Characterization of early defence responses in rust-infected sunflower

By

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The manner in which one endures what must be endured is more important than the thing that must be endured. - Dean Acheson

It is the tension between creativity and skepticism that has produced the stunning and unexpected findings of science. - Carl Sagan

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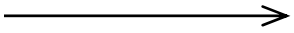
Abbreviations



A

ACC	1-aminocyclopropane-1-carboxylate
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
Avr	Avirulence

B

BA2H	Benzole acid-2 hydroxylase
BAG	Benzole acid glucoside
BFB	Bromophenol blue
BLAST	Basic local alignment search tool
BSA	Bovine serum albumine
BTH	Benzothiadiazole

C

CA	Cinnamic acid
CARD	Caspase recruitment domain
CBL	Calcineurin B-like
CC	Coiled-coil
CDPK	Calcium-dependent protein kinases
CHRK1	Chitinase-related RLK
CHS	Chalone synthase
CIPK	CBL-interacting protein kinase
CK1	Casein kinase 1
CR	Control resistant
CS	Control susceptible
CTAB	Cetyltrimethylammonium bromide

D

dATP	Deoxyadenosinetriphosphate
dCTP	Deoxycytidine triphosphate
DDRT-PCR	Differential display RT-PCR
DMPC	Dimethyl pyrocarbonate
dNTPs	Deoxynucleotidetriphosphates
DTT	Dithiothreitol
DTE	Dithioerythritol
DUF26	Domain of unknown function 26

E

EDS1	Enhanced disease susceptibility
EDTA	Ethylenedinitrilotetraacetic acid
EFE	Ethylene-forming enzyme
EGF	Epidermal growth factor
ESTs	Expressed sequence tags

F

FHA	Forkhead-associated phosphopeptide ligands
FLS2	Flagellin-sensing-2
F_m	Maximal fluorescence of a dark-adapted sample
F_m'	Maximum steady state fluorescence
F_o	Minimal fluorescence of a dark-adapted sample
F_s	Minimum steady state fluorescence
F_v/F_m	Maximum quantum yield
ϕPSII	Quantum yield of PSII

G

GADPH	Glyceraldehyde-3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GP	Glutathione peroxides
GSK3	Glycogen synthase kinase-3
GST	Glutathione S-transferase
GTP	Guanosine triphosphate

H

H_2O_2	Hydrogen peroxide
HDPase	Hydroxyperoxide dehydrase
HO_2^-	Hydroperoxyl radical
h.p.i.	Hours post infection
HR	Hypersensitive reaction

I

INA	Dichloroisonicotinic acid
IPTG	Isopropyl-β-D-thiogalactopyranoside
IR	Infected resistant
IS	Infected susceptible

J

JA	Jasmonic acid

L

LB	Luria Bertani
LOX	Lipoxygenase

LRRs	Leucine rich repeats

M

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

N

NB	Nucleotide binding
NBS	Nucleotide binding site
NDR1	Nonrace specific disease resistance
NO	Nitric oxide
Nod	Nucleotide-binding oligomerization domain
NPQ	Non-photochemical quenching

O

O_2^-	Superoxide
OGA	Oligalacturonide fragments
OGA-R	Oligalacturonide fragments and receptor
OH	Hydroxyl radical

P

P	Phosphatase
PAL	Phenylalanine ammonia lyase
PC	Plastocyanin
PCR	Polymerase chain reaction
PERK	Proline-rich extension-like protein kinase

PGases	Polygalacturonases
PGIPS	Plant polygalacturonic acid inhibitor proteins
Phe	Phenylalanine
PQ	Plastoquinone
PR	Pathogenesis related
PR5K	PR5-like receptor kinase
PSI	Photosystem one
PSII	Photosystem two
PUFAs	Poly-unsaturated fatty acids
PVP	Polyvinylpyrrolidone

Q

*q*P	Portion of open PSII

R

R	Resistant
RACE	Rapid Amplified cDNA Ends
RbcL	Rubisco large subunit
RbcS	Rubisco small subunit
R-genes	Resistant genes
RLK	Receptor-like protein kinase
ROS	Reactive oxygen species
Rp	Plant receptor protein
RPP	Resistance for *P. patasitica*
RPW8	Resistance to powdery mildew 8
RT-PCR	Reverse transcriptase polymerase chain reaction
Rubisco	Ribulose-1,5-bisphosphate carboxylase/oxygenase
RuBP	Ribulose 1,5-biphosphate

S

S	Susceptible
SA	Salicylic acid
SAG	Salicylic acid glucoside
SAR	Systemic acquired resistance
SDS	Sodium dodecyl sulphate
SIPK	Salicylic induced protein kinase
SLG	S-locus glycoprotein
SOD	Superoxide dismutase
SRK	S-locus receptor kinase

T

Temed	Tetramethylethylendiamine
TIR	Interleukin (IL)-1 receptors
TMAC	Tetramethylammonium chloride
TMV	Tobacco mosaic virus
Tris-HCl	Tris(hydroxymethyl)aminomethane
Tween20	Polyoxyethylenesorbitanmonolaurat

U

U	Units
UV	Ultra violet
UTR	Untranslated region

W

WAK	Wall-associated receptor kinase
WIPK	Wound induced protein kinase

X

X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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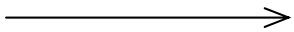
Chapter One:



Introduction

1.1 Introduction.

Plants are continually affected by changes in their environment including invasion by pathogens and pests. Through evolutionary adaptations, plants are equipped with defence systems which enable plants to defend themselves effectively against such changes. These well orchestrated systems enable the plant to recognise any potential threat at an early stage, thus allowing the plant to survive.

Gene-for-gene mediated plant disease resistance involves two basic interactions. A specific interaction between a resistance gene present in the plant genome and an avirulence gene from the pathogen genome, leads to a incompatible interaction which provides disease resistance in the plant (Flor, 1971). Several such resistance genes have been identified. The majority of these encode a protein which contains a nucleotide binding site followed by a series of highly conserved leucine-rich repeats. The nucleotide binding site domain is able to bind and hydrolyse ATP as it contains a conserved motif (Tameling *et al.*, 2002). An example of such a resistance gene is the *RPW8* gene indentified from *Arabidopsis* which provides resistance against *Erysiphe cruciferarum* (Xiao *et al.,* 2001). The leucine-rich repeat region on the other hand is able to modulate the defence response activation as it contains specific residues needed for bacterial recognition. An example of such a resistance gene is the *Xa21* gene isolated from the rice genome which provides resistance against *Xanthomonas oryzae* pv. *oryzae* (Song *et al.,* 1995). While a number of resistance proteins have very unique structures, several like *Xa21* and *LRK10*, form part of the larger class of receptor-like protein kinases.

In the defence signalling pathways various factors are present which regulate the downstream signal transduction. The first in line are the receptor-like protein kinases. This is a diverse group of proteins that spans the plasmamembrane where they act as secondary receptors of the invading pathogen (Morris and Walker, 2003). Members of the receptor protein kinase family all share highly conserved catalytic domains that have been shown to phosphorylate serine and

threonine residues (Mu *et al.,* 1994). Activation of these receptors depends on the binding of ligands called elicitors, which include cell wall breakdown products produced as a result of attack by invaders. The activation will trigger the activation of subsequent downstream signalling factors (Shiu and Bleecker, 2001).

