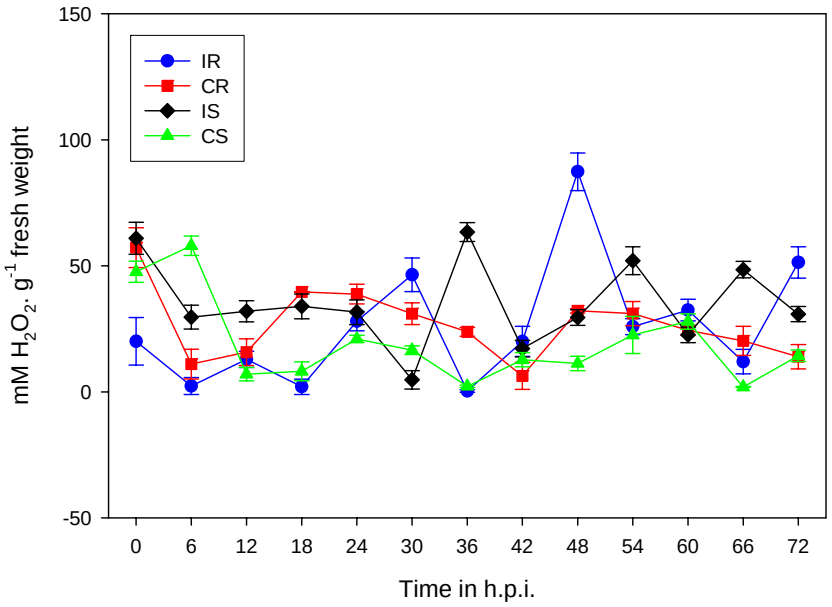


Fig. 4.5: The internal H_2O_2 levels of stripe rust infected wheat plants. A and B represent the two sets of experiments conducted. The level of H_2O_2 is indicated as mM $H_2O_2.g^{-1}$fresh weight for each time interval given in hours post inoculation [h.p.i.]. Abbreviations are IR, infected resistant; IS, infected susceptible; CR, control resistant; CS, control susceptible.

4.2.2. Involvement of Glutatione Peroxidases

One of the secondary defense mechanisms that are directly linked to the oxidative burst, more specifically to the production of H_2O_2, is cell wall bound glutatione peroxidases. Glutatione peroxidases are activated by elevated H_2O_2 levels.

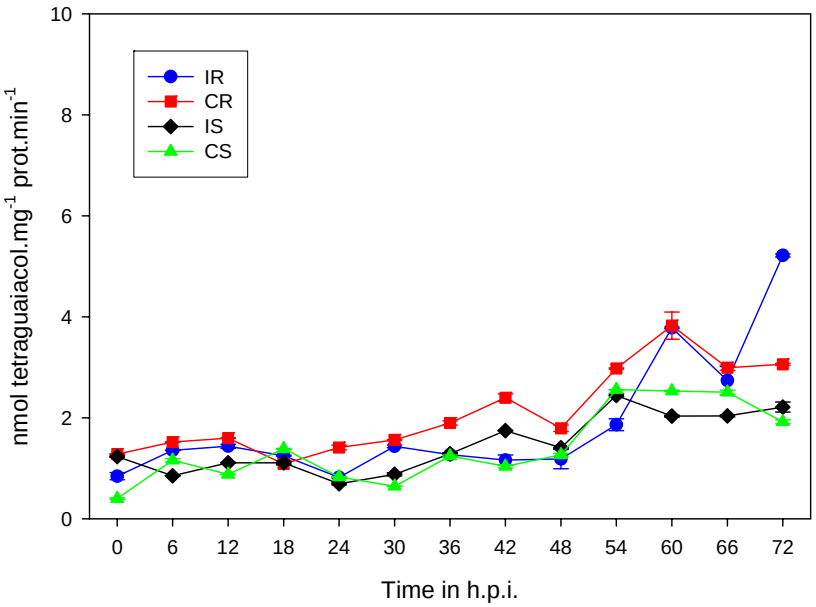
To determine whether the secondary defenses were activated, the activity of glutatione peroxidases was determined (Fig. 4.6). In the second experiment, a strong induction in peroxidase activity in the IR plants starting at 24 h.p.i. was found reaching a peak at 66 h.p.i. The levels obtained at 66 h.p.i. were nearly 9 times that of time 0. Although in IS plants, an increase in peroxidase activity starting at 48 h.p.i. occurred, this increase was comparable to that of the control plants. In the first experiment an increase in peroxidase activity in the IR was observed starting at 48 h.p.i. reaching a peak at 72 h.p.i. but a similar increase was also present in the CR and control susceptible [CS] plants. However, the sustained increase was not observed in the CR and CS plants (Fig. 4.6 A). A difference was observed between the first and second experiment in that a faster increase of peroxidase activity, in both infected and control plants, was seen in the second experiment compared to the first.

4.2.3. Involvement of Protein Kinases

All signal transduction cascades rely on phosphorylation events that not only transduce the signal, but also amplify it. Therefore, total protein kinase activity was measured to determine and confirm the involvement of protein kinases in the interaction between *P. striiformis* and *T. aestivum.* This would also indicate the earliest time of recognition by the plant of the intruding pathogen.

The total kinase activity was measured for both experiments (Fig. 4.7). Increased protein kinase activity was detected in the infected resistant cultivar of both sets of plants. Again, it was clear that the response of the first infection was slower compared to the second infection.

A



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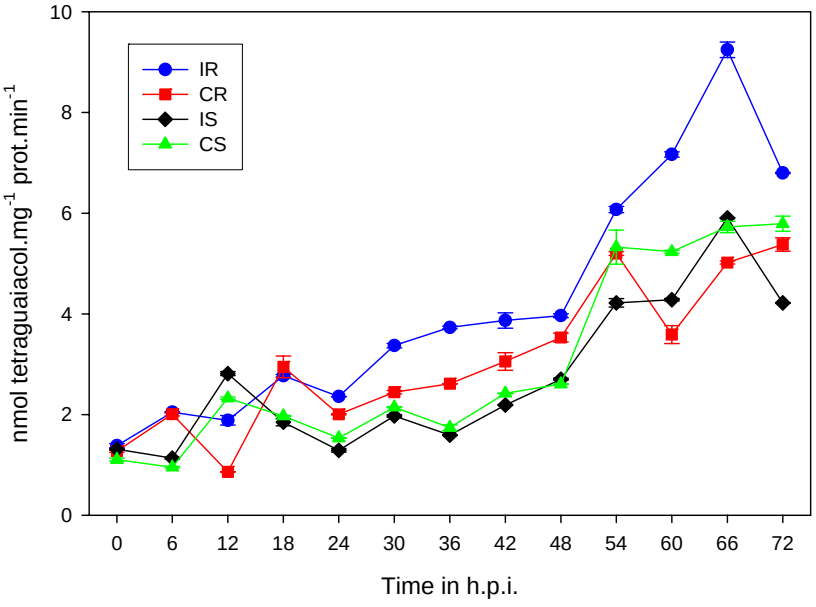

Fig. 4.6: The peroxidase activity of stripe rust infected wheat plants. A and B represent the two sets of experiments conducted. Specific peroxidase activity is indicated as nmol tetraguaiacol.mg^{-1}prot.min^{-1} for each time interval given in hours post inoculation [h.p.i.]. Abbreviations are IR, infected resistant; IS, infected susceptible; CR, control resistant; CS, control susceptible.

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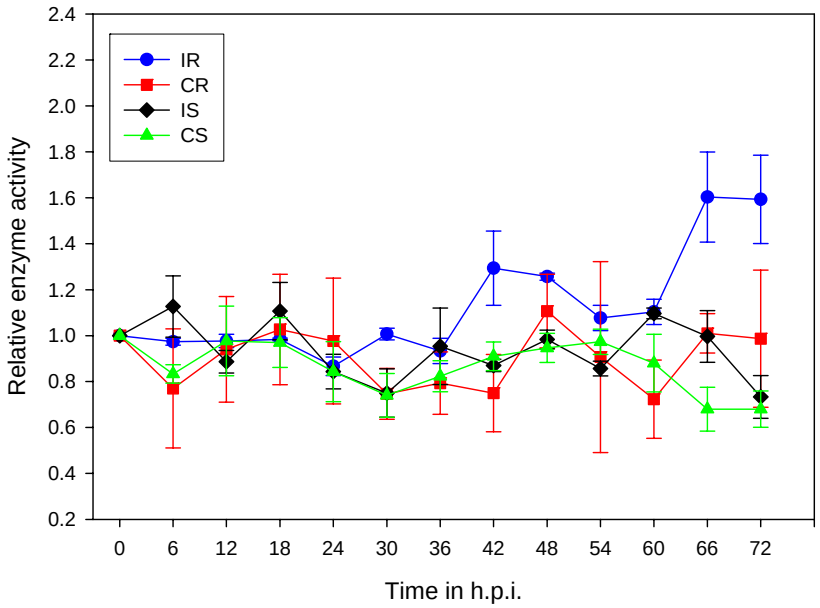

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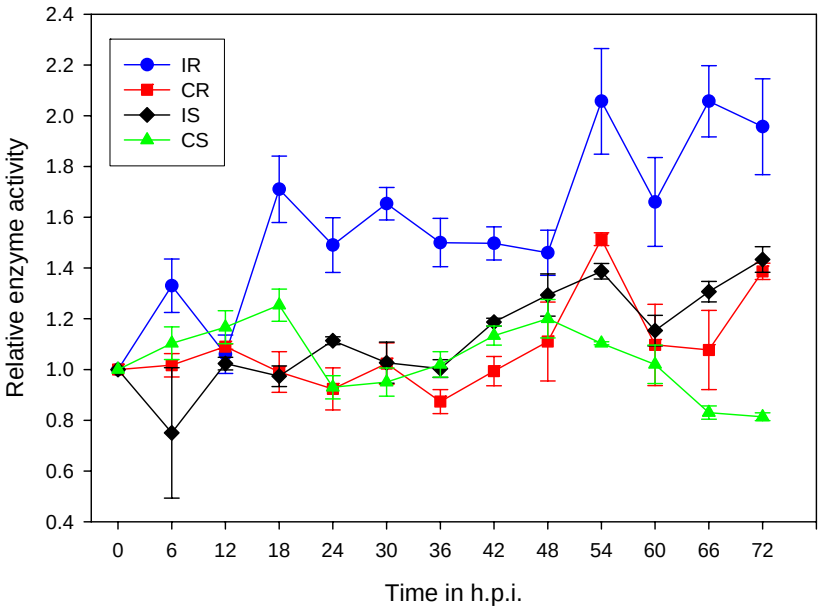


Fig. 4.7: Total protein kinase activity of stripe rust infected wheat plants. A and B represent the two sets of experiments conducted. The level of protein kinase activity was expressed relative to time zero for each time interval indicated in hours post inoculation [h.p.i.]. Abbreviations are IR, infected resistant; IS, infected susceptible; CR, control resistant; CS, control susceptible.

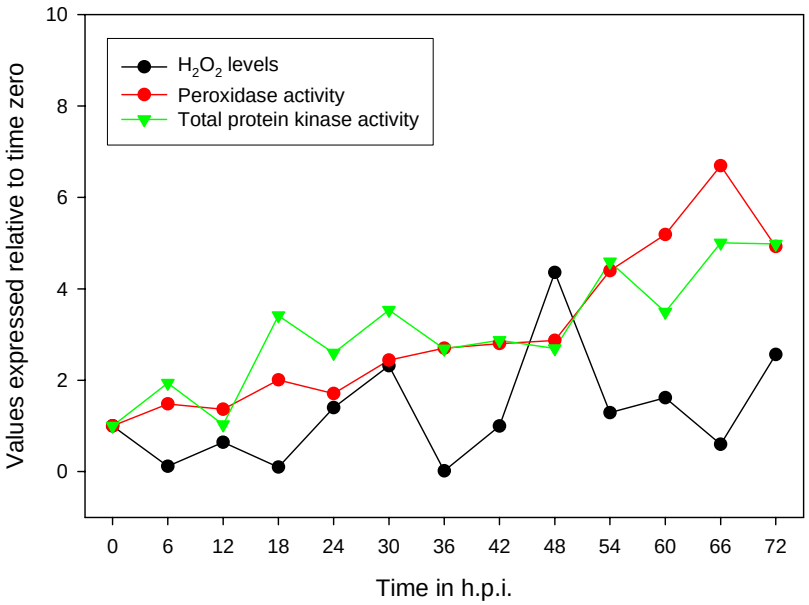
In the first infection (Fig. 4.7 A) an increase in protein kinase activity was seen in the IR plants starting at 36 h.p.i. with a further increase in activity after 60 h.p.i. The initial increase was relative small compared to the increase later on, which was 1.6 times higher than the activity of time zero. It is notable that the activity of these two specific time intervals was more than double that of the controls and IS plants. The IS and control plants did not show any significant increase or variation in protein kinase activity.

During the second infection (Fig. 4.7 B) an increase in protein kinase activity occurred as early as 18 h.p.i. which was nearly double that of the control and IS plants. A more significant induction in activity occurred from 54 h.p.i. till the end of the trial period. This elevated protein kinase activity was maintained in the IR plants from 18 - 72 h.p.i. An increase in the protein kinase activity of the CR plants occurred at 54 and 72 h.p.i. For the IS and its control plants, no dramatic increase in protein kinase activity was observed. However, at 36 h.p.i. an increase in activity of the IS plants occurred until 54 h.p.i. Notably the activity of the IS plants was twice that of the CS plants at 66 and 72 h.p.i.

The biochemical defense responses of the second infection are summarised for IR and IS plants in Fig. 4.8 A and B respectively. All the values are expressed relative to time zero. An increase in total protein kinase activity of three times that of time zero was observed at 18 h.p.i. in the IR plants. This increase in kinase activity was sustained until 48 h.p.i. whereafter a further increase was observed. This increase in kinase activity at 48 h.p.i. coincided with increased peroxidase activity and H_2O_2 levels. Compared to the IR plants, IS plants showed no increased enzyme activity or H_2O_2 levels until 54 h.p.i. Peroxidase activity increased from 54 h.p.i. reaching a peak at 66 h.p.i. The peak in peroxidase activity in the IS plants was 4 times that of time zero compared to the seven fold increase in the IR plants.