Several other protein kinases have also been shown to be involved in the earliest perception of stress signals. The calcium-influx and protein kinase activity have been reported to be required in many plant-pathogen systems for further downstream signaling (Lecourieux *et al.,* 2002). The calcium-dependent protein kinases (CDPK) comprise of a family of plant-specific and multi-functional serine/threonine kinases which contains a regulatory calcium-binding domain (Harmon *et al.,* 2000). CDPKs are therefore ideally suited to sense changes in intracellular calcium levels and translating them into kinase activity (Harmon *et al.,* 2000). A CDPK was identified from tobacco cells which expressed the *Cf-9* resistance gene from tomato as a transgene (Romeis *et al.,* 2000). The CDPK was activated upon elicitation by the fungal-derived *avirulence* gene product Avr9 (Romeis *et al.,* 2000). This indicated that the up-regulated expression of this *CDPK* was specific in the *Avr9/Cf9* interaction.

Other factors that contribute to downstream signal transduction are the mitogen-activated protein kinases (MAPKs) (Garrington and Johnson, 1999). MAPK cascades are composed of three kinase modules namely MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and MAPK. The three components of the cascade transfer the signal through the process of phosphorylation and dephosphorylation to a final downstream acceptor. Receptor-mediated activation of MAPKKK can occur via physical interaction and/or phosphorylation by the receptor itself (Garrington and Johnson, 1999). MAPKKK activates MAPKK through phosphorylation of two serine/threonine residues in a conserved S/T-X_{3-5}-S/T motif. The MAPK is then phosphorylated on threonine and tyrosine residues in the T-X-Y motif (Garrington and Johnson, 1999). The final acceptors of the MAPK signal cascades include a variety of substrates such as transcription

factors, protein kinases and cytoskeletal proteins. The specificity of MAPKs are enhanced by the presence of MAPK docking domains on various components of the MAPK modules also involved in the signal transduction cascade (Tanoue *et al.,* 2001).

The constant process whereby a plant defends itself against a pathogen is very cost-intensive. As the invading pathogen needs living tissue for growth and reproduction, the plant is under tremendous stress to provide nutrients to sustain itself, despite the presence of the pathogen (Heil and Bostock, 2002). This was shown when plant-pathogen interactions were studied to determine the photosynthetic capacity of the infected plant. In many cases, it has been found that the photosynthetic capacity of the plant was negatively influenced during the incompatible interaction (Scharte *et al.,* 2005; Bonfig *et al.,* 2006). A decrease in photosynthesis was detected in the incompatible interaction between barley and *Blumeria graminis* (Swarbrick *et al.,* 2006). It was also shown that the expression of sugar-regulated photosynthetic genes such as the small subunit of ribulose-1,5-biphosphate carboxylase/oxygenase and chlorophyll *a,b* binding protein was down-regulated after pathogen infection (Swarbrick *et al.,* 2006). A further negative effect on the plant regarding the down regulation of photosynthesis, is the increased need for assimilates. This situation easily leads to source-tissue becoming sink-tissue (Roitsch *et al.,* 2003). In tobacco infected with the tobacco mosaic virus, an increased level of soluble sugars was noted (Herbers *et al.,* 2000). Many studies further confirmed that the regions surrounding the sites of pathogen infection contained higher sugar levels than the uninfected regions (Chou *et al.,* 2000). Thus, a common relationship exists between the carbohydrate status of the plant and the development of disease resistance.

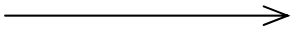
Sunflower is an important crop that is used world wide for oilseed production. A pathogen that invades the plant negatively influences the crop and has the ability to potentially lower the yield (Muro *et al.,* 2001). The aim of this study was to characterise some aspects of the early defence response of sunflower to the leaf

rust fungus (*Puccinia helianthi*). A putative protein kinase gene previously identified from sunflower that was infected with the leaf rust pathogen (M. Bezuidenhout; M.Sc. unpublished), would be characterized in an attempt to define its role during this particular plant pathogen interaction.

Since no resistance genes have been isolated from sunflower, an attempt would be made to isolate cDNA sequences that contain the nucleotide binding site of resistance genes from *P. helianthi* infected sunflower. The characterization of such genes would contribute to the understanding of the sunflower response upon pathogen infection.

Finally, the effect that the leaf rust fungus has on sunflower photosyntesis will be determined by using chlorophyll fluorescence. It would be determined whether an impaired photosynthetic capacity would influence the resistance response of the plant in a negative manner.

Chapter Two:



Literature Review

2.1 Introduction.

Plants are constantly challenged with fluctuations in their environmental conditions as well as with pathogens and pests. Plant cells use receptors located on the cell membrane to sense these changes (Walker, 1994). The early recognition of a pathogen by a plant is essential to mount an appropriate defence response. Plant cells are able to sense pathogen invasions by recognizing molecules derived from either a damaged plant cell (endogenous) or molecules originating from the pathogen (exogenous). These signal components are called elicitors. A complete set of plant defence reactions can be induced by some elicitors when they interact with specific plant receptors (Martin, 1999). Elicitors can be either specific or nonspecific. Specific elicitors are the end products of avirulence (*Avr*) genes that are recognized by the encoded products of resistance (*R*) genes that can ultimately lead to plant defence activation. Nonspecific elicitors can trigger various plant defence responses independently of any resistance gene. Recognition is probably due to various high-affinity receptors located in the plasma membrane. These general elicitors include substances typically associated with basic microbial metabolism such as cell wall glucans, fatty acids, sterols and glycopeptides (Wang, 2004; Shah, 2005).

2.2 Disease resistance genes.

Disease resistance in plants commonly requires two complementary genes. This immune system involves an allele-specific genetic interaction between a host *R* and a pathogenic *Avr*-gene. This is called a gene-for-gene interaction which leads to resistance due to an appropriate and timely activation of the plant defence response (Flor, 1971).

Each *R*-gene confers resistance to a specific strain of the pathogen (Ellis and Jones, 1998). *R*-genes provide surveillance for the plant against pathogens. The universal distribution of disease resistance genes which can target a large array of potential pathogens shows that the evolution of resistance genes is a high priority in plants (Richter *et al.,* 1995). A common feature of *R*-genes is

that they are frequently clustered on the same chromosomal regions of crops and could therefore undergo recombination events which lead to extensive sequence exchange (Bent, 1996). Recombination or gene conversion between tandem clusters of resistance genes may be a general feature in the generation of novel specificities that complement new pathogenic *Avr*-genes (Michelmore and Meyers 1998; Hulbert *et al.,* 2001).

The majority of *R*-genes encode a protein that has a nucleotide-binding site (NBS) and a series of highly conserved leucine rich repeats (LRRs) (Hulbert *et al.,* 2001). Their most noticeable structural feature is the variable number of carboxy-terminal LRRs (Dangl and Jones, 2001). A genome-wide survey regarding *R*-gene polymorphism in *Arabidopsis* showed that LRR regions were highly polymorphic for protein variants. This suggested that plants may generate many alleles that are maintained for short time periods (Bakker *et al.,* 2006).

Each NB-LRR protein has a conserved NBS for adenosine triphosphate (ATP) binding and hydrolysis (Tameling *et al.,* 2002). The NB-LRR class can be subdivided based on the N-terminal structural features of the polypeptides. The first class has a domain with sequence homology to the intercellular signalling domains of *Drosophila* Toll and mammalian interleukin (IL)-1 receptors (TIR). These resistant genes are called TNL genes. TNL genes have been found mostly in dicots (Meyers *et al.,* 1999; Pan *et al.,* 2000). The second class of NB-LRR R proteins does not contain a TNL region (Ellis and Jones, 1998; Meyers *et al.,* 1999), but commonly has putative coiled-coil domains in the N terminal region. These are called CC-NB-LRR or CNL genes. The LRR domains in NB-LRR proteins are thought to mediate the direct or indirect interaction between the R-protein and pathogen-derived molecules (Ellis *et al.,* 1999). The specificity of the R-proteins is believed to reside in the LRRs, which are constantly under selection pressure to be diverse in their amino-acid variability (Dangl and Jones, 2001).

The evolution of *R*-genes and the recombination within gene clusters are poorly understood, but one of the best-studied plant-pathogen interactions is

the tomato-*Cladosporium fulvum* interaction (Rivas and Thomas, 2002). *C. fulvum* is a biotrophic pathogen of tomato (*Lycopersicon*) species. Resistance to *C. fulvum* has been introduced from wild tomato species. The *Cf*-9 resistance gene originated from *L. pimpinellifolium* which confers resistance to strains of *C. fulvum* that secrete the Avr9 protein (Jones *et al.,* 1994). Plants with the *Cf-9* resistance gene displayed a hypersensitive reaction (HR) at the site of infection upon Avr9 recognition thereby restricting the growth of the fungus (Jones *et al.,* 1994). Other members also belonging to this large family of homologous *C. fulvum* resistance genes include *Cf-2, Cf-4* and *Cf-5* (Parniske and Jones, 1999). In addition, two *Avr*-genes namely *Avr4* and *Avr9* were identified from the fungus (Jones *et al.,* 1994; Dixon *et al.,* 1996; Thomas *et al.,* 1997; Takken *et al.,* 1998).

Cf-9 was found to be a member of the *Hcr9* gene family (homologs of *Cladosporium fulvum* resistance gene *Cf-9*) (Parniske *et al.,* 1997). This family encodes proteins with extracellular LRRs, a hydrophobic transmembrane domain and a short cytoplasmic tail which lacks an obvious signaling signature (Rivas and Thomas, 2002). Several clusters of *Hcr9* are present in tomatoes with up to five homologs per cluster of which *Hcr9-9C* is the functional *Cf-9* gene (Parniske *et al.,* 1997). When the *Hcr9* clusters were analysed comparatively, results suggested that point mutation, unequal recombination, gene conversion and translocation contributed to the diversity (Parniske and Jones, 1999). Van der Hoorn *et al.* (2001) identified a natural *Cf*-9 variant, *9DC*, in *L. pimpinellifolium* which has identical acitivity and specificity when conferring HR-resistance in response to *Avr9*. This suggests that *9DC* was ancestral to *Cf-9* (Van der Hoorn *et al*., 2001). Further research has shown that *9DC* and *Cf-9* share certain homologies which might show that these genes originated from the same ancestor (Kruijt *et al.,* 2004). After the comparison of the *9DC* and *Cf-9* clusters, it was hypothesized that the three *9DC* genes were generated by subsequent and unequal recombination events (Kruijt *et al.,* 2004).

Downstream defence signaling upon the Cf-9-mediated recognition of Avr9 has been studied in transgenic tobacco plants (Cai *et al.,* 2001). An oxidative

burst, the activation of MAPKs as well as ion fluxes were found to be involved in the regulation of Cf-9/Avr9 initiated defence responses (Cai *et al.,* 2001). Each *Cf-Avr* gene combination resulted in the arrest of hyphen growth at a distinct stage of colonization, either within the substomatal cavity or in the adjacent mesophyll cell layers (Hammond-Kosack and Jones, 1996*)*.

The *Pto* gene from tomato is another example of a resistance gene in tomato which encodes a functional cytoplasmic serine/threonine protein kinase that interacts directly with the Avr-Pto avirulence protein to confer resistance to bacterial speck disease (Zhou *et al.,* 1997). The 'guard hypothesis' suggests that a more complicated interaction among proteins activates defence (Dangl and Jones, 2001; van der Biezen and Jones, 1998). It has been proposed that Pto is required in a defence pathway of nonspecific elicitors of phytopathogenic bacteria. The function of Avr-Pto is to refrain Pto in this defence pathway. Physical interaction between Pto and Avr-Pto have been confirmed (Tang *et al.,* 1999), but Pto does require the NB-LRR protein, Prf, to activate the defence response. Prf 'guards' Pto and detects its association with Avr-Pto. This will lead to the defence system being activated.

Bacterial blight, one of the most devastating diseases in rice is caused by *Xanthomonas oryzae* pv. *oryzae*. Several resistance genes that confer resistance to this disease have been identified, but only three have been cloned. These are *Xa21* (Song *et al.,* 1995)*, Xa1* (Yoshimura *et al.,* 1998) and *Xa26* (Yang *et al.,* 2003). *Xa1* encodes a NB-LRR protein whereas *Xa21* and *Xa26* both encode a LRR receptor-like protein kinase with an extracellular domain, a transmembrane region and a cytoplasmic protein kinase domain (Sun *et al.,* 2004). The highest sequence homology has been reported between Xa26 and Xa21 (Song *et al.,* 1995). A small but distinguishable structural difference between the two exists regarding the amount of LRRS each contain where *Xa26* contain 26 LRRs and *Xa21* 23 LRRs (Song *et al.,* 1995). The difference in resistance conferred by the two genes lies in the solvent-exposed amino acids which are exposed when creating a surface for ligand interaction in the LRR domain (Kobe and Deisenhofer, 1995).

2.3 Receptor-like Protein Kinases.

Receptor-like protein kinases (RLKs) play fundamental signaling roles in a variety of processes that include the regulation of growth, development and defence responses (Morris and Walker, 2003). RLKs are a diverse group of proteins which acts as cell receptors which span the plasma membrane that allow cells to recognize and respond to their changing extracellular environment (Van der Geer *et al.,* 1994).

RLKs are structurally defined by the presence of a ligand-binding extracellular domain, a single membrane-spanning domain and a cytoplasmic kinase domain (Fig. 2.1) (Walker, 1994). Members of the plant receptor protein kinase family share highly conserved catalytic kinase domains. These have been shown to phosphorylate serine and threonine residues as well as occasionally tyrosine residues (Shiu and Bleecker, 2001). The general mechanism of receptor protein kinase function is that the binding of an extracellular ligand induces receptor dimerization. This triggers the subsequent activation of the intracellular kinase domain via auto-phosphorylation. The activated kinases then trans-phosphorylate substrate proteins within the cell, resulting in the transduction of the signal (Zhang, 1998).

The extracellular domain of RLKs allowed the grouping of plant RLK subfamilies based on the structures present. These include the LRR, S-domain, wall-associated receptor kinase (WAK), proline-rich extension-like receptor kinase (PERK), DUF26, PRK5, LRK10 and CHRK1 groups (Shiu and Bleecker, 2001). A brief description of the different RLK classes implicated in plant defence will now be given.