This summary of the biochemical responses was done to determine the earliest response time of *T. aestivum* infected with *P. striiformis*. Tissue, harvested from IR plants in this time period (from 18 h.p.i. onwards) was then used for the identification of genes involved in defense signaling through DDRT–PCR.

A



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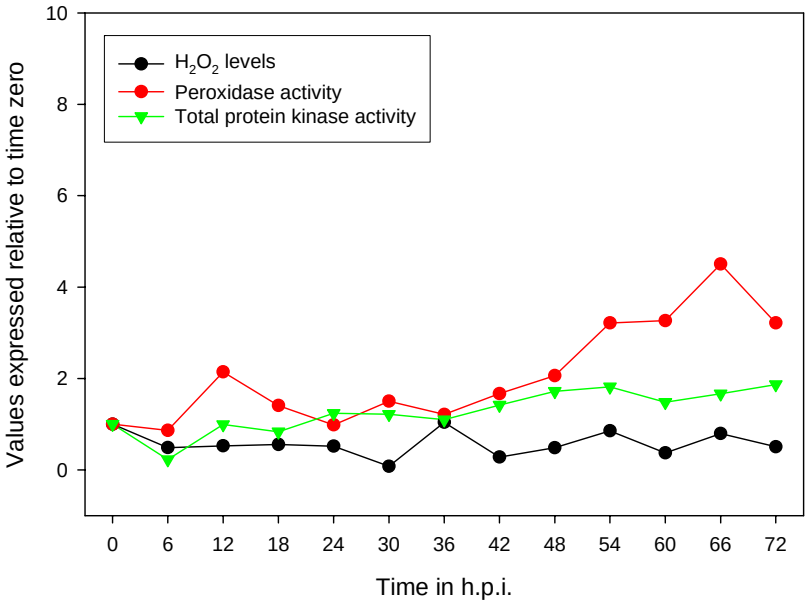


Fig. 4.8: Summary of the of the biochemical response of wheat infected with stripe rust. A en B represent the specific responses in IR and IS plants respectively. Values are indicated relative to time zero for each time interval indicated in hours post inoculation [h.p.i.]. Abbreviations are IR, infected resistant; IS, infected susceptible; CR, control resistant; CS, control susceptible.

4.3. Identification of Differentially Expressed Gene Fragments

Since a better infection efficiency was observed from the second infection as well as a stronger response to infection, the tissue harvested from those plants were used for the identification of differentially expressed genes. DDRT–PCR was used to identify putative protein kinase genes whose expression was induced shortly after infection. For this, total RNA was extracted from Yr1 plants infected with stripe rust (Fig. 4.1). The extracted RNA was of good quality and intact, since the rRNA bands were sharp and not broken down (Fig. 4.9).

The background smear indicated the mRNA present in all samples. The RNA concentration of all the extracts was constant, indicating accurate concentration determination.

The intact total RNA was then used for the DDRT-PCR to identify differentially expressed putative serine/threonine protein kinases. A partial result of the DDRT–PCR is indicated in Fig. 4.10. As can be seen for the particular cDNA fragment, the expression thereof was induced starting at 42 h.p.i. The transient expression of the fragment lasted until 48 h.p.i., whereafter the expression was repressed. The fragment was again induced from 66 h.p.i. until the end of the time trial.

In total 8 cDNA fragments visually exhibited differential expression after infection with stripe rust. Five of these cDNA fragments were successfully re-amplified with the monocot / subdomain VIII primer combination and were designated M1 – M5. Another 3 cDNA fragments were amplified with the dicot / subdomain VIII primer combination and were designated D1–D3. The earliest induced expression was for M1 occurring at 30 h.p.i. Several of the cDNA fragments showed induced expression for extended periods of time from 36 h.p.i. until the end of the trial peeriod. In other instances, e.g. M5, the cDNA fragment was induced for short periods of time being present at 42, 48 h.p.i. and again at 66 and 72 h.p.i.

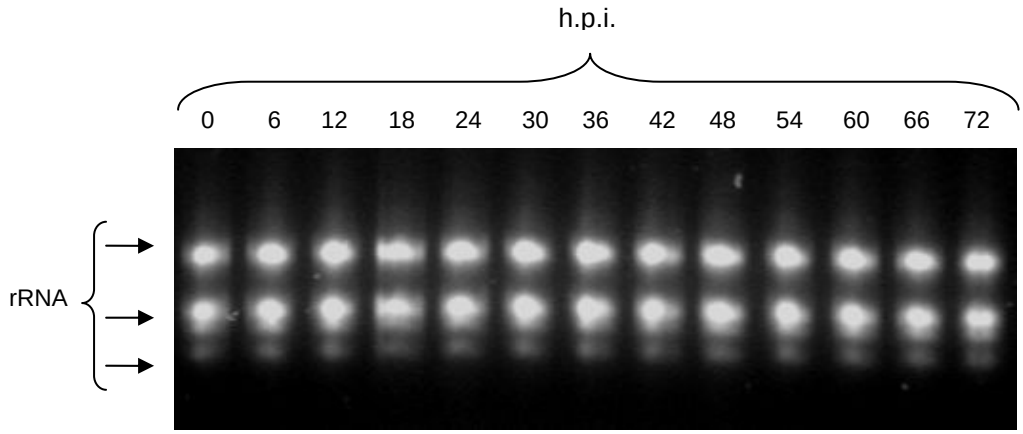


Fig. 4.9: Total RNA extracted from resistant Yr1 wheat at different time points after infection with stripe rust. The different rRNA fragments are indicated with arrows. The harvesting times of the tissues are indicated as hours post inoculation [h.p.i.] at the top of the image.

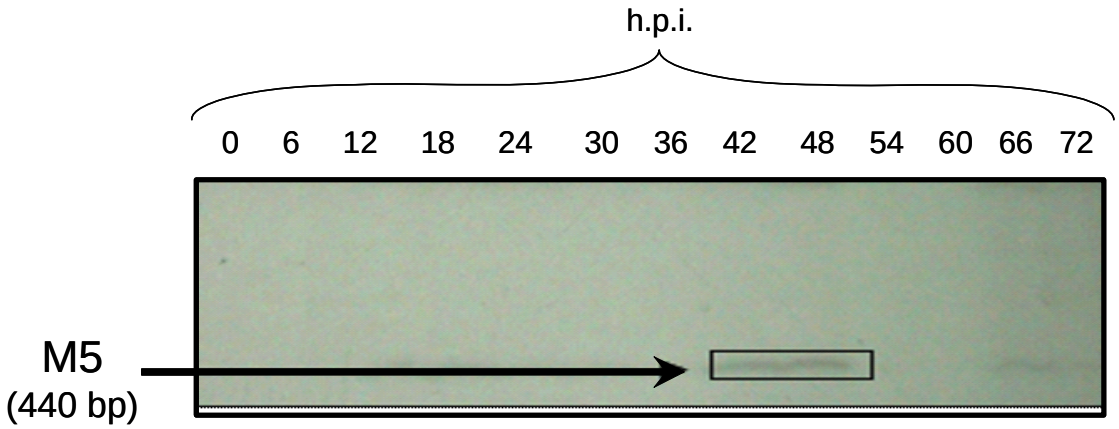


Fig. 4.10: A partial result of the DDRT-PCR from resistant Yr1 wheat at different time points after infection with stripe rust. A cDNA fragment, which was differentially expressed, is indicated as M5. The time intervals are indicated as hours post inoculation [h.p.i.], at the top of the image.

4.4. Cloning of Isolated Gene Fragments

After the amplification of the identified cDNA fragments, the fragments were cloned into the pGEM-T easy vector. After transforming competent *E. coli* JM 109 cells with the ligation reaction, the selection for recombinant plasmids was done on LB plates containing Ampicillin, IPTG and X-gal (Fig. 4.11).

On the plate, both white and blue colonies were seen. The blue colonies represent *E. coli* cells containing an empty pGEM-T plasmid without an insertion, while the white colonies represent *E. coli* cells containing a pGEM-T plasmid with a cloned cDNA insert. A white colony representative of each cloned cDNA fragment was selected.

To confirm that the white colonies indeed contained recombinant plasmids, isolated plasmid DNA was digested with *Eco*RI, since the pGEM-T easy vector contains two *Eco*RI restriction sites flanking the polycloning site (Fig. 4.12).

The first two lanes in Fig. 4.12 A and B contained undigested and *Eco*RI digested plasmid DNA isolated from a blue colony. The digested plasmid DNA formed a single DNA fragment representing the linearized plasmid DNA. The undigested DNA formed the characteristic plasmid banding pattern of the three different forms. The rest of the lanes in Fig. 4.12 A and B represent the digested plasmid DNA isolated from white colonies. Most of the recombinant plasmids contained an insert identical in size to that found for the DDRT–PCR, since the fragments decreased in size from M2 to M5 and from D1 to D3. M1 and M4 were the exceptions in that the cloned insert was smaller than that of M2 and M3 respectively. This could be due to an internal *Eco*RI restriction site in the cDNA fragment itself. *Eco*RI digestion could therefore produce two smaller DNA fragments instead of a single larger fragment.

4.5. Confirmation of Differentially Expressed Genes

DDRT–PCR at best could lead to false positive results, necessitating the confirmation of the obtained results. To confirm results obtained by RT–PCR, the induced expression of all 8 clones were tested using reverse Northern blots.

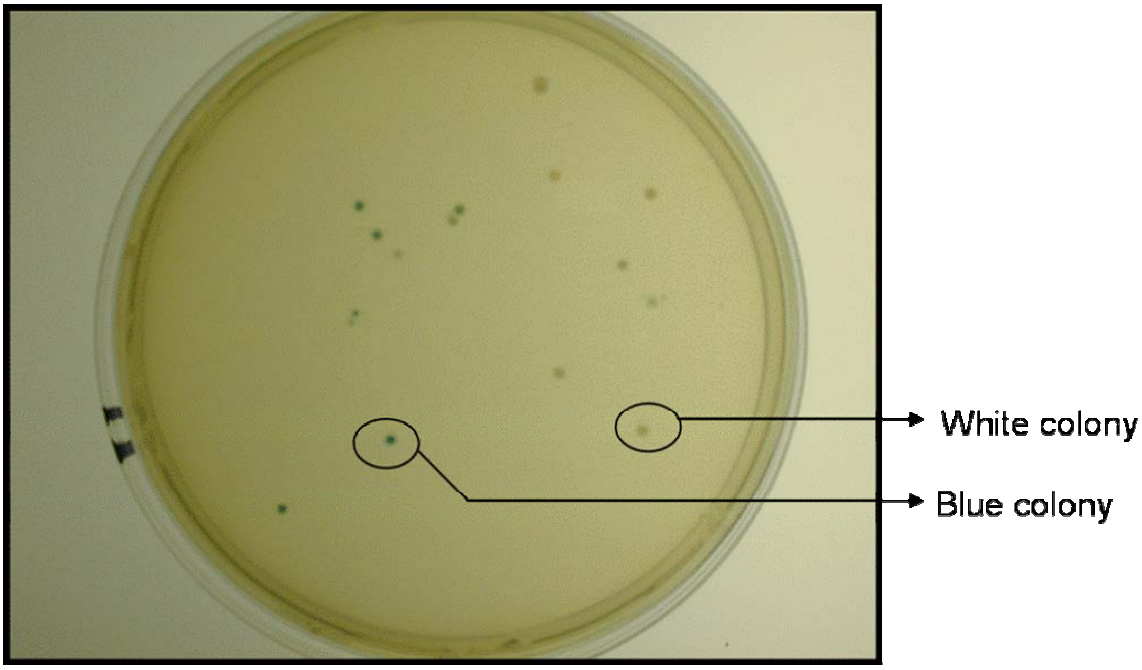


Fig. 4.11: The selection of *E. coli* cells containing recombinant plasmid DNA. Indicated in the figure are blue colonies (contain non-recombinant plasmids) and white colonies (contain recombinant plasmids).

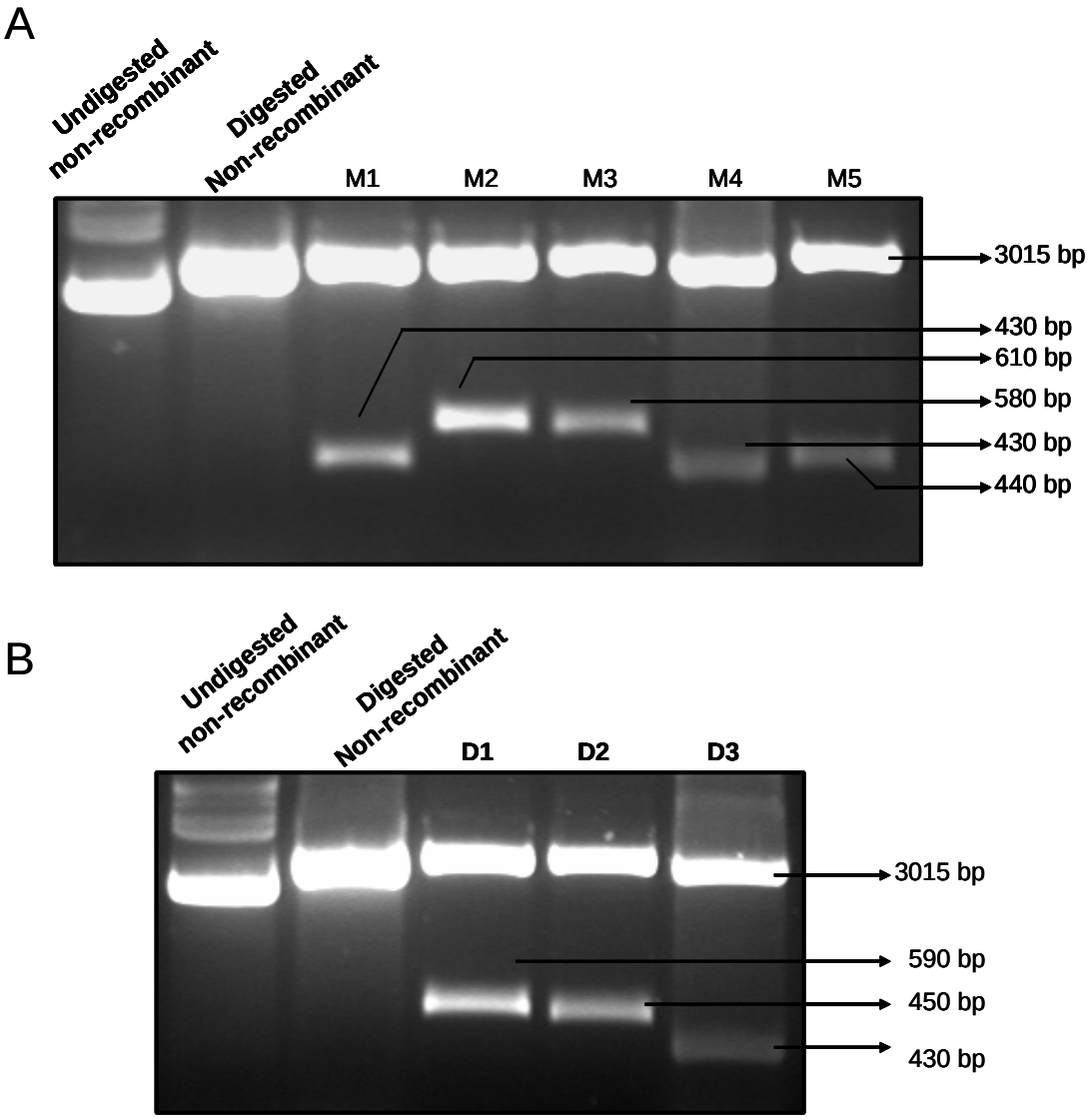


Fig. 4.12: Restriction digestion of isolated recombinant plasmids. A and B represents the different gene fragments isolated with the monocot and dicot primer combinations respectively. The plasmid as well as the fragment sizes are indicated.

Since the induced gene expression was mostly detected from 36 h.p.i. in the trial, it was decided to use extracted RNA from tissue harvested at 24 h.p.i. and later for the preparation of cDNA probes. Again the quality and quantity of the RNA was assessed (Fig. 4.13). RNA from both IR and IS plants at the different time intervals used were intact and of good quality.

The induced expression of all 8 clones was tested in both IR and IS plants (Fig. 4.14). The first impression is that all 8 clones were induced at some stage in both IR and IS plants, with one exception in the IS plants. However, there were variations in the levels of expression, as well as the time of induction between the different clones. In comparing the different clones to one another in the IR and IS plants, it seemed that the induction of M1 and M2 were higher in the IR plants compared to the IS plants. Contrary to this, it would seem that M5, D1 and D2's induction levels were higher in the susceptible plants.

At 24 h.p.i., the expression level of M1 was double that of time zero in the IR plants, and double that of M1's expression in the IS plants at the same time period. A peak in expression was seen at 36 h.p.i. in the IR plants, while in the IS plants M1's expression reached a peak at 42 h.p.i. The peak in the resistant plants was not only at an earlier time period, but it was also twice as high compared to the IS plants. While the expression level of M1 dropped at 48 h.p.i. in both IR and IS plants, the expression remained induced in the IR plants, while the level of expression in the IS plants was comparable to time zero.

M2's expression followed a similar expression pattern in both the IR and IS plants, with it expression being induced at 24 h.p.i. and reaching a peak at 36 h.p.i. in the resistant plants. The expression of M2 reached a peak in the IS plants at 48 h.p.i. However, when comparing the expression levels, M2's expression in IR plants was double that of the IS plants.

Clones M3 and M4 had similar expression patterns in both IR and IS plants. In the IR plants, the expression of both clones increased gradually from time zero to 48 h.p.i., reaching a maximum expression level of at least 2 fold that of time zero at 48 h.p.i. Comparable expression of the two clones was found in the IS plants, where a maximum level of expression of 2 times time zero was reached. The difference however was that this peak in expression in the IS plants was reached at 42 h.p.i.

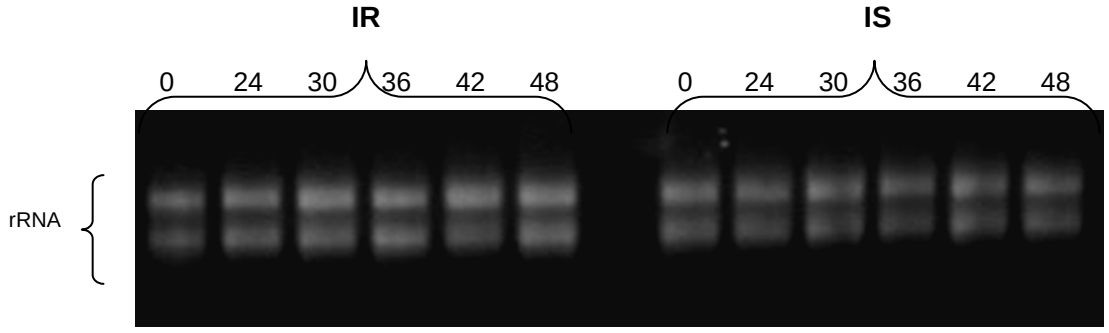
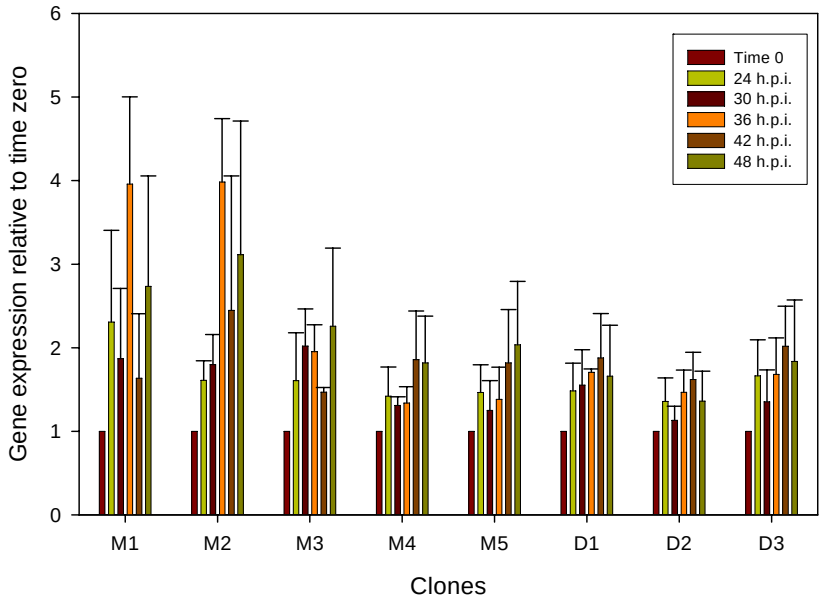


Fig 4.13: Total RNA extracted from resistant Yr1 and Avoset-S wheat cultivars at different time points after infection with stripe rust. The different rRNA fragments are indicated. The harvesting times of the tissues are indicated as hours post inoculation [h.p.i.] at the top of the image.

A



B

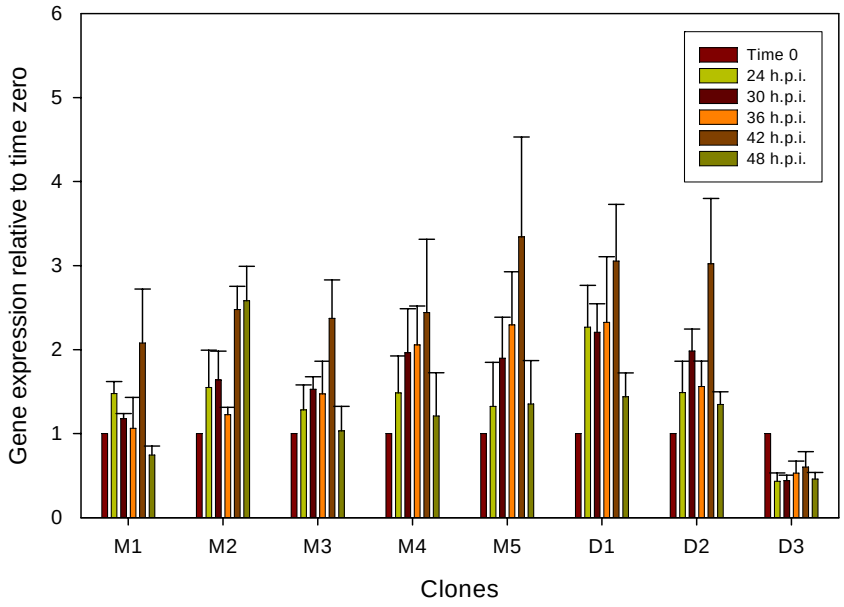


Fig 4.14: Expression profiles for all isolated cDNA fragments using reverse Northern blots. A and B represents the expression of the respective clones in the IR and IS plants respectively. Expression is indicated relative to time zero after normalization with actin gene expression. Time given as hours post inoculation [h.p.i.]

Clone M5 had a similar expression pattern as M3 and M4 in the IR plants, where its expression was gradually induced to peak at 48 h.p.i. In the IS plants however, M5 expression at 42 h.p.i. was more than 3 times that of time zero. This expression level was higher than that of the IR plants. A similar pattern was found for clone D1 that was also induced to higher expression levels in the IS plants than in the IR plants.

Clone D2 was induced in the IS plants starting at 30 h.p.i. and reaching a maximum in expression at 42 h.p.i. When compared to the expression of D2 in the IR plants, no significant induction occurred. Significantly was the fact that clone D3 was shown to be suppressed in the IS plants, while its expression was induced in the IR plants. The induction in the resistant plants was only twice that of time zero, but the fact that D3's expression was suppressed in the IS plants made it notable.

4.6. Sequence Analysis of Isolated Gene Fragments

Once the cloned cDNA fragments were sequenced, they were renamed; 05WVZ01 to 05WVZ05 for clones M1 to M5 and 05WVZ06 to 05WVZ08 for clones D1 to D3. The sequencing and BLAST results for the clones 05WVZ01 to 05WVZ08 are given in Fig. 4.15 to 4.22. After translating the cDNA sequences, the amino acid sequence for each clone was used in an amino acid similarity BLAST analysis to establish whether they show any homology to any known polypeptides present on data bases on the internet.

The amino acid sequence of the cDNA fragment 05WVZ01 showed a 34% homology to a nodulation protein B (fragment) from *Rhizobium* sp. with an E–value of only 0.006 (Fig. 4.15). This low homology is reflected in an amino acid alignment of the two polypeptides (Fig. 4.15 D).

The blast results for 05WVZ02 – 05WVZ04 as well as 05WVZ07 and 05WVZ08 all showed high homology with the TOBAC Hypothetical protein from *Nicotiana tabacum* (Fig. 4.16 – 4.18, 4.21 and 4.22). The identity between the polypeptides and the TOBAC protein ranged between 86% (05WVZ07) and 97% (05WVZ04), while the E – values ranged between 3 e^{-38} and 3 e^{-22}.

A

GAATTCCGGTGCAACAAGTGGCCGGAAGGTAATGTAGTGACCAATCCAGTAGCGACGCGCGGAGCAGCACCAATGGTATATG
AGGTGACCAACCGAGTGGCGACACCCCCCAGTGGAGAGGCGCGGAGAGGCCAAGCGGAGATGAGGTGACCAACCAAATGGCG
ATGGGAGGAGCAGCTAGGTGGTGATGAGGTGACCAACCAAATGACGACGCGCGGCACCGGCCGAGTGGTGATGAGGTGACCA
AACGAATGGCGACGCACCGGGCGGCCCAGACCAGATGAGGATGATCAAACCAGTGGCGACGCACTGAGCGGCCGAGTGGAGA
GGAGGTGATCAACTCAGAGACGACACGAGGGACGGCCACAATGGAGAGGAGGTGACCAATCCAGTGGTGACTCGAGTAGCGG
CACGAACGGATATGAGGTGACCACCCAAGATACGTCGCGTCGGACATCACTAGA**GAATTC**

B

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

C

Clone	Homology to:	Genbank accession nr.	Homology	E - value
05WVZ01	Nodulation protein B (fragment) – *Rhizobuim* sp.-	Q9Z3N4	34%	0.006

D

```
05WVZ01    : 33  VPRVVSELITSSPLGRSVRRHWFDHPHLVWAARCVAIRLVTSSPLGRCRASSFGWSPHHH 92
                 V R+VSE       G  V  H   HP L   +R    R +  +   R   S+   +   H
Nod prot B : 28  VQRIVSE-------GHGVANHTMTHPDLALCSRPQVEREIDEA--NRALLSACAGASIRH 78

05WVZ01    : 93  LAAPPIAIW----LVTSSPLGLSAPLHWGVSPLGWS 124
                 + AP    W    LV S+ LGL AP+HW V P  WS
Nod prot B : 79  IRAP-YGKWTEEALVKSASLGL-APVHWSVDPRDWS 112
```

Fig. 4.15: Sequence analysis of 05WVZ01. (**A**) Nucleotide sequence with *Eco*RI restriction sites indicated in bold at each end. (**B**) Translated sequence of the largest open reading frame (ORF). (**C**) BLAST analysis of the amino acid sequence. (**D**) Alignment between the Nodulation protein B fragment and 05WVZ01. Identical amino acids are indicated with gray letters and conserved substitutions as (**+**).