2.3.1 LRR-class.

The LRR class of RLKs represents the largest group and comprise approximately half of the predicted RLKs in *Arabidopsis* (Shiu and Bleecker, 2001). LRRs are common motifs in signal transduction proteins that mediate

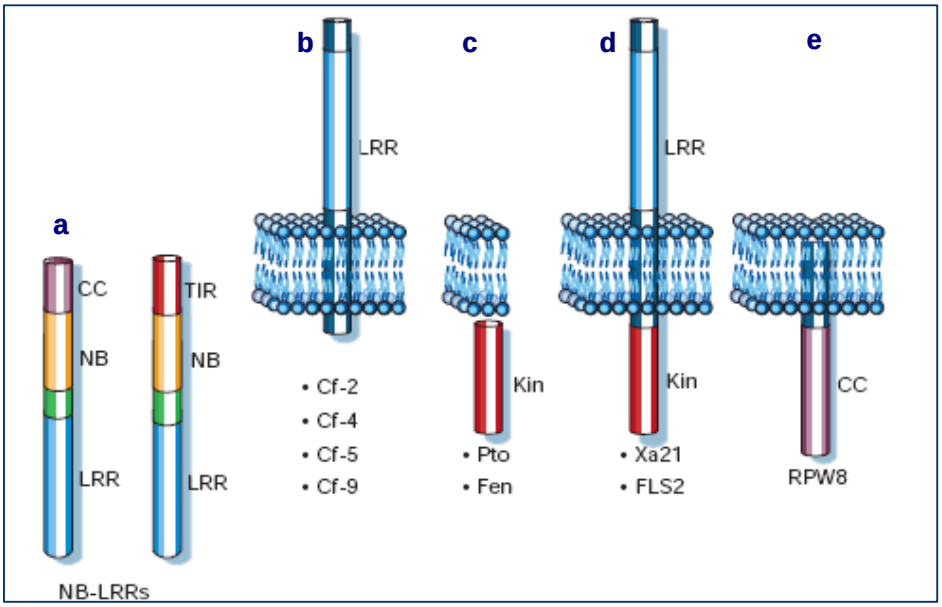


Figure 2.1 Illustration of the structure and location of the five main classes of plant disease resistance proteins (Dangl and Jones, 2001). In a), the largest class of R-proteins carry N-terminal domains and are cytoplasmic. In b), the Cf-X proteins carry transmembrane domains and have extracellular LRRs. In c), the *Pto* gene encodes a cytoplasmic Ser/Thr kinase. In d), the Xa21 and FLS2 proteins carry extracellular LRRs, transmembrane domains and Ser/Thr kinase domain. In e), the RPW8 gene product carries a putative signal anchor at the N terminus.

protein-protein interactions, peptide-ligand binding and protein-carbohydrate

interaction (Jones and Jones, 1996; Kajava, 1998). The LRR-RLKs class contain proteins that are involved in a range of biological functions. It has been found that the LRR receptor kinases act as receptors for polypeptides originating from pathogens (Gomez-Gomez *et al.,* 1999) and that it is involved in developmental processes (Becraft, 1998).

Flagellin-sensing-2 (FLS2) is one of the best-studied LRR-RLKs that are involved in the plant defence pathway (Gomez-Gomez *et al.,* 1999). An early transcriptional activation of gene expression was found in *Arabidopsis* after treatment with flg22, a peptide corresponding to the most conserved domain of flagellin (Navarro *et al.,* 2004). *FLS2* encodes a RLK with 28 LRRs in the extracellular domain (Fig. 2.1) (Gomez-Gomez and Boller, 2000). This extracellular domain has homology with the *Cf*-gene family of resistance genes that confers resistance to various strains of *Cladosporium fulvum* (Gomez-Gomez and Boller, 2000). In response to flagellin, FLS2 induces a signaling pathway that includes a complete plant MAPK cascade and transcription factors in *Arabidopsis* (Asai *et al.,* 2002).

A 160-kDa systemin cell-surface receptor that was identified in *Lycopersicon peruvianum* (Scheer and Ryan, 2002) is a LRR-RLK that also plays a role in disease resistance. Systemin, a polypeptide hormone, activates a cascade of intracellular signaling events. These trigger the release of linolenic acid from membranes which are then converted to oxylipin molecules that signal defence gene expression (Scheer and Ryan, 2002). SR160 has shown similarity with other protein kinases regarding the percentage of amino acids which are conserved in its kinase domains (Morris and Walker, 2003).

2.3.2 S-domain class.

The S-domain class of RLKs comprises a smaller group of RLKs which are believed to be involved in the self-incompatibility phenotype (Nasrallah, 1997). The S-class of RLKs is required for the ability of the stigma to inhibit self-pollination. Two highly polymorphic genes are required namely the *S*-locus glycoprotein (*SLG*) gene and the *S*-locus receptor kinase (*SRK*) which

encodes a RLK that belongs to the family of serine/threonine receptor kinases (Stein *et al.,* 1996).

The expression of *SFR2*, another S-domain RLK, is induced in response to wounding and pathogen infection (Dwyer *et al.,* 1994; Tobias and Nasrallah, 1996). *SFR2* mRNA also accumulates rapidly after treatment with salicylic acid (SA) (Pastuglia *et al.,* 1997). This SFR2 induction pattern indicates that this gene plays a role in the defence signal transduction pathway.

2.3.3 Wall-associated receptor kinases.

The first contact of a pathogen with a plant cell must include some form of interaction with the cell wall (Gaulin *et al.,* 2006). The plant cell wall and extracellular matrix form a complex arrangement of carbohydrates and proteins (Zheng-Hui *et al.,* 1996). The *Arabidopsis* WAKs with 23 members is a class that represents protein kinases with epidermal growth factor (EGF) repeats in the extracellular domain (Kohorn *et al.,* 1992; He *et al.,* 1999; Shiu and Bleecker, 2001). Initially, five genes were isolated (*WAK1-WAK5*) that were arranged in a tandem cluster (Zheng-Hui *et al.,* 1996). WAKs are plasma membrane associated proteins that are also tightly bound to the cell wall (Anderson *et al.,* 2001). The members of the WAK family have been implicated to play a role in defence responses (He *et al.,* 1999; Anderson *et al.,* 2001). *WAK1* is expressed upon treatment with the bacterial pathogen *Pseudomonas syringae* (Zheng-Hui *et al.,* 1996). In addition, treatment with defence-inducing compounds, SA and dichloroisonicotinic acid (INA), resulted in increased levels of *WAK1, WAK2, WAK3* and *WAK5* transcripts (He *et al.,* 1998, 1999).

2.3.4 PERK.

The PERK in *Arabidopsis* has a predicted extracellular domain that is proline-rich that shares sequence similarity with extensins (Silva and Goring, 2002). The PERK-family has only a single membrane-spanning region with no predicted signal peptide at the N-terminus (Silva and Goring, 2002). It had

been shown that PERK1 is ubiquitously expressed in *Brassica* and that mRNA levels increased rapidly in response to wounding and infection with the fungal pathogen *Sclerotinia sclerotiorum* (Silva and Goring, 2002).

2.3.5 DUF26.

The DUF26 class of RLKs is related to the S-domain class and is known as the cysteine-rich repeat class. This class currently consists of 38 members (Takahashi *et al.,* 1998; Chen, 2001; Shiu and Bleecker, 2001). The homology of these extracellular domains is restricted to a 60 amino acid region that contains four highly conserved cysteine residues (Du and Chen, 2000; Shiu and Bleecker, 2001). These conserved cysteine residues may act to maintain a three-dimensional structure or to form zinc-finger motifs to mediate protein-protein interactions. It may also be involved in sensing redox changes in the extracellular space during plant defence responses (Hardie, 1999, Chen, 2001). The DUF26 receptor kinase genes are inducible upon pathogen infection or treatment with reactive oxygen species (ROS) and SA (Du and Chen, 2000).

2.3.6 PR5K.

The only member of this class is the *Arabidopsis* PR5-like receptor protein kinase (PR5K). The thaumatin extracellular domain of PR5K is very similar to the acidic PR5-proteins that accumulate in the extracellular spaces of plants challenged with pathogenic micro-organisms (Wang *et al.,* 1996). Structural similarity between the extracellular domain of PR5K and the antimicrobial PR5-proteins suggests a possible interaction with common or related microbial targets.