A

GAATTCGTATTCTGGCGCGTATGTAACCAGGGTGGCCTTAGCTGGTGATCCGGGCTGTTTCCCTCTCGACGATGAAGCTTAT
CCCCCATCGTCTCACTGGCCGACCTTGACCCCTGTTATTTTTGGGTCATATCTAGTATTCAGAGTTTGCCTCGATTTGGTAC
CGCTCGCGCAGCCCGCACCGAAACAGTGCTTTACCCCTAAATGTCCAGTCAACTGCTGCGCCTCAACGCATTTCGGGGAGAA
CCAGCTAGCTCTGGGTTCGAGTGGCATTTCACCCCTAACCACAACTCATCCGCTGATTCTTCAACATCAGTCGGTTCGGACC
TCTGCTTAGTTTCATCCAAGCTTCATCCTGGTCATGGATAGATCACCCAGGTTCGGGTCCATAAGCAGTGACAATCGCCCTA
TTAAGACTCGCTTTCGCTACGGCTCCGGTGGGTTCCGTTCCCTTAACCAAGCCACTGCCTATGAGTCGCCGGCTCATTCTTC
AACAGGCACGCGGTCAGAGATCACTTTCCCCTCCCACTGCTTGGGAGCTCAGCGCGGTTTCACGTTCTATTTCACTATCCAC
TGGGGGTTCTTTTCACCTTTCCCTCACGGTACTACTTCGCTATCGGCCACCCTGGGTATATTGCACCAGAAATCACTAGT**GA**
ATTC

B

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

C

Clone	Homology to:	Genbank accession nr.	Homology	E – value
05WVZ02	TOBAC Hypothetical protein orf106b *-Nicotiana tabacum-*	Q5M9Z9	88%	6 e^{-32}

D

```
05WVZ02: 36  PVIFGSYLVFRVCLDLVPLAQPAPKQCFTPKCPVNCCASTHFGENQLALGSSGISPLTTT 95
         P+I  SYLVFRVCLDLVPL++PAPK CFTP+CPVNCCASTHFGENQLALG+SGISPLTTT
TOBAC  : 15  PLILRSYLVFRVCLDLVPLSRPAPKPCFTPRCPVNCCASTHFGENQLALGASGISPLTTT 74

05WVZ02: 96  HPLILQHQSV 105
         HPLILQHQSV
TOBAC  : 75  HPLILQHQSV 84
```

Fig. 4.16: Sequence analysis of 05WVZ02. (**A**) Nucleotide sequence with *Eco*RI restriction sites indicated in bold at each end. (**B**) Translated sequence of the largest ORF. (**C**) BLAST analysis of the amino acid sequence. (**D**) Alignment between the TOBAC Hypothetical protein *Nicotiana tabacum* and 05WVZ02. Identical amino acids are indicated with gray letters and conserved substitutions as (**+**).

A

GAATTCGATTTCCGGTGCGATATAGCCTGGGTGGCCGATAGCGAAGTAGTACCGTGAGGGAAAGGTGAAAAGAACCCCCAGT
GGGTAGTGAAATAGAACGTGAAACCGTGCTGAGCTCCCAAGCAGTGGGAGGGGAAAGTGATCTCTGACCGCGTGCCTGTTGA
AGAGTGAGCCGGCGACTCATAGGCAGTGGCTTGGTTAAGGGAACGGAACCCACCGGAGCCGTAGCGAAAGCGAGTCTTAATA
GGGCGATTGTCACTGCTTATGGACCCGAACCTGGGTGATCTATCCATGACCAGGATGAAGCTTGGATGAAACTAAGCAGAGG
TCCGAACCGACTGATGTTGAAGAATCAGCGGATGAGTTGTGGTTAGGGGTGAAATGCCACTCGAACCCAGAGCTAGCTGGTT
CTCCCCGAAATGCGTTGAGGCGCAGCAGTTGACTGGACATCTAGGGGTAAAGCACTGTTTCGGTGCGGGCTGCGCGAGCGGT
ACCAAATCGAGGCAAACTCTGAATACTAGATATGACCCAAAAATAACAGGGGTCAAGGTCGGCCAGTGAGACGATGGGGGAT
AAGCTTCATCGTCGAGAGGGCCACCCAGGATACATTGCACCGGAAATCACTAGT**GAATTC**

B

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

C

Clone	Homology to:	Genbank accession nr.	Homology	E - Value
05WVZ03	TOBAC Hypothetical protein orf106b *-Nicotiana tabacum-*	Q5M9Z9	90%	3 e^{-32}

D

```
05WVZ03: 19 PVIFGSYLVFRVCLDLVPLAQPAPKQCFTPRCPVNCCASTHFGENQLALGSSGISPLTTT 78
            P+I  SYLVFRVCLDLVPL++PAPK CFTPRCPVNCCASTHFGENQLALG+SGISPLTTT
TOBAC   : 15 PLILRSYLVFRVCLDLVPLSRPAPKPCFTPRCPVNCCASTHFGENQLALGASGISPLTTT 74

05WVZ03: 79 HPLILQHQSV 88
            HPLILQHQSV
TOBAC   : 75 HPLILQHQSV 84
```

Fig. 4.17: Sequence analysis of 05WVZ03. (**A**) Nucleotide sequence with *Eco*RI restriction sites indicated in bold at each end. (**B**) Translated sequence of the largest ORF. (**C**) BLAST analysis of the amino acid sequence. (**D**) Alignment between the TOBAC Hypothetical protein *Nicotiana tabacum* and 05WVZ03. Identical amino acids are indicated as gray letters and conserved substitutions as (**+**).

A

GAATTCGCATTTCTGGTGCGATGTACCCAGGGTGGCCGAATAGCGAAGTAGTACCGTGAGGGAAAAGGTGAAAAGAACCCCC
AGTGGGTAGTGAAATAGAACGTGAAACCGTGCTGAGCTCCCAAGCAGTGGGAGGGGAAAGTGATCTCTGACCGCGTGGCTGT
TGAAGAATGAGCCGGCGACCCATGGGCAGTGGCTTGGTTAAGGGAACGGAACCCACCGGAGCCGTAGCGAAAGCGAGTCTTA
ATAGGGCGATTGTCACTGCTTATGGACCCGAACCTGGGTGATCTATCCATGACCAGGATGAAGCTTGGATGAAACTAAGCAG
AGGTCCGAACCGACTGATGTTGAAGAATCAGCGGATGAGTTGTGGTTAGGGGTGAAATGCCACTCGAACCCAGAGCTAGCTG
GTTCTCCCCGAAATGCGTTGAGGCGCAGCAGTTGACTGGCCACCCAGGATACGTCGCACCGGAAATCACTAGT**GAATTC**

B

W P V N C C A S T H F G E N Q L A L G S S G I S P L T T T H P L I
L Q H Q S V R T S A **Stop**

C

Clone	Homology to:	Genbank accession nr.	Homology	E – Value
05WVZ04	TOBAC Hypothetical protein orf106b *-Nicotiana tabacum-*	Q5M9Z9	97%	3 e^{-22}

D

```
05WVZ04: 2  PVNCCASTHFGENQLALGSSGISPLTTTHPLILQHQSV 39
         PVNCCASTHFGENQLALG+SGISPLTTTHPLILQHQSV
TOBAC  : 47 PVNCCASTHFGENQLALGASGISPLTTTHPLILQHQSV 84
```

Fig. 4.18: Sequence analysis of 05WVZ04. (**A**) Nucleotide sequence with *Eco*RI restriction sites indicated in bold at each end. (**B**) Translated sequence of the largest ORF. (**C**) BLAST analysis of the amino acid sequence. (**D**) Alignment between the TOBAC Hypothetical protein *Nicotiana tabacum* and 05WVZ04. Identical amino acids are indicated as gray letters and conserved substitutions as (**+**).

A

GAATTCGATTTCTGGTGCAATATAGCCTGGGTGGCCGAAATGGTGGTGATGTGACCAATCAAGTAGCGACGCGCGGAGCAGC
CGAATGGTTATGAGGTGACCAACCGAGTGGCGACACCCCCGAGTGGCGACGCGCGGAGAGGCCGAGCGGTGATGAGGTGACC
AACCAAGTGGCGATGGGCGGAGCAGCTAGGTGGTGATGAGGTGACCAACGAAGTGGCGACGCGCGGACCGGCCGAGTGGTGA
TGAGGTGACCAACCGAGTGGCGACGCACGGGCGGCCCAGAGGTGATGAGGTGATCAACCGAGTGGCGACGCACTGAGCGGCC
GAGTGGTGATGAGGTGATCAACAAAGAGACGACGCGCGGGACGGCCCAATGGTGATGAGGTGACCAATCAAGTGGTGACTCG
TGGAGCGGCCGAATGGTTATGAGGTGGCCACCCAGGATACATCGCACCGGAAATCACTAGT**GAATTC**

B

V R C I L G G H L I T I R P L H E S P L D W S P H H H W A V P R V
V S L L I T S S P L G R S V R R H S V D H L I T S G P P V R R H S
V G H L I T T R P V R A S P L R W S P H H H L A A P P I A T W L V
T S S P L G L S A R R H S G V S P L G W S P H N H S A A P R V A T
Stop

C

Clone	Homology to:	Genbank accession nr.	Homology	E – Value
05WVZ05	Hypothetical protein [P0758B01.19] *-Oryza sativa-*	Q6YVF9	33%	0.009

D

```
05WVZ05           :33  PRVVSLLITSS-PLGRSVRRHSVDHLITSGPPVRRHSVGHLITTRPVRASPLRWSPHHHL
91
                       PRVV LL+  + P   S RR S D       P+   + GHLI   P+ ++P    PH
HYPOTHETICAL PROT :48  PRVVGLLLLLALPTSSSSRRQSPDPPSLRRQPLPPCAAGHLILLAPLISAP----PH---
100


05WVZ05           :92  AAPPIATWLVTSSPLGLSARRHSGVSPLGWSPH 124
                        A  ++  L+ +SP   S+ RH  + PL  + H
HYPOTHETICAL PROT :101 -AASLSLLLLLASPAACSSSRHQPLIPLRATGH 132
```

Fig. 4.19: Sequence analysis of 05WVZ05. (**A**) Nucleotide sequence with *Eco*RI restriction sites indicated in bold at each end. (**B**) Translated sequence of the largest ORF. (**C**) BLAST analysis of the amino acid sequence. (**D**) Alignment between a Hypothetical protein from *Oryza sativa* and 05WVZ05. Identical amino acids are indicated as gray letters and conserved substitutions as (**+**).

A

GAATTCTGGTGCAATATAACCTGGGTGGCCGAATGGTGATGATGTGACCAATCAAGTAGCGACGCGCGGAGCAGCCGAATGG
TTATGAGGTGACCAACCGAGTGGCGACACCCCCGAGTGGCGACGCGCGGAGAGGCCGAGCGGTGATGAGGTGACCAACCAAG
TGGCGATGGGCGGAGCAGCTAGGTGGTGATGAGGTGACCAACGAAGTGGCGACGCGCGGACCGGCCGAGTGGTGATGAGGTG
ACCAACCGAGTGGCGACGCACGGGGCGGCCCAGAGGTGATGAGGTGATCAACCGAGTGGCGACGCACTGAGCGGCCGAGTGG
TGATGAGGTGATCAACAAAGAGACGACGCGCGGGACGGCCCAATGGTGATGAGGTGACCAATCAAGTGGTGACTCGTGGAGC
GGCCGAATGGTTATGAGGTGGCCACCACGGTTACATTGCACCGGAAATCACTAGT**GAATTC**

B

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

C

Clone	Homology to:	Genbank accession nr.	Homology	E – Value
05WVZ06	HGWP repeat containing protein *-Oryza sativa-*	Q6YPC3	34%	0.035

D

```
05WVZ06: 22  LDWSPHHH--------WAVPRVVSLLITSSPLGRSVRRHSVDHLITSGPPRASP------ 67
             +D S HHH        W  PR+   ++T   LG  V         T  PPR  P
HGWP   : 276 MDQSEHHHRLAVVAADWC-PRLYGWVVTPPLLGVVV--------FTDRPPRPPPTGVFAY 326

05WVZ06: 68  -LGWSPHHHSAGPRVATSLVTSSPPSCSAHRHLV 100
              +GWS HH  A         TSSPP+C   R LV
HGWP   : 327 IVGWSHHHCWAS--------TSSPPACLHRRRLV 352
```

Fig. 4.20: Sequence analysis of 06WVZ06. (**A**) Nucleotide sequence with *Eco*RI restriction sites indicated in bold at each end. (**B**) Translated sequence of the largest ORF. (**C**) BLAST analysis of the amino acid sequence. (**D**) Alignment between a HGWP repeat containing protein from *Oryza sativa* and 05WVZ06. Identical amino acids are indicated as gray letters and conserved substitutions as (**+**).

A

GAATTCGTATTTCCGGTGCAATGTATCCTGGGTGGCCGAATAGCGAAAGTAGTACCGTGAGGGCAAAGGTGAAAAGAACCCC
CAGTGGGTAGTGAAATAGAACGTGAAACCGTGCTGAGCTCCCAAGCAGTGGGAGGGGAAAGTGATCTCTGACCGCGTGCCTG
TTGAAAAATGAGCCGGCAACTCATAGGCGGTGGCTTGGTTAAGGGAACGGAACCCACCGGAGCCGTAGCGAAAGCGAGTCTT
AATAGGGCGATTGTCACTGCTTATGGACCCGAACCTGGGTGATCTATCCGTGACCAGGATGAAGCTTGGATGATACTAAGCA
GAGGTCCGAACCGACTGATGTTGAAGAATCAGCGGATGAGTTGTGGTTAGGGGTGAAATGCCACTCGAACCCAGAGCTAGCT
GGTTCTCCCCGAAATGCGTTGAGGCGCAGCAGTTGACTGGACATCTAGGGGTAAAGCACTGTTTCGGTGCGGGCTGCGCGAG
CGGTACCAAATCGAGGCAAACTCTGAATACTAGATATGACCCAAAAATAATAGGGGTCATAGGTCGGCCTGTGGGACGATGG
GGGATAAGCTTCATCGTCGAGAGGGCCACCCAGGGGACATCGTACCTTAAATTACTAGT**GAATTC**

B

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

C

Clone	Homology to:	Genbank accession nr.	Homology	E - Value
05WVZ07	TOBAC Hypothetical protein orf106b *-Nicotiana tabacum-*	Q5M9Z9	86%	3 e^{-38}

D

```
05WVZ07: 1  MKLIPHRPTGRPMTPIIFGSYLVFRVCLDLVPLAQPAPKQCFTPRCPVNCCASTHFGENQ 60
            MKLIPHR T RP  P+I  SYLVFRVCLDLVPL++PAPK CFTPRCPVNCCASTHFGENQ
TOBAC  : 1  MKLIPHRLTSRPCPPLILRSYLVFRVCLDLVPLSRPAPKPCFTPRCPVNCCASTHFGENQ 60

05WVZ07: 61 LALGSSGISPLTTTHPLILQHQSV 84
            LALG+SGISPLTTTHPLILQHQSV
TOBAC  : 61 LALGASGISPLTTTHPLILQHQSV 84
```

Fig. 4.21: Sequence analysis of 05WVZ07. (**A**) Nucleotide sequence with *Eco*RI restriction sites indicated in bold at each end. (**B**) Translated sequence of the largest ORF. (**C**) BLAST analysis of the amino acid sequence. (**D**) Alignment between the TOBAC Hypothetical protein *Nicotiana tabacum* and 05WVZ07. Identical amino acids are indicated as gray letters and conserved substitutions as (**+**).

A

GAATTCGATTCGCGATGTAGCCTGGGTGGCCAGTCAACTGCTGCGCCTCAACGCATTTCGGGAAGAACCAGCTAGCTCTGGG
TTCGAGTGGCATTTCACCCCTAACCACAACTCATCCGCTGATTCTTCAACATCAGTCGGTTCGGACCTCTGCTTAGTTTCAT
CCAAGCTTCATCCTGGTCATGGATAGATCACCCAGGTTCGGGTCCATAAGCAGTGACAATCGCCCTATTAAGACTCGCTTTC
GCTACGGCTCCGGTGGGTTCCGTTCCCTTAACCAAGCCACTGCCTATGAGTCGCCGGCTCATTCTTCAACAGGCACGCGGTC
AGAGATCACTTTCCCCTCCCACTGCTTGGGAGCTCAGCACGGTTTCACGTTCTATTTCACTACCCACTGGGGGTTCTTTTCA
CCTTTCCCTCACGGTACTACTTCGCTATCGGCCACCCAGGATACATTGCACCGGAAATCACTAGT**GAATTC**

B

P V N C C A S T H F G K N Q L A L G S S G I S P L T T T H P L I L
Q H Q S V R T S A **Stop**

C

Clone	Homology to:	Genbank accession nr.	Homology	E - Value
05WVZ08	TOBAC Hypothetical protein orf106b *-Nicotiana tabacum-*	Q5M9Z9	94%	3 e^{-22}

D

```
05WVZ08: 1  PVNCCASTHFGKNQLALGSSGISPLTTTHPLILQHQSV 38
         PVNCCASTHFG NQLALG+SGISPLTTTHPLILQHQSV
TOBAC  : 47 PVNCCASTHFGENQLALGASGISPLTTTHPLILQHQSV 84
```

Fig. 4.22: Sequence analysis of 05WVZ08. (**A**) Nucleotide sequence with *Eco*RI restriction sites indicated in bold at each end. (**B**) Translated sequence of the largest ORF. (**C**) BLAST analysis of the amino acid sequence. (**D**) Alignment between the TOBAC Hypothetical protein *Nicotiana tabacum* and 05WVZ08. Identical amino acids are indicated as gray letters and conserved substitutions as (**+**).

Alignment done on amino acid level with the TOBAC Hypothetical protein and the above mentioned polypeptides all confirmed the high homology.

The amino acid sequence of 05WVZ05 showed a 33% homology to a hypothetical protein from *Oryza sativa* with a 0.009 E - value in a blast similarity search (Fig. 4.19). The sequence of 05WVZ06 showed similarity of 34% and an E–value of 0.035 to a HGWP repeat containing protein from *Oryza sativa* (Fig. 4.20). In an alignment between 05WVZ05 and the hypothetical protein (Fig. 4.19 D), and 05WVZ06 and the HGWP repeat containing protein (Fig. 4.20 D), respectively, the low homology was confirmed.

Since 05WVZ02 to 05WVZ04, 05WVZ07 and 05WVZ08 all showed similarities to a hypothetical protein from tobacco, their sequences were aligned on nucleotide level (Fig. 4.23). The sequences showed almost 100% homology with one another with only a few bases, highlighted in red, differing between the sequences. Since the above mentioned sequences showed high homology to a TOBAC hypothetical protein, their amino acid sequences were aligned (Fig. 4.24).

In the figure, the areas where homology occurred are highlighted in light gray. It can be seen that the highest homology occurred more or less in the middle of the TOBAC protein with 38 amino acids being identical. This specific region of homology was also indicated on the nucleotide sequence, highlighted in dark gray (Fig. 4.23). Interestingly, this region occurs in the beginning of the isolated gene fragments as indicated in Fig. 4.23, with various potential stop codons located in this region, highlighted in black. In the amino acid alignment two conserved substitutions occurred, first between the TOBAC and 05WVZ08 sequences and secondly between the TOBAC and the rest of the sequences with a glutamate being substituted for a lysine and a serine being substituted for an alanine (Fig. 4.24).

Since 05WVZ02 to 05WVZ04, 05WVZ07 and 05WVZ08 all showed very high similarity to each other only 05WVZ03 was used for further analysis. 05WVZ03 was chosen because it had the largest ORF of the five sequences in question (Fig. 4.17).

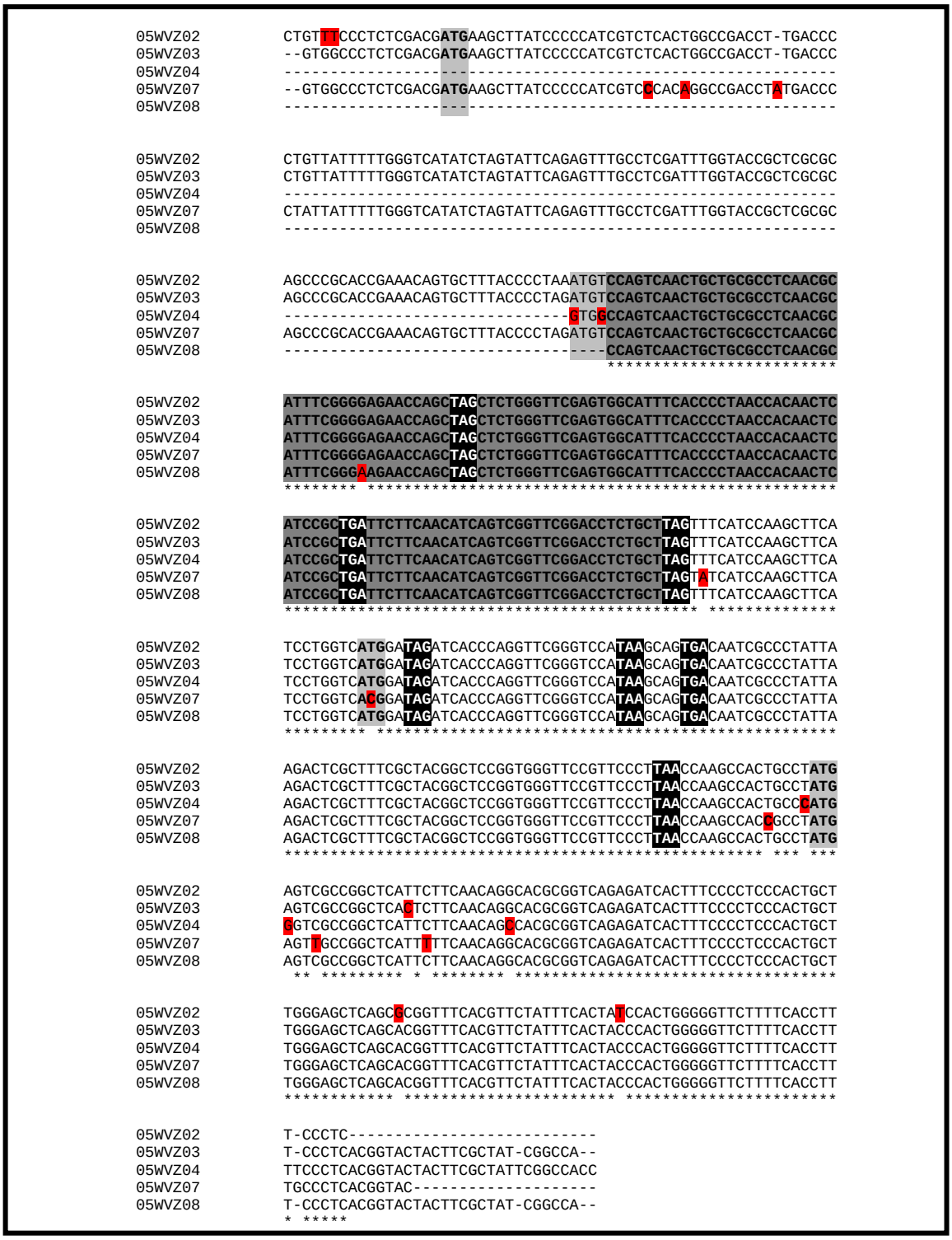


Fig. 4.23: Alignment of the nucleotide sequences of 05WVZ02 – 05WVZ04, 05WVZ07 and 05WVZ08. Identical nucleotides are indicated as (*). Start codons are highlighted in light gray and stop codons are highlighted in black. Differences between the sequences are highlighted in red. The area highlighted in dark gray shows homology to the ORF of a TOBAC hypothetical protein from *N. tabacum.*

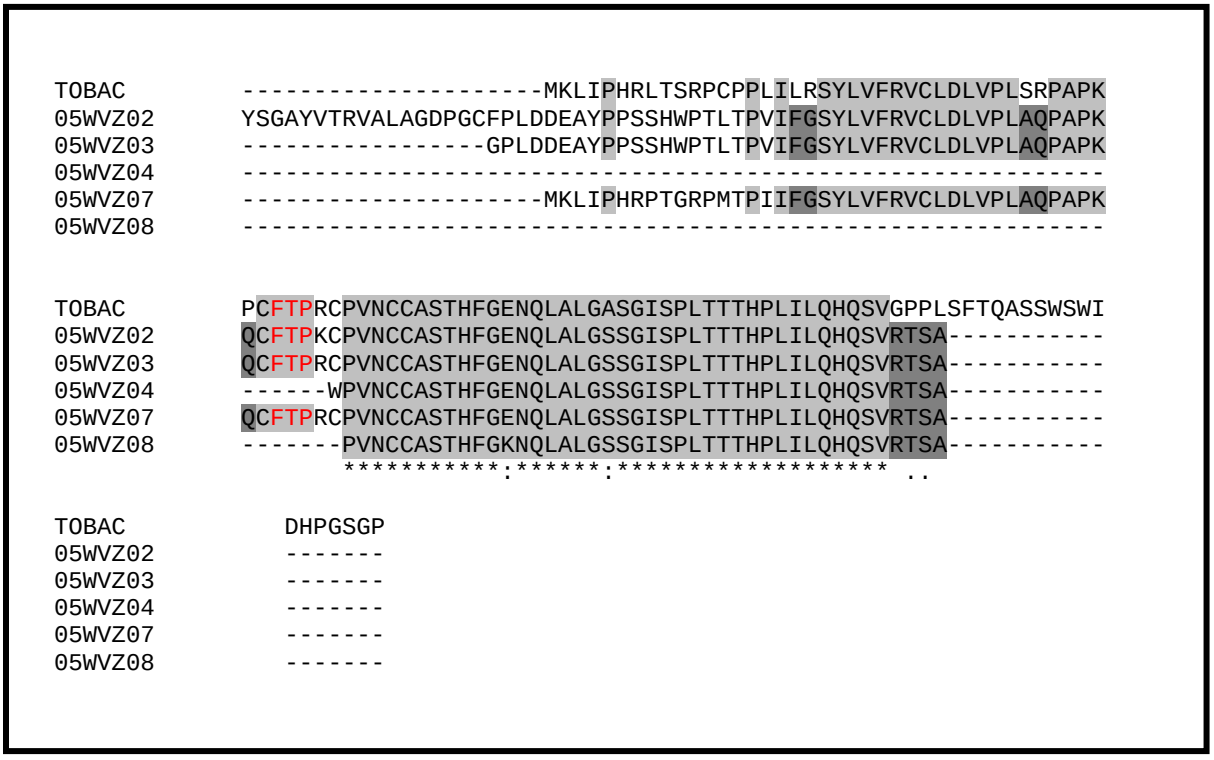


Fig. 4.24: Alignment of the amino acid sequences of 05WVZ02 – 05WVZ04, 05WVZ07 and 05WVZ08 with the complete TOBAC Hypothetical polypeptide from *N. tabacum*. Identical amino acids are indicated as (*), conserved substitutions as (**:**) and semi – conserved substitutions as (**.**). Light gray indicates homology between all the given sequences, while dark gray indicates homology between all the sequences except the TOBAC sequence. In red, a protein kinase C phosphorylation site is indicated

A domain and pattern search was done on the amino acid sequences of the identified gene fragments and the results are shown in Fig. 4.25. No known domains were found in the sequences although some frequent patterns were present. Protein kinase C phosphorylation sites were the most abundant with 2 sites in 05WVZ01, 2 sites in 05WVZ03, 4 sites in 05WVZ05 and 3 sites in 05WVZ06. 05WVZ01 contained a casein kinase II phosphorylation site, as did 05WVZ05 and 05WVZ06. Three cAMP- and cGMP dependent phosphorylation sites occurred in 05WVZ05, while only one such site was present in 05WVZ06. Furthermore, an XYPPX repeat occurred in 05WVZ03, an N–glycosylation site in 05WVZ05 and an N–myristoylation site in 05WVZ06.

Notable was that overlapping occurred between some protein kinase C phosphorylation sites and cAMP- and cGMP dependent phosphorylation sites. These incidents are indicated where the sequences are underlined in Fig. 4.25.