2.3.7 LRK10.

LRK10 contains a unique type of extracellular domain which is not found in any other known plant or animal receptor protein kinases (Feuillet *et al.,* 1997). *LRK10* was mapped to the leaf rust *Lr10* disease resistance locus in

wheat (Feuillet *et al.,* 1997). The *LRK10* gene encodes a kinase that is involved in the plant-pathogen interaction. Several conserved amino acid sequences present in S-domain glycoproteins and receptor-like kinases were also found in LRK10. The LRK10 kinases consist of a hydrophobic signal sequence at the amino-terminus, putative extracellular domain, transmembrane sequence and a Ser/Thr kinase domain (Feuillet *et al.,* 1997).

2.3.8 CHRK1.

A chitinase-related *RLK* (CHRK1) was isolated from tobacco (*Nicotiana tabacum)* (Kim *et al.,* 2000). The extracellular domain is closely related to the class V chitinases from tobacco as well as to microbial chitinases. The amino acid sequence analysis revealed that the chitinase-like domain of CHRK1 lacks the essential glutamic acid residue that is required for chitinase activity. *CHRK1* mRNA accumulation is significantly stimulated by fungal pathogens and tobacco mosaic virus (TMV) infection (Kim *et al.,* 2000), suggesting that CHRK1 may be involved in pathogen signaling.

2.4 MAPKs involved in signal transduction.

In order for a plant to provide resistance against a pathogen attack, a system is required to first recognise the invading pathogen by means of cell wall receptors. Recognition must then be followed by a cascade of events that includes signal transduction through the cytoplasm that eventually leads to a biochemical defence response.

The mechanism of signal transduction is known for the processes of molecules being phosphorylised and dephosphorylised. These events enable the plant to convert extracellular signals from a pathogen attack into an intracellular response to pathogen derived molecules (Jonak *et al.,* 2002). During the process of phosphorylation ATP is hydrolised to adenosinediphosphate (ADP) where the phosphate group within the kinase domain is transferred to serine, threonine or tyrosine residues of the protein kinase.

MAP kinases are activated by MAPKK through dual phosphorylation of threonine and tyrosine residues of a TXY motif located between subdomain VII and VIII of the catalytic kinase domain (Sessa and Martin, 2000). In turn, MAPKK is activated by phosphorylation by a MAPKKK. Recent studies provided evidence that MAP kinases are also involved in plant signaling pathways, particularly during the activation of stress-associated responses (Asai *et al.* 2002).

OsBIMK1 is a MAPK gene which is expressed in *Oryza sativa* (Song and Goodman, 2002). OsBIMK1 contains all eleven of the conserved MAPK subdomains and shares high similarity with other MAPK genes. The gene is expressed upon treatment with chemical and biological inducers in both compatible and incompatible interactions of rice and *Magnaporthe grisea*. The expression of *OsBIMK1* is rapidly activated upon treatment with benzothiadiazole (BTH), as well as with INA, probenazole, jasmonic acid (JA) or wounding. BTH treatment induced a systemic activation of *OsBIMK1* expression. These results suggest that *OsBIMK1* plays an important role in rice disease resistance, again emphasizing the importance of protein kinases in the plant defence response.

Protein phosphorylation is thus responsible for the modification of enzyme activity and subcellular location. It is a crucial factor in the integration of signals within the cell and determines the extent and duration of the response and the effectiveness thereof (Sessa and Martin, 2000).

2.5 Biochemical defences.

A plant under pathogen attack (Fig. 2.2) triggers a multicomponent defence response (Hammond-Kosack and Jones, 1996). The activation of this response requires the initial recognition of the pathogen by the plant which then leads to a signal transduction event. The final step in this process is the induced expression of defence related genes whose encoded products are responsible for the physiological processes that cause disease resistance.

Resistance is manifested by the appearance of necrotic lesions and localized cell death at the site of infection. This localized cell death is called the HR (Heath, 2000). The HR limits the spread of the pathogen throughout the infected plant by killing infected plant cells, thus depriving the supply of nutrients to the pathogen.

The biochemical response associated with the HR includes the production of ROS, the transient opening of ion channels, cell wall fortifications, the production of antimicrobial phytoalexins and the synthesis of pathogenesis-related (PR) proteins such as glucanases and chitinases (Hammond-Kosack and Jones, 1996). Once the earliest defence responses have been activated, complex secondary biochemical pathways within the responding cells are activated as new signaling molecules are generated. A brief description of some of the biochemical responses will now be given.

2.5.1 Calcium and ion channels.

Transient ion fluxes across the plasma membrane are some of the most rapid changes measured in plant cell cultures upon stimulation with pathogen-derived elicitors (Blumwald *et al.,* 1998). With the application of elicitors to several plant species, an efflux of Cl^- and K^+ ions and an influx of H^+ were registered (Nürnberger *et al.,* 1994). Ion channels that are activated as a result of an activated receptor together with an increased Ca^{2+} uptake, were shown to precede the oxidative burst (Zimmerman *et al.,* 1997). The intensity and duration of the increase in Ca^{2+} depends on the eliciting agent and can differ between two different pathogen-derived elicitors that bind to the same cellular protein (Grant *et al.,* 2000; Lecourieux *et al.,* 2002).

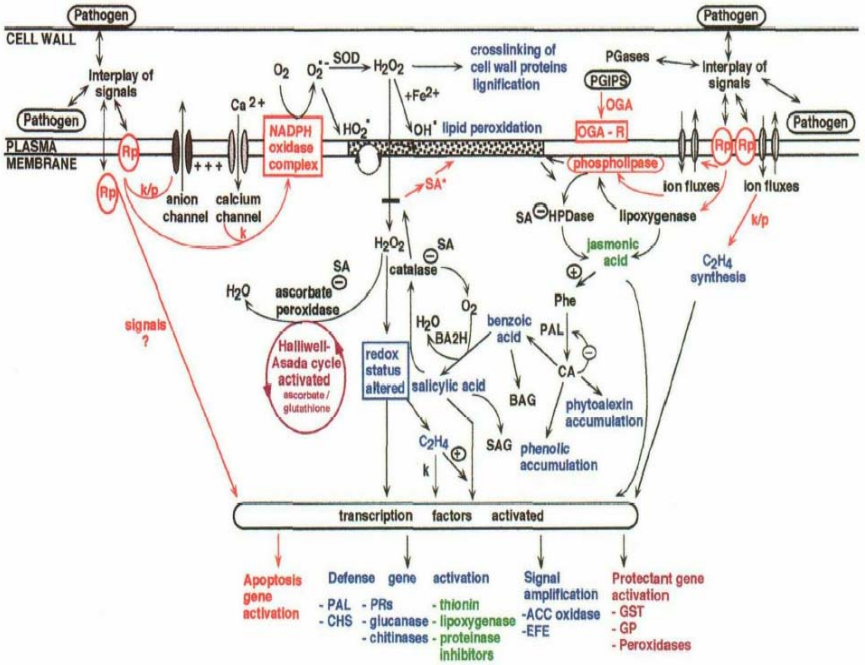


Figure 2.2 Complexity of signalling events controlling activation of defence responses. Abbreviations: ACC oxidase, 1-aminocyclopropane-1-carboxylate oxidase; BAG, benzole acid glucoside; BA2H, benzole acid-2 hydroxylase; CA, cinnamic acid; CHS, chalcone synthase; EFE, ethylene-forming enzyme; HO_2, hydroperoxyl radical; HPDase, hydroxyperoxide dehydrase; GP, glutathione peroxidase; GST, glutathione S-transferase; k, kinase; O_2^-, superoxide anion; OH^-, hydroxyl radical; OGA and OGA-R, oligalacturonide fragments and receptor; P, phosphatase; PAL, phenylalanine ammonia-lyase; PGases, polygalacturonases; PGIPS, plant polygalacturonic acid inhibitor proteins; Phe, phenylalanine; PR, pathogenesis related; Rp, plant receptor protein; SA and SAG, salicylic acid and salicylic acid glucoside; SA (Homand-Kosack and Jones, 1996).