4.7. Southern Blot Analysis of cDNA Fragments

In order to determine the gene copy number of the isolated cDNA fragments and search for possible polymorphisms between resistant and susceptible wheat cultivars, Southern blots were done using the respective cDNA fragments as probes. Together with the susceptible Avoset-S cultivar, the presence of the genes were tested in five resistant cultivars, namely Yr1, Yr5, Yr10, Yr15 and Yr18 (Fig. 4.26). All the isolated cDNA fragments were present as single copy genes in all the cultivars tested. 05WVZ01 and 05WVZ05 hybridized with genomic DNA fragments with estimate sizes of 11173 bp and 11481 bp, while 05WVZ03 hybridized with a genomic DNA fragment with an estimated size of 4184 bp. 05WVZ06 hybridized with a genomic DNA fragment estimated to be 14125 bp. No polymorphisms were present for all the fragments tested, since the hybridized fragments were the same size for all four clones.

05WVZ01

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

05WVZ03

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

05WVZ05

V R C I L G G H L I **T I R** P L H E **S P L D** W S P H H H W A V P R V
V S L L I T S S P L G R **S V R R H S** V D H L I T S G P P V **R R H S**
V G H L I **T T R** P V R A S P L R W S P H H H L A A P P I A T W L V
T S S P L G L **S A R R H S** G V S P L G W S P H **N H S A** A P R V A T
Stop

05WVZ06

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

Fig. 4.25: Analysis of amino acid sequences of the identified gene fragments. Different patterns are indicated as follows; red for protein kinase C phosphorylation sites, light gray for casein kinase II phosphorylation sites, turquoise for XYPPX repeats, green for cAMP- and cGMP–dependent protein kinase phosphorylation sites, dark gray for N-glycosylation sites and yellow for N–myristoylation sites.

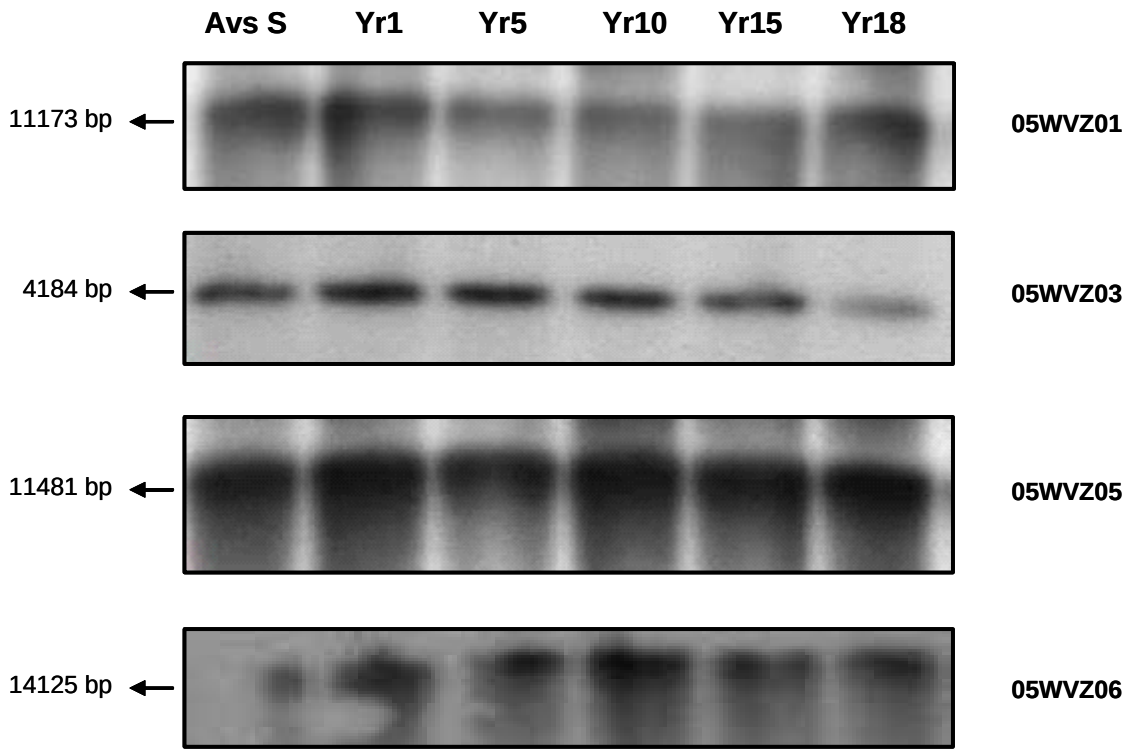


Fig. 4.26: Southern blot analysis of isolated cDNA fragments. The figure indicates the presence of the isolated cDNA fragments in various resistant cultivars. Sizes of genomic fragments that hybridized with the different fragments are indicated.

4.8. Expression Analysis of Isolated cDNA Fragments

A northern blot was done for the entire trial period of 72 h to assess the expression pattern of the cloned cDNA fragments in both IR and IS plants. The intact total RNA used for the Northern blots were included in Fig. 4.27 as a standard to indicate the equal loading of the RNA on the gels.

Results of the Northern blots differed from that of the reverse Northern blot (Fig. 4.14). Variation in the transient expression of all the clones in IR and IS plants was observed (Fig. 4.27).

The cDNA fragment, 05WVZ01, hybridized to an mRNA with an estimated size of 5365 bp. This gene was slightly induced 6 h.p.i. in the IR plants whereafter expression was gradually suppressed until 54 h.p.i. Contrary to this, induced expression was observed in the IS plants starting at 18 h.p.i. For the time intervals 54 h.p.i. and 66 h.p.i. of the IS plants the RNA seemed to be either of a lower concentration compared to the rest of the loaded RNA or degraded on the membrane. Taking this into consideration, an increased pattern in gene expression would probably be present in 05WVZ01 from the IS plants.

The cDNA fragment 05WVZ03 hybridized with two mRNA species with estimate sizes of 3937 bp and 2606 bp respectively. Both these genes showed constitutive expression in IR plants from 0 h.p.i. to 66 h.p.i. whereafter the expression was induced at 72 h.p.i. In the IS plants the larger fragment was constitutively expressed, while the expression of the smaller fragment was transiently inducibly expressed at 30 h.p.i. with three further inductions later on.

An mRNA with an estimated size of 11045 bp hybridized to 05WVZ05. This gene was strongly expressed from time zero in the IR plants whereafter it was gradually suppressed to much lower levels at 72 h.p.i. Expression in the IS plants was different to that of the IR plants. It seemed that 05WVZ05 was only expressed from 18 h.p.i., being present at very low levels and gradually increasing to a maximum at 72 h.p.i.

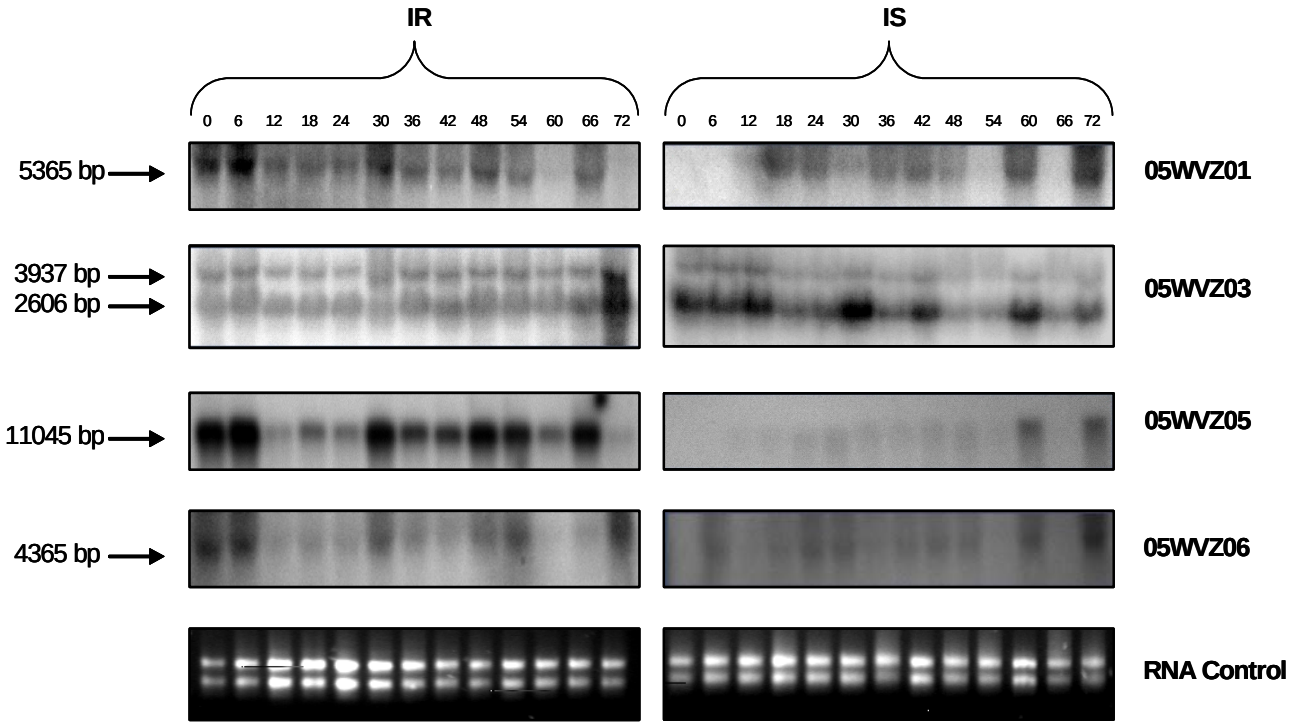


Fig. 4.27: Northern blot analysis of isolated cDNA fragments in IR and IS plants at different time points after infection. Calculated sizes of the different mRNA's that hybridized with the specific cDNA fragments are indicated. A RNA control is included and the time intervals are indicated as hours post inoculation [h.p.i].

A similar expression profile was found for 05WVZ06, which hybridized with a 4365 bp mRNA fragment.

Since there were some differences in the expression patterns between the Northern blots and the previously done reverse Northern blots, it was decided to do an expression analysis using RT–PCR. This however proved troublesome and only one of the four cDNA fragments, 05WVZ03, was successfully analyzed (Fig. 4.28). The result from the RT–PCR gave one clear band which showed a similar expression pattern as was obtained for the larger mRNA during the Northern blot (Fig. 4.27). Interestingly enough was the fact that although 05WVZ03 was shown to have a constitutive expression pattern in both IR and IS plants for the first 66 h.p.i., suppression in gene expression occurred at 36 h.p.i. in the IR plants according to the RT–PCR result. This suppression was also present in the IS plants although it occurred at 42 h.p.i. and lasted until 54 h.p.i.

The other three cDNA fragments proved difficult to test, since after several attempts with RT-PCR, no results were obtained. Future studies would entail the final characterization of expression of these cDNA fragments using RT-PCR.

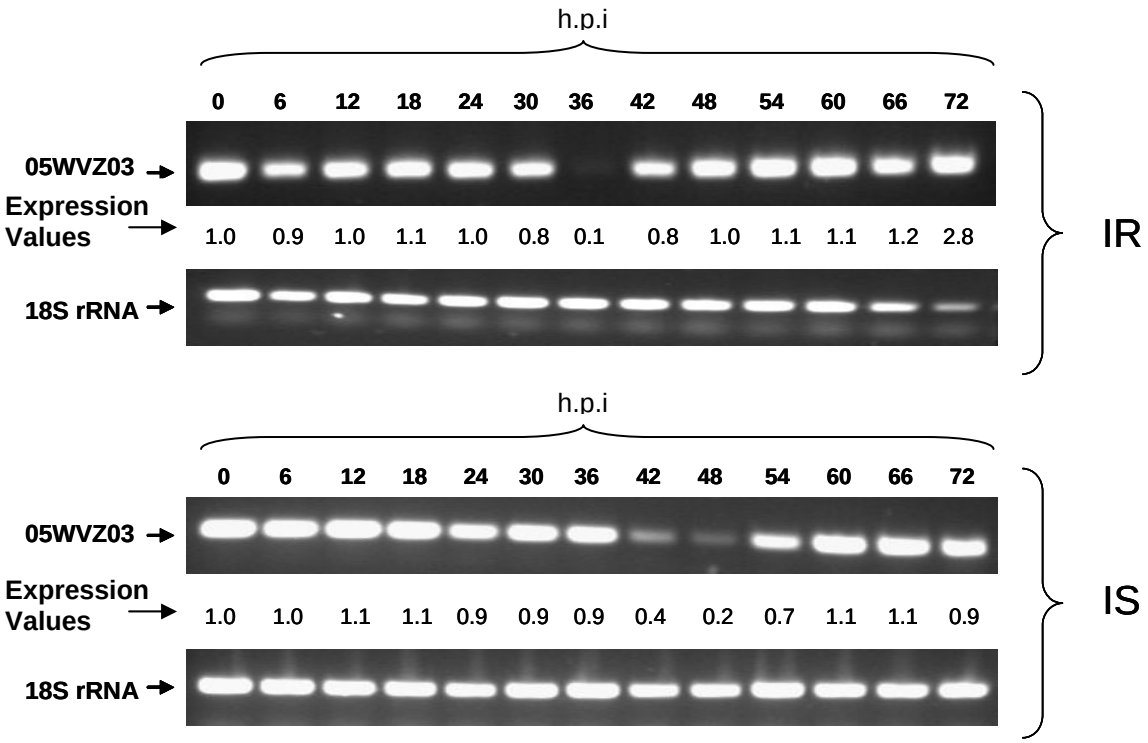


Fig. 4.28: RT–PCR analysis of 05WVZ03 in both IR and IS plants at different time points after infection. Values representing expression for each band are included. Values are relative to time zero and were normalized with 18S rRNA as a control. Time intervals are indicated as hours post inoculation [h.p.i].

CHAPTER 5

Discussion

DISCUSSION

The response of plants infected with fungal pathogens is very complex with a large number of different proteins playing crucial roles that allow the plant to survive the challenge (Vera-Estrella *et al*., 1994; Martin *et al*., 2003). Often, the responses of resistant and susceptible plants overlap with common proteins playing key roles. However, the ability of resistant plants to survive such infections relies on the presence of a few key proteins within the resistant plant. Such proteins include amongst others disease resistant genes (Hammond-Kosack and Jones, 1996) and transcription factors (Singh *et al*., 2002).