2.5.2 Reactive oxygen species.

The production of ROS is another early response detected shortly after an attack by an avirulent pathogen (De Gara *et al.,* 2003) which is later followed by a second prolonged ROS production stage. This was first reported by Doke (1983) who demonstrated that potato tuber tissue generated superoxide (O_2^-) following the infection with an avirulent race of *Phytophtohora infestans.* The virulent race of the same pathogen failed to produce O_2^-. This two part ROS production is typical of the HR and usually found in the incompatible plant-pathogen interactions (Bolwell, 1999). The primary ROS production system in plant cells is a membrane-bound NAD(P)H oxidase (Fig. 2.2). Plant NAD(P)H oxidase transfers reducing equivalents from cytosolic NAD(P)H to extracellular oxygen, generating O_2^-. Superoxide dismutase (SOD) iso-enzymes are then responsible for hydrogen peroxide (H_2O_2) production by means of superoxide dismutation (Fig. 2.2).

Both O_2^- and H_2O_2 are only moderately reactive, but can cause cellular damage (Levine *et al.,* 1994). H_2O_2 leads to the induction of a battery of cellular protectant genes and at higher doses, cell death (Hu *et al.,* 2003). Protonation of O_2^- yields the hydroperoxyl radical (HO_2^-). It can cross membranes easily and has the ability to attack fatty acids directly, resulting in membrane damage (Van Camp *et al.,* 1998).

Nitric oxide (NO) is well known as a signalling compoud in immune, nervous and vascular systems of vertebrates (Durner *et al.,* 1998). NO is generated when arginine is split into citrulline and NO (Delledonne *et al.,* 1998). NO synthase activity was detected in plants and fungi (Delledonne *et al.,* 1998). When animal NO synthase or NO-releasing compounds were infiltrated into tobacco leaves, the expression of two defence related genes, *PR-1* and phenylalanine ammonia lyase (*PAL*), was activated (Delledonne *et al.,* 1998). Two important downstream components (cyclic GMP and cyclic ADP-ribose) were shown to further stimulate the NO-activated *PR-1* and *PAL* gene expression. It is also known that NO interacts with O_2^- to form peroxynitrite radicals, which cause cellular destruction and the triggering of apoptotic cell

death. Thus, NO could act as a 'master signal' to induce HR and defence gene activation (Durner *et al.,* 1998).

2.5.3 Cell wall fortification.

The fortification of the plant cell wall can increase resistance against microbes (Hammond-Kosack and Jones, 1996). For extracellular biotrophs such as *Pseudomonas syringae* and *Cladosporium fulvum*, sealing of the cell wall could impede leakage of cytoplasmic contents, thereby reducing nutrients available to the pathogen (Tenhaken *et al.,* 1995). Necrotrophs such as *Botrytis cinerea* on the other hand, rely on the hydrolysis of the plant cell wall in advance of the hyphal growth. The peroxidase-mediated oxidative cross-linking of structural proteins and possibly other polymers makes the cell wall less fragile to digestion by microbial enzymes (Fig. 2.2). These rapid modifications may enhance the effectiveness of the cell wall as a barrier to slow pathogen spread prior to the deployment of transcription-dependent defences such as the production of phytoalexins, lytic enzymes and other antimicrobial proteins (Tenhaken *et al.,* 1995). Moreover, rapid oxidative cross-linking may also serve to trap pathogens in cells destined to undergo hypersensitive cell death, thereby enhancing the effectiveness of host cell suicide in pathogen restriction.

2.5.4 Lipoxygenase.

The rapid increase of lipoxygenase (LOX) enzyme activity and/or mRNA and protein levels, is frequently found to be specifically associated with *Avr-R* mediated incompatibility (Montillet *et al.,* 2002). Poly-unsaturated fatty acids (PUFAs) are susceptible to oxidation by free radicals. These can contribute to generating secondary metabolites such as jasmonates and oxylipins (Fig. 2.2). The accumulation of these harmful metabolites generated by LOX increases the induction of hypersensitive cell death (Montillet *et al.,* 2002).

2.5.5 Jasmonates.

The plant hormone jasmonic JA and its methyl ester, methyl jasmonate (MeJA), collectively referred to as jasmonates, represent the well characterized class of endogenous signals involved in the plant defence response (Szczegielniak *et al.*, 2005). When an insect or microbial pathogen attacks a plant, an interaction between elicitors and receptors follows which initiates the octadecanoid-based pathway (Blechert *et al.,* 1995). In this pathway, JA is formed from the C_{18} fatty acid linolenic acid. JA has been implicated in defence gene expression in response to the peptide hormone systemin, wounding and pathogen-derived oligosaccharide elicitors (Doares *et al.,* 1995).

The rapid synthesis of JA induces phytoalexin accumulation in suspension-cultured cells of several plant species (Boller and Keen, 1999). This suggests that JA acts as a second messenger to induce phytoalexin synthesis (Fig. 2.2). JA might however not be tied uniquely to defence related responses, as it is involved in signal transduction pathways in response to wounding and ultra violet (UV) radiation of intact plants (Creelman and Mullet, 1997).

2.5.6 Salicylic acid.

SA is a phenolic acid that plays a key role in the plants defence response upon primary infection and is instrumental in the activation of *PR*-gene expression (Klessig *et al.,* 2000). The overproduction of SA in plants using bacterial transgenes enhanced pathogen resistance and defence gene expression (Brodersen *et al.,* 2005). Studies have shown that SA is involved in a feedback loop both upstream and downstream of cell death (Zottini *et al.,* 2007). SA treatment leads to signal transduction and the expression of a number of *PR*-genes. Following TMV infection of tobacco, endogenous SA levels increased specifically during the resistance response (Malamy *et al.,* 1990). Moreover, the induction of *PR-1* gene expression paralleled the rise in SA levels in leaves of infected resistant plants (Gaffney *et al.,* 1993). In susceptible plants, neither an increase in endogenous SA nor the induction of *PR*-gene expression was observed. Elevated *PR*-gene expression, such as

PR-1 and *PR-2* (Ryals *et al.,* 1996) has also been reported after treatment with the SA analog BTH. These results suggested that SA plays a role in the development of systemic as well as local resistance (Chen *et al.,* 1995).

2.5.7 Phytoalexins.

Phytoalexins are low molecular weight, lipophilic, antimicrobial compounds that accumulate rapidly around sites of avirulent pathogen infections (Blechert *et al.,* 1995). It accumulates in response to an extensive array of biotic and abiotic elicitors (Hammerschmidt, 1999). Phytoalexin biosynthesis occurs after a diversion of primary metabolic precursors into novel secondary metabolic pathways. This diversion often arises from the activity of enzymes such as PAL that control key branch points in biosynthetic pathways (Tsuji *et al.,* 1992) (Fig. 2.2). Highly co-ordinated activities of numerous enzymes are required in the attacked cells to successfully establish this type of response.

2.5.8 G-proteins.

G-proteins act as molecular signal transducers whose active or inactive states depend on the binding of GTP or GDP respectively (Scheel, 1998). The G-proteins include two major subfamilies, the heterotrimeric G-proteins and the small G-proteins (Scheel, 1998). The heterotrimeric G-proteins contain an alpha subunit (Gα) that has two domains. One contains a predominantly alpha helical secondary structure, while the other contains a GDP/GTP binding site, GTP hydrolytic activity and a covalently attached lipid that anchors this subunit to the bi-layer via lipid modification at its carboxy-terminus (Jones, 2002). The small G-proteins appear to be similar to free α subunits operating without the $\beta\lambda$ heterodimer. Generally, it is the α subunit of the heterotrimeric G-protein that has the receptor-binding region and it possesses a guanosine nucleotide binding site and GTPase activity. Both classes of G-proteins use the GTP/GDP cycle as a molecular switch for signal transduction. Interaction of the G-protein with the activated receptor promotes the exchange of GDP for GTP and the subsequent dissociation of the α-GTP complex from the $\beta\lambda$ heterodimer. Thus, the activation of defence responses could be G-protein

mediated through plasma-membrane delimited pathways (Scheel, 1998).

2.5.9 Pathogenesis-related proteins.

PR-proteins (Table 2.1) are proteins encoded by the host plant whose gene expression is induced specifically in pathological or related situations (Fritig *et al.,* 1998). These enzymes do not only accumulate locally in the infected leaves, but are also induced systemically when associated with the development of systemic acquired resistance (SAR). PR-proteins belong to the family of 'stress-inducible' proteins and were first described in tobacco (Legrand *et al.,* 1987). These proteins include enzymes like chitinases, glucanases, lectin-like and proteinase-inhibitor-like antifungal proteins (van Wees *et al.,* 2000). The glucanase enzymes lead to the degradation or alteration of the fungal cell wall that could arrest or severely impair fungal growth. The constitutive expression of PR-proteins of known or unknown function in transgenic plants has led to increased resistance to some fungal pathogens (Hwang *et al.,* 2003). PR-proteins are generally low molecular weight proteins which can be extracted in acidic buffers. They are inducible and/or show a certain tissue specificity and developmental regulation of expression (Jung *et al.,* 1993).

The term 'hypersensitive' was first applied by Stakman (1915) to describe the rapid and localized plant cell death induced by rust fungi in rust-resistant cereals. The HR can be described as a process which includes both cell death and defence gene expression. For biotrophic fungal pathogens, the initiation of the HR requires the pathogen to have an *Avr*-gene that matches the *R*-gene in a gene-for-gene relationship (Flor, 1971).

Ion fluxes and the production of ROS appear to be early steps in the HR (Higgins *et al.,* 1998). An increase in cytosolic calcium precedes and also seems to be necessary for cell death triggered by rust fungi (Xu and Heath, 1998).

Table 2.1. The families of pathogenesis-related proteins (Van Loon and Van Strien, 1999).

Family	Type member	Properties
PR-1	Tobacco PR-1a	unknown
PR-2	Tobacco PR-2	B-1,3-Glucanase
PR-3	Tobacco P,Q	chitinase type I,II,IV,V,VI,VII
PR-4	Tobacco 'R'	chitinase type I,II,IV,V,VI,VII
PR-5	Tobacco S	thaumatin-like
PR-6	Tomato Inhibitor I	protease-inhibitor
PR-7	Tomato P69	endoproteinase
PR-8	Cucumber chitinase	chitinase type III
PR-9	Tobacco 'lignin-forming peroxidase'	peroxidase
PR-10	Parsley 'PR1'	ribonuclease-like
PR-11	Tobacco class V chitinase	chitinase type I
PR-12	Radish Rs-AFP3	defensin
PR-13	*Arabidopsis* THI2.1	thionin
PR-14	Barley LYP4	lipid-transfer protein

2.6 The hypersensitive reaction.

The production of H_2O_2 resembles inflammation responses in the immune system of mammals and the mechanism of H_2O_2 action in HR death may be related to apoptotic cell death (Hammond-Kosack and Jones, 1996). Other chemicals that form part of the signaling pathway such as JA and SA are also involved in the activation of HR and play a crucial role in disease resistance (Hammond-Kosack and Jones, 1996).

2.7 Systemic acquired resistance.

SAR is a secondary defence response that plays an important role in the ability of plants to defend themselves against pathogens (Ryals *et al.,* 1996). After the formation of necrotic lesions due to HR, SAR is activated throughout the plant to act as a protective mechanism by activating a broad spectrum of systemic defences to prevent the spreading of infection. Various experiments that have been conducted suggests that SAR signals which have been produced in an infected leaf, travels trough the phloem of the plant to upper leaves of the plant to convey the signal (Sticher *et al.,* 1997). Components involved in SAR are ROS, lignification and the forming of PR-proteins (Sticher *et al.,* 1997). The signaling molecules needed for SAR include SA, jasmonates, systemin and ethylene (Sticher *et al.,* 1997). SAR can be distinguished from other disease resistance responses by both the spectrum of pathogen protection and the associated changes in gene expression (Ryals *et al.,* 1996).

It has often been suggested that SAR is very costly for the plant to have the defence response switched on at all times (Heidel *et al.,* 2004). This theory is supported when tobacco mutant plants tend to be reduced in its physical size and has a loss of apical dominance. It showed a loss of fertility when *PR*-gene expression and SA levels were permanently elevated in the resistance reaction against infection (Heil and Baldwin, 2002). The overexpression of *NPR1* triggers lesion development and chlorosis under certain environmental conditions. Thus can be concluded that the constant activation of SAR can be

detrimental to the plant.

2.8 The interaction between sunflower and leaf rust.

2.8.1 *Helianthus annuus.*

The sunflower, *Helianthus annuus* L., is a member of the Compositae family of flowering plants (Kolte, 1985). Sunflower probably originated in the South Western United States and Mexico area and its seed was used for food by the local inhabitants. Sunflower was introduced to Europe in the sixteenth century (Weiss, 2000). It is an annual herb with a basic chromosome number of 20 (2n=40) (Kolte, 1985). The sunflower head is an inflorescence composed of about 1000 – 2000 individual flowers joined to a common base, called the receptacle. Cross-pollination occurs via insects, particularly bees. The oil-content of the seed is more than 40%.

Sunflower became a major international oilseed primarily due to the introduction of short-stemmed, high-yielding hybrid cultivars and is currently being cultivated in many countries. The largest market for sunflower oil is Europe which uses approximately two-thirds of all oil traded (Weiss, 2000). The main commercial production of sunflower is in regions with a warm climate, but breeding and selection produced cultivars adapted to a wide range of environments. Sunflower is therefore an important crop plant. It is however a prime target for pathogens that can lower its yield (Weiss, 2000).

2.8.2 *Puccinia helianthi.*

A common and serious disease of sunflower is leaf rust caused by *P. helianthi* (Staples, 2001). Infection by this fungus can lead to significant yield and quality loss on susceptible sunflower hybrids which can be a major factor limiting sunflower production (Kolte, 1985). *P. helianthi* has been reported from every region where either cultivated or wild sunflower is found. The first signs of rust usually appear when the plants have reached maximum size and formed a dense canopy. The plants are then either at or past bloom. Leaf rust has a more profound effect on pre-bloom plants. Favorable conditions for rust

infection include free water on the leaves, either from rainfall or dew and warm temperatures. A minimum of only two hours of wet leaves is sufficient for rust infection while six to eight hours of wetness will result in maximum infection. Once the germinated spores have penetrated the leaf, the surrounding temperature is the only inhibitor on its growth (Kolte, 1985).