In the recent past, many efforts have been made to identify the genes encoding these unique proteins (Lawrence *et al*., 1995; Kim *et al*., 2002; Van der Biezen *et al*., 2002). Once these genes were cloned, they were exploited to increase resistance of the plant against that particular as well as other, pathogens (Stuiver and Custers, 2001).

For this study, the aim was to establish the oxidative burst and the involvement of protein kinases in the early responses involved in the resistance of a resistant wheat cultivar (Yr1) to *Puccinia striiformis*, thereby establishing the earliest point of recognition and the onset of defense responses to the intruding pathogen by the plant. This time period was then used in an attempt to clone genes involved in the downstream signaling. The earliest responses of plants upon infection include the phosphorylation/dephosphorylation of target proteins through protein kinases and phosphatases (Chandra and Low, 1995; Haffani *et al*., 2004) as well as the oxidative burst (Clarke *et al*. 2000a).

Therefore, in this study the occurrence of the oxidative burst was first established, whereafter the activation of protein kinases was determined. It was then assumed that the overlapping time intervals of the two responses should represent the earliest response time of the infected wheat plants.

The oxidative burst is a very fast response in reaction to various stresses, including pathogen infection in plants (Mlickova *et al*., 2004). In plant-pathogen interactions such as the tomato-*Oidium neolycopersici* interaction, increased ROS levels was detected within a period of 4 – 12 h after infection (Mlickova *et al*., 2004). Elevated H_2O_2 levels were detected only 4 h after the wounding of tomatoes (Orozco-Cardenas

et al., 2001). In the interaction between barley and powdery mildew, H_2O_2 was already detected 15 h after inoculation (Thordal–Christensen *et al.*, 1997). Thus, it is assumed that the oxidative burst usually occurs within the first 24 h after inoculation of the plant with the pathogen.

Various sources of ROS have been proposed. Producers of ROS include the plasma membrane NADPH oxidase (Jiang and Zhang, 2002), pH dependent peroxidase (Bestwick *et al*., 1997), germin-like oxalate oxidase (Dumas *et al*., 1995), mitochondria (Tiwari *et al*., 2002) and chloroplasts (Field *et al*., 1998).

Although all of the above mentioned sources have been shown to produce ROS, Bolwell and Wojtaszek (1997) proposed that the plasma membrane NADPH oxidase alongside the pH dependent peroxidase, function as the major sources of ROS during the oxidative burst, while the others only contribute small amounts of ROS. Various reports have implicated NADPH oxidase as the major producer of ROS (Keller *et al*., 1998; Pellinen *et al*., 1999; Jiang and Zhang, 2002).

The first response following the oxidative burst is the activation of ROS scavenging enzymes. Included amongst these enzymes are cell wall bound glutatione peroxidases (Noctor and Foyer, 1998). These peroxidases are involved in mediating the cross-linking of cell wall proteins (Bradley *et al*., 1992) and have been shown to be activated by elevated H_2O_2 levels during infection (Thordal-Christensen *et al*., 1997; Mohammadi and Kazemi, 2002).

In general it is thought that a phosphorylation cascade precedes most signals in the initiation of the defense reaction of plants against external stresses (Grant *et al*., 2000). CDPK's (Xing *et al*., 1996) and MAPK's (Ligterink *et al*., 1997) are actively involved in the activation of defense responses when tomatoes are infected by *Cladosporium fulvum* or treated with pathogen elicitors respectively. Thus, a great variety of kinases seems to be involved in downstream signaling from the initial recognition reaction to the activation of defense responses.

The plasma membrane NADPH oxidase system from mammalian phagocytotic cells has been shown to be activated through phosphorylation (Babior, 1999). It is thought that the plant homologue is activated similarly, but it is yet to be established. What is known is that phosphorylation does indeed play a major role in the elicitation of the oxidative burst (Chandra and Low, 1995).

In search for the oxidative burst in infected wheat, it was initially thought that NADPH oxidase was primarily responsible for the production of ROS. However, this enzyme did not seem to function early on in this specific plant-pathogen interaction, since there was no activation detected in either NADPH oxidase nor SOD activity in both IR and IS plants (Fig. 4.3 and 4.4). It is however possible that the NADPH oxidase system might function at a later stage in this plant-pathogen interaction.

Nevertheless, two transient increases in H_2O_2 levels, at 24 h.p.i. and 48 h.p.i., resembling an oxidative burst were detected in the IR plants of the second experiment (Fig. 4.5 B). It would thus seem that another source of ROS was functioning as the major contributor to the oxidative burst in this specific interaction.

As a confirmation of the oxidative burst, cell wall bound glutatione peroxidase activity was assayed. In Fig. 4.6 B, a peak in peroxidase activity was observed at 66 h.p.i. The peroxidase activity in the IR plants of the second experiment started to increase at 24 h.p.i., which coincided with the first increase in H_2O_2 levels (Fig 4.5 B). The peak in peroxidase activity at 66 h.p.i. coincided with the second and more significant increase in H_2O_2 levels. Thus, this confirmed that an oxidative burst have taken place at ± 48 h.p.i. with increased H_2O_2 levels and increasing peroxidase activity coinciding.

Furthermore, a significant increase in protein kinase activity was already observed at 18 h.p.i. in the IR plants with a further and even more significant increase at 54 h.p.i. (Fig. 4.7 B). This again coincided with the increased peroxidase activity. These increases in protein kinase activity were significant since the IS, CR and CS plants did not exhibit the same increases in activity, but rather remained stable with very little variation in activity overall.

These biochemical responses were summarized in Fig. 4.8, where the response of the IR plants was compared with the IS plants. It is very clear that a definite response is apparent in the IR plants, but absent in the IS plants. It is a possibility that the first increase in protein kinase activity at 18 h.p.i. is linked to a recognition event of the plant towards the intruding fungus. The kinase activity is further linked to two oxidative bursts, one lasting from 18 – 36 h.p.i. and another starting at 36 h.p.i. and lasting until 54 h.p.i. In addition to this, peroxidase activity started to increase constantly from 24 h.p.i. reaching a maximum at 66 h.p.i.

The second increase in protein kinase activity could possibly also be in response to the production of H_2O_2, since the role of H_2O_2 as a second messenger in the activation of defense responses are well documented (Orozco–Cardenas *et al*., 2001).

According to Moldenhauer *et al*. (2005), infection structures were visible on infected wheat leaves infected with stripe rust at 72 h.p.i. It is therefore possible that with the penetration of the stomata by the infection hyphae, recognition of the pathogen by the plant might have occurred. This recognition could therefore occur even before any cells were damaged by fungal penetration and/or infection structures. From Fig 4.8 B, it is clear that within the same time interval, the IS plants did not activate any defense responses, except for a small increase in peroxidase activity later on. This compared very favorably to several of the studies where it was proposed that the difference of resistance versus susceptibility relies on the speed with which the resistant plant recognized and reacts to the infection (Maleck *et al*., 2000; Gay and Tuzun, 2000).

Guo *et al*. (2004) investigated the role of an endogenous NO burst during the resistance response of wheat to stripe rust and found it to be at 24 h.p.i. The synergism between NO and H_2O_2 have been documented in other plant–pathogen interactions (Delledonne *et al*., 2001; De Gara *et al*., 2003) and is an active field of study. In this specific interaction, it would then also seem that NO and H_2O_2 play integral roles in the defense response. The investigation of synergism between NO and H_2O_2 in the interaction of wheat and stripe rust would be an interesting future aspect especially in unraveling the signaling cascade from the recognition to the initiation of defense responses. It would also promote the understanding of the complexity of signal transduction in plant–pathogen interactions.

The two experimental setups in this study showed good resemblance to one-another, with the second experiment only showing a quicker response compared to that of the first experiment. Successful infection was characterized by necrotic lesions on the IR plants and excessive pustule formation on the IS plants fifteen days after inoculation (Fig. 4.1). It was however apparent that a more efficient infection took place on the second experimental set of plants compared to the first (Fig. 4.1 A versus B), since the occurrence of necrotic lesions were more localized.

To conclude, the time period between 18 and 54 h.p.i. was identified as the period crucial for signaling events in the IR plants that was necessary for the activation of defense response. Therefore, to clone genes involved in these signaling events, tissue harvested from the second experimental set of IR plants was used for the DDRT-PCR reactions.

Various receptor proteins have been implicated to play a part during plant–pathogen interactions. Amongst these are LRK10 (Feuillet *et al*., 1997), FLS2 (Gomez-Gomez and Boller, 2000) and Xa21 (Song *et al*., 1995). All these receptors contain a serine/threonine kinase domain and play crucial roles in the recognition of the pathogen by the plant.

Other proteins that function in downstream signaling events include the CDPK's (Evans *et al*., 2001), MAPK's (Frey *et al*., 2001) and protein phosphatases (Braun *et al*., 1997). Another set of proteins involved in the onset of the plant defense response are the transcription factors such as ERF (Riechmann *et al*., 2000) and WRKY (Robatzek *et al*., 2002).

In general, it would seem that serine/threonine kinases are important players in the plant's response to external stresses, especially to intruding pathogens. The catalytic domain of plant serine/threonine protein kinases can be divided into 12 defined subdomains. These subdomains are recognized as being invariant or nearly invariant throughout the superfamily of kinases and are hence strongly implicated to play essential roles in enzyme function (Hanks and Hunter, 1995). According to Hanks *et al*. (1988) of all the 12 subdomains, subdomain VIb and VIII are the most conserved. For this reason, subdomain VIb and VIII were used for the generation of degenerate primers in order to clone putative protein kinase genes involved in the defense signaling events after infecting wheat with stripe rust through DDRT–PCR.

The DDRT–PCR technique was developed in 1992 by Liang and Pardee and have been used to identify differentially expressed mRNA in humans (De Vries *et al*., 2000) and plants (Kuno *et al*., 2000). However, the method as originally developed had limitations, including the fact that the differential expressed pattern of fragments could not always be reproduced on Northern blots. This was due to false positives that arose with high frequency, making it necessary to confirm the results obtained with for example Northern blotting.

In this study, several cDNA fragments were initially found to be differentially expressed. Eight such cDNA fragments, which were differentially expressed during the allocated time period of 18 h.p.i. to 54 h.p.i., were identified and cloned into pGEM–T.

Sequencing of the identified cDNA fragments revealed that five of the clones, namely 05WVZ02, 05WVZ03, 05WVZ04, 05WVZ07 and 05WVZ08 were highly homologous to each other (Fig. 4.23 and Fig. 4.24), although they varied in length. A similarity BLAST search done on amino acid level indicated that the above mentioned polypeptides shared very high homology with a TOBAC hypothetical protein from *Nicotiana tabacum* (Fig. 4.16 – 4.18, 4.21 and 4.22), indicating that they coded for the same protein. The alignment of the nucleotide sequences of these five cDNA fragments showed that there was almost 100% homology between the five sequences (Fig. 4.23) with a number of base substitutions.

When the encoded polypeptide were aligned with the complete polypeptide sequence of the TOBAC hypothetical protein, a region of 38 amino acids were identical between the different polypeptides and the TOBAC hypothetical protein (Fig. 4.24). It could thus be possible that this region could be a conserved domain determining the function of the protein. In contrast, both the carboxyl and amino terminal regions of the isolated clones differed from that of the TOBAC protein. The TOBAC protein itself has however yet to be characterized.

An analysis of the polypeptide sequence of 05WVZ03 as a representative of these five clones revealed that no known domains occurred in the sequence. However, two protein kinase C phosphorylation sites and a XYPPX repeat were found (Fig. 4.25). The XYPPX repeat is present in a wide variety of proteins and generally consists of the motif XYPPX where X can be any amino acid. The function of this repeat is still unknown.

The other three cDNA fragments that were sequenced namely, 05WVZ01, 05WVZ05 and 05WVZ06, showed very low sequence homology to a nodulation protein B (fragment) from *Rhizobium* sp., a hypothetical protein from *Oryza sativa* and a HGWP repeat containing protein also from *Oryza sativa* respectively (Fig. 4.15, 4.19 and 4.20).

The nodulation protein B from *Rhizobium* sp. with which 05WVZ01 shared low homology is known to function in carbohydrate metabolism, where it cleaves carbon–nitrogen, but not peptide bonds (Wernegreen and Riley, 1999). Analysis of the polypeptide sequence of 05WVZ01 indicated the presence of two protein kinase C and one casein kinase II phosphorylation site (Fig. 4.25).

The cDNA fragment 05WVZ05 showed the best sequence homology to a hypothetical protein from *Oryza sativa*, although the homology was low (Fig. 4.19). Amino acid analysis revealed the presence of various phosphorylation sites namely, protein kinase C, casein kinase II and cAMP- and cGMP – dependent protein kinase sites. In addition, an N-glycosylation site was also present (Fig. 4.25).

The last cDNA fragment sequenced, 05WVZ06, shared homology with a HGWP repeat containing protein from *Oryza sativa* (Fig. 4. 22). A HGWP repeat is a short 30 amino acid repeat found in a number of plant proteins. It contains a conserved HGWP motif, hence its name. The function of these proteins is still unknown (Nagaki *et al*., 2004). Various phosphorylation sites such as protein kinase C, casein kinase II and cAMP- and cGMP–dependent protein kinase phosphorylation sites as well as a N–myristoylation site were found to be present on the polypeptide sequence of 05WVZ06 (Fig. 4.25).

The presence of various phosphorylation sites, as mentioned on the different polypeptides, indicate the possibility that all these polypeptides could be regulated through phosphorylation. Although the N-glycosylation and N-myristoylation sites present on the polypeptides of 05WVZ05 and 05WVZ06, respectively, are not a unique or characteristic sites of any one protein it does play a role in membrane localization.

In general, many integral membrane proteins and secretory proteins contain covalently bound oligosaccharide chains. Several proteins also depend on the addition of a myristic acid for membrane localization and function (Nimchuk *et al*., 2000). One example of such a protein is Pto (Shan *et al*., 2000), although there is widespread speculation as to whether myristoylation is required for it’s functioning in the recognition of AvrPto (Loh *et al*., 1998). This then indicates that 05WVZ05 and 05WVZ06 could be either membrane associated or secreted proteins containing various phosphorylation sites.

None of the identified and cloned cDNA fragments showed any resemblance to or had any homology with serine/threonine protein kinases. The fact that degenerate primers specific for subdomain VIb and VIII were used for the DDRT-PCR and that no serine/threonine protein kinases were found, could probably be related to a lack of specificity of the RT-PCR. This could lead to the binding of the primers to various phosphorylation sites encoded on the DNA and hence the amplification of genes other than protein kinases. This needs to be addressed in the future by using another set of more specific primers.

All four cDNA fragments occur most probably as single copy genes within the wheat genome, since a single fragment hybridized when probed with the respective fragments (Fig. 4.26). The fact that the *Eco*RI digestion produced a single hybridizing fragment in all cases, however does not exclude the possibility that multiple copies of the genes with identically placed *Eco*RI sites, occur. What is however more important, is that all four genes were present in the susceptible, as well as five different resistant cultivars carrying different resistance genes. This clearly indicates that the four genes cannot be uniquely linked to the resistance locus present in these resistant cultivars.

Once the identities of the cloned cDNA's had been established, their expression had to be analyzed. This was first done using reverse Northern blots. In Fig. 4.14 it could be seen that all the clones were inducibly expressed in the IR plants. While 05WVZ01 and 05WVZ02 were strongly induced in the IR plants, their expression in the IS plants were much lower. In contrast, clones 05WVZ04 – 05WVZ07, had a stronger induction in the IS plants compared the IR plants. The exception was 05WVZ08 whose expression was suppressed in the IS plants.

Even though clones 05WVZ02 – 05WVZ04, 05WVZ07 and 05WVZ08 coded for the same protein, the results of the reverse Northern for the five cDNA fragments differed considerably from each other in both the IR and IS plants. In order to clarify this difference in expression pattern, clone 05WVZ03 was chosen as the representative of this group for a Northern blot, since it was the longest cDNA fragment. Two mRNA species, 3.9 and 2.6 kb in size respectively, hybridized to 05WVZ03 (Fig. 4.27). Since 05WVZ03 is present as a single copy gene in wheat (Fig. 4.26), it implicates that this specific gene was differentially transcribed. Genes being expressed as multiple transcripts in plants are known. One such example is *Inrpk1*, a gene encoding a putative receptor-like protein kinase that was isolated from the Japanese morning glory, *Ipomoea* (*Pharbitis*) *nil* Roth. cv. Violet (Bassett *et al*., 2000).

It is however also possible, as previously mentioned, that multiple identical copies of the gene exist and that they are differentially expressed. This could explain the different expression patterns on the reverse Northern blot (Fig. 4.14). Furthermore, the smaller fragment on the Northern blot is inducibly expressed in the IS plants at 30, 42, 60 and 72 h.p.i., but not in the IR plants. In contrast, the larger fragment was expressed constitutively in the IS and IR plants except at 72 h.p.i. in the IR plants, where induction in gene expression occurred.

In order to try to resolve the expression pattern of 05WVZ03, RT-PCR was done using two gene specific primers since it is a more sensitive technique to evaluate expression (Fig. 4.28). Despite its constitutive expression in both IS and IR plants, the expression of 05WVZ03 was suppressed at 36 h.p.i. in the IR plants and at 42 – 48 h.p.i. in the IS plants. The induction present on the Northern blot for the larger fragment in the IR plants was also seen in the RT-PCR. Thus, the expression pattern of the larger fragment in the Northern blot was mostly similar to that found with the RT-PCR.

Therefore, it is clear that the expression of clone 05WVZ03 differed between the reverse Northern blot, Northern blot and RT–PCR expression analysis. The major difference with the results obtained from the Northern blot and RT–PCR was the fact that with the Northern blot, two mRNA species hybridized with 05WVZ03 as probe, while with the specific primers designed for 05WVZ03, only one band was observed with the RT–PCR.

Since the reverse Northern blot differed vastly from the results obtained with both the Northern blot analysis and the RT–PCR done for 05WVZ03, the reverse Northern blot results were deemed as unreliable. An attempt was made to do RT-PCR reactions for all the clones, but due to problems with the designed primers and a time constraint, only the analysis of clone 05WVZ03 was finished.

The expression profile obtained from the Northern blots indicated that 05WVZ01 was gradually suppressed in the IR plants while being induced in the IS plants from time zero until 72 h.p.i. (Fig. 4.27). The expression profile of both 05WVZ05 and 05WVZ06 were similar to the expression profile of 05WVZ01 in both IR and IS plants (Fig. 4.27).

The fact that all three these genes were initially expressed in the IR plants and then subsequently repressed after infection, clearly indicate that they are not involved in the early signaling events leading to the defense response of wheat infected with

stripe rust. They do however seem to play some role during the defense response in the IS plants, since their expression was induced. More work must however be done to elucidate this role.

Of all four cDNA fragments analyzed, only one showed a possibility of being involved in the later defense response of wheat infected with stripe rust namely, 05WVZ03. Although the expression pattern of 05WVZ03 only showed induction at 72 h.p.i. it might yet play an important role in the secondary defense responses. The fact that it was most probably present in various transcriptional forms strengthens the idea, since this makes it a very versatile protein with the possibility of more than one function.

Thus, no substantial evidence was obtained to conclude that any of the identified genes are directly involved in downstream signaling events leading to the onset of defense responses in wheat. Since a time period when signaling events could have taken place, had been identified, it is recommended that the DDRT-PCR should be repeated. For this, the stringency of the amplification step of the DDRT-PCR should be adapted for better results. The possibility of using another conserved domain, such as the NBS domain, for the identification of induced genes should also be investigated since proteins containing this motif have been shown to play critical roles in the defense responses of plants (Tornero *et al*., 2002).

Future studies should also include a thorough investigation regarding the oxidative burst to establish the source of ROS production in the specific plant-pathogen interaction, since it differed from the accepted norm.

To conclude, an oxidative burst was established in the resistant Yr1 cultivar at 48 h after infection with *P. striiformis* after a possible recognition event occurring at 18 h.p.i. The oxidative burst coincided with elevated total protein kinase activity and increased glutathione peroxidase activity.

It was also established that the NADPH oxidase system, proposed to be the major producer of ROS, was not involved in the recognized oxidative burst. Thus, the possibility exists that pH dependent peroxidases could be primarily responsible for the production of ROS during the oxidative burst early on during infection.

Furthermore, four cDNA fragments showing homology with a nodulation protein B (fragment) from *Rhizobium* sp., a TOBAC hypothetical protein from *Nicotiana tabacum*, a hypothetical protein from *Oryza sativa* and a HGWP repeat containing protein also from *Oryza sativa* respectively, were cloned. Only one of these cDNA fragments, 05WVZ03, showed potential of being involved in the defense response of wheat infected with *P. striiformis*, but only later on.

CHAPTER 6

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SUMMARY

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The aim of this study was to establish the oxidative burst and the involvement of protein kinases in the early responses involved in the resistance of a resistant wheat cultivar (Yr1) to *Puccinia striiformis* f. sp. *tritici*, thereby establishing the earliest point of recognition and the onset of defense responses to the intruding pathogen by the plant. This time period was then used in an attempt to clone genes involved in the downstream signaling.

Plants are continuously challenged by a variety of pathogens. To survive these challenges, plants possess an arsenal of defenses, which are activated upon the recognition of the pathogen through certain signaling events. In some cases the difference between resistance and susceptibility lies in the timely activation of signaling. Early signaling events include protein phosphorylation and dephosphorylation and the production of reactive oxygen species (ROS).

To establish the earliest time of recognition and the activation of defense responses, the occurrence of the oxidative burst in the plant was first established, whereafter the activation of protein kinases were determined.

The oxidative burst was assessed through various enzyme activities e.g. NADPH oxidase, superoxide dismutase (SOD) and peroxidase (POX) activity as well as H_2O_2 levels. The earliest response of the plants was 18 h.p.i. when total protein kinase activity almost doubled in the infected resistant plants. This was followed by a similar increase in activity 48 h.p.i. Both increases in total protein kinase activity were accompanied by increases in H_2O_2 levels and glutathione peroxidase activity. However, the second increase at 48 h.p.i. was more significant and is therefore concluded to be the oxidative burst. The recognition of the pathogen, as well as the activation of the defenses therefore occurred between 18 and 48 h.p.i.

Once this reaction time was established, differentially expressed cDNA fragments were amplified using DDRT-PCR. Eight different gene fragments were isolated, cloned and sequenced. These isolated cDNA fragments showed different levels of homology to four known polypeptides namely, a Nodulation protein B (fragment) from *Rhizobuim* sp, a TOBAC Hypothetical protein from *Nicotiana tabacum*, a Hypothetical

protein from *Oryza sativa* and a HGWP repeat containing protein from *Oryza sativa* respectively.

Although no unique motifs were present on the respective polypeptide sequences, the presence of various phosphorylation sites indicate possible regulation through phosphorylation. An XYPPX repeat was present on the polypeptide sequence of 05WVZ03, while an N-glycosylation and an N-myristoylation was present on 05WVZ05 and 05WVZ06 respectively.

The isolated cDNA fragments were present as single copy genes in various resistant cultivars, as well as in the susceptible cultivar, Avoset-S, indicating that these genes are not unique to any one resistance cultivar. While naturally expressed in the IR plants, three genes (05WVZ01, 05WVZ05 and 05WVZ06) showed induced expression in the IS plants. The fourth gene (05WVZ03) was apparently expressed as multiple copies within wheat. The expression profiles of none of the clones however indicated a real involvement in signaling.

OPSOMMING

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Die doel van hierdie studie was om die oksidatiewe uitbarsting vastestel en die proteïen kinases wat daarby betrokke is te identifiseer by die vroeë seintransduksie en aanskakeling van verdedigingsreaksies in ‘n weerstandbiedende koring-kultivar (Yr1) na infeksie met *Puccinia striiformis* f. sp. *tritici* (streep-roes). Daardeur was die vroegste punt van herkenning van die patogeen deur die plant bewerkstellig. Daardie tyd periode was dan gebruik om gene te identifiseer wat moontlik betrokke kon wees by vroeë seintransduksie.

Plante word voortdurend deur patogene bedreig, maar besit die vermoë om hulself te verdedig deur verskeie verdedigingsmeganismes te gebruik. Hierdie meganismes word deur spesifieke seine in die plant na die herkenning van die patogeen geaktiveer. In sekere gevalle lê die verskil tussen weerstandbiedendheid en vatbaarheid vir spesifieke siektes, in die reaksietyd van die plant om die betrokke verdedigingsmeganismes aan te skakel. Proteïen fosforilering en defosforilering, tesame met die produksie van reaktiewe suurstofspesies (die oksidatiewe uitbarsting) vorm deel van vroeë seinoordraging in weerstandbiedende plante na infeksie.

Eerstens is bepaal hoe vinnig na infeksie, die patogeen deur die plant herken word. Verder is ook bepaal wanneer die plant se verdedigingsmeganismes aangeskakel is deur vas te stel wanneer die oksidatiewe uitbarsting plaasgevind het, asook deur die betrokkenheid van proteïen kinases in die verdedigingsreaksie te ondersoek.

Die oksidatiewe uitbarsting was bepaal deur die aktiwiteite van ‘n verskeidenheid ensieme te bestudeer nl. NADPH oksidase, superoksied dismutase (SOD) en peroksidase (POX). Die H_2O_2 vlakke is ook gemeet. Die vroegste reaksie in geïnfekteerde weerstandbiedende plante was 18 uur na infeksie waargeneem met ‘n toename in kinase aktiwiteit. ‘n Verdere toename in kinase aktiwiteit was 48 uur na infeksie waargeneem. Beide hierdie toenames was deur toenames in H_2O_2 vlakke en glutatioon-peroksidase aktiwiteit gevolg. Daar is tot die gevolgtrekking gekom dat die oksidatiewe uitbarsting 48 uur na infeksie plaasgevind het, aangesien die toename in kinase aktiwitiet en H_2O_2 vlakke hier meer beduidend was. Dus was die vroegste herkenning tesame met die aktivering van die verdedigingsresponse vasgestel as die periode tussen 18 en 48 uur na infeksie.

Na die vasstelling van bogenoemde, is cDNA fragmente wat geinduseerd tot uiting kom geamplifiseer d.m.v. DDRT-PCR. Agt cDNA fragmente is geïsoleer, gekloneer en die nukleotied volgordes daarvan bepaal. Hierdie cDNA fragmente het homologie getoon met vier reeds bekende polipeptiede nl. ‘n noduleerings proteïen B fragment van *Rhizobuim* sp, ‘n TOBAC hipotetiese protein van *Nicotiana tabacum*, ‘n hipotetiese protein van *Oryza sativa* en ‘n protein met ‘n HGWP motief van *Oryza sativa* onderskeidelik.

Geen unieke proteïen motiewe was op die onderskeie polipeptiedes gevind nie, alhoewel daar verskeie fosforileringsgebiede teenwoordig was. Die teenwoordigheid van fosforileringsgebiede dui op die moontlike regulering deur fosforilering. ‘n XYPPX herhalende volgorde was teenwoordig op die 05WVZ03 polipeptied, terwyl ‘n N-glukosilerings - en ‘n N-miristolerings – gebied onderskeidelik op 05WVZ05 en 05WVZ06 teenwoordig was.

Die geïsoleerde cDNA fragmente is as enkelkopie gene in verskeie weerstandbiedende kultivars asook in die vatbare Avoset-S kultivar teenwoordig. Dit dui daarop dat hierdie gene nie uniek is tot enige van die weerstandbiedende kultivars is nie. Hoewel al die gene tot uiting kom in die weerstandbiedende plante, het drie van hierdie gene nl. 05WVZ01, 05WVZ05 en 05WVZ06 geïnduseerde uiting in die vatbare geïnfekteerde plante vertoon. Die geen, 05WVZ03 word moontlik as veelvuldige kopiëe in koring uitgedruk. Die uitingsprofiele van al die klone toon egter dat nie een ‘n direkte betrokkenheid by vroeë seinoordrag het nie.