Cinnamon-brown pustules (uredia) first occur on the lower leaves which then spread to the upper leaves. Eventually rust will occur on the petioles, stems and the back of the flowerhead. Uredial pustules occur on both the upper and lower leaf surfaces and are roughly circular. It can be surrounded by a chlorotic (yellow) border. The uredial pustules contain unicellular urediospores. A single pustule can produce 1000 or more urediospores. These are easily dislodged from the pustules and can be blown in the wind over great distances. New infections occur every 10 to 14 days (Kolte, 1985).

With the onset of cooler weather, the uredial pustules change into telial pustules. These are characteristically dark brown or black (Kolte, 1985). The telial pustules contain two-celled teliospores, which are the overwintering stage of the fungus. The teliospores do not dislodge easily from the leaf. In early spring, the teliospores germinate to produce basidiospores that infect sunflower seedlings. The first signs of infection are aecial (yellow-orange) pustules on either the upper or lower surfaces of cotyledons and leaves of seedlings.

Urediospores usually germinate within 4 hours post infection (hpi). A germ tube is issued from one of the equatorial germ spores. Germ tubes form irregularly shaped appressoria over the stomata of a leaf. A penetration peg grows from the lower surface of the appressorium and penetrate through the stomata into the substomatal cavity. An H-shaped vesicle is formed into which the appressorium then empties its contents. Vesicle formation occurs about 12 hpi (Sood and Sackston, 1970).

Haustorium development follows at 24 hpi. The tip of an intercellular hypha, which is in contact with a mesophyll cell, will elongate. A septum is laid down

and a very fine tube enters the host cell and enlarges to form a round or knob-shaped haustorium. Haustoria in resistant hosts remain round and are fewer in number compared to susceptible plants (Sood and Saxton, 1970).

Mycelial growth is rapid in susceptible plants and reaches the lower epidermis within 96 hpi. Mature hyphae form cushions of sporogenous tissue under the upper and lower epidermis. Urediospores are formed at about 144 hpi. As it grows, it becomes oval and the colour turns reddish-brown. It will eventually invade the host epidermis. Mycelial growth is slower and more restricted in resistant plants (Sood and Saxton, 1970). This indicates a reaction at cellular level between host and fungus.

2.9 Biochemical defences in sunflower.

Several biochemical substances were found to be involved in the resistance response of sunflower to leaf rust (Mohase *et al.,* 2006). The first line of defence starts with the changes in the ion permeability of the plasma membrane. This involves an increase of ion fluxes across the membrane to activate downstream defence responses (Nürnberger and Scheel, 2001). The ion fluxes are followed by a release of ROS such as O_2^- and H_2O_2. NADPH oxidase has been shown to be responsible for ROS accumulation during some defence responses (Torres *et al.,* 2006). ROS cause damage to proteins, lipids and DNA and must be strictly controlled. Several other enzymes such as SOD and catalase play an important role in this line of defence (Moller, 2001). Peak activities of these membrane-bound proteins upon infection, are indeed an indication that it is involved in the sunflower-rust interaction. NO plays an important role in the induction of plant defence. The increased production of NO is at the onset of HR, but is not sufficient to activate the HR. The HR is triggered by the balance of production of NO and ROS (Neill *et al.,* 2002).

An increased activity of β-1,3-glucanase and chitinase have been reported in the interaction between leaf rust and sunflower (Mohase *et al.,* 2006). The activity of PR-proteins were higher in resistant than in susceptible plants. As

the intercellular PR-proteins accumulate in the apoplast and since the apoplast is where fungal structures differentiate, it can be assumed that PR-proteins play an important role in the defence response (Mohase *et al.,* 2006).

PAL is a key player in the plant defence response as it synthesizes phenolic compounds including SA, phytoalexins and lignin monomers which are all involved in defence responses (Hahlbrock and Scheel, 1989). Increased PAL activity has been indicated in the incompatible plant-pathogen reaction of sunflower. Therefore, the assumption can be made that PAL plays a role in the resistance reaction of sunflower to leaf rust (Mohase *et al.,* 2006).

Chapter Three:

Characterization of a putative CIPK from sunflower

3.1 Introduction

Plants need an efficient surveillance system to detect any potential pathogen attack. Such a surveillance system is based on the early recognition of the invading pathogen which is followed by the activation of an appropriate defence response. A timely defence response could result in the arrest of the pathogen invasion, allowing the plant to provide resistance and hence survive the infection.

During plant-pathogen interactions, protein kinases play a central role in the recognition and subsequent activation of plant defence mechanisms (Romeis, 2001). A major function of protein kinases is to mediate the signalling required for the induction of systemic defence responses (Dangl and Jones, 2001). Protein kinases have been shown to be involved in signalling in both race and non-race specific interactions. Several protein kinases participate in the direct perception of the *Avr*-gene product while others mediate the signal required for the initiation of the defence response (Gomez-Gomez *et al.,* 1999).

The plant protein kinase family all have catalytic kinase domains that are highly conserved (Hanks *et al.,* 1988). Nearly all of these protein kinases have been shown to phosphorylate serine and/or threonine residues, while a single example phosphorylates tyrosine residues (Mu *et al.,* 1994). Several plant protein kinases are known to provide disease resistance against pathogens. An example is the well studied serine/threonine protein kinase Pto (Zhou *et al.,* 1997). This protein kinase from rice has been shown to interact with the avirulence Avr-Pto protein from *Pseudomonas syringae* causing bacterial speck disease (Tang *et al.,* 1999). In order for Pto to provide resistance against the pathogen, it first needs to interact with the Prf protein. Pto in association with Prf lead to the interaction with Avr-Pto. The interaction between these proteins then leads to the hypersensitive reaction (Zhou *et al.,* 1995).

The extracellular domain structures of these kinases are very diverse allowing them to be grouped into 15 subfamilies based on the properties of these domains (Shiu and Bleecker, 2001). The variation within the extracellular domains would lead to a response to a wide range of external signals.

RLK resistance genes include *Xa21* (Song *et al.,* 1995) and *Xa26* (Yang *et al.,* 2003) which are both involved in the interaction between *Xanthomonas oryzae* pv. *oryzae* and rice when the pathogen infects the plant. Both these genes encode an RLK with an LRR extracellular domain, a transmembrane domain and a cytoplasmic protein kinase domain (Sun *et al.,* 2004).

Not only are protein kinases involved in the direct perception of the elicitor or pathogenic products, they are also involved in the downstream mediation of these signals (Martin, 1999). MAPK cascades from several plant species have been shown to be the major pathways by which extracellular stimuli are transduced into intracellular responses leading to the activation of the defence response (Meskiene and Hirt, 2000). These enzymes are multifunctional as they can be activated by race and non-race elicitors (Romeis *et al.*, 1999). MAPK phosphorylation cascades involves complex protein-protein interactions. Within the cascade, subsequent genes are present which are responsible for conveying the signal throughout the cascade.

The MAPK cascade consists of a three-kinase module (Widmann *et al.,* 1999). The last kinase in the cascade is MAPK. It is activated when the threonine and serine residues are phosphorilated by the TXY motif of MAPKK. MAPKK in turn is activated by MAPKKK (Dan *et al.,* 2001). MAP kinases have been associated with cell death and the HR (Zhang and Klessig, 2001). This was shown in tobacco cells treated with xylanase and *Arabidopsis* cells treated with harpin (Suzuki *et al.,* 1999; Desikan *et al.,* 1999). The activation of salicylic acid-induced protein kinase (SIPK) and the wound-induced protein kinase (WIPK) have also been shown to be involved in HR-related cell death upon induction by fungal elicitors (Zhang *et al.,* 2000).

In order to better understand disease resistance in sunflower, clone D15 was isolated from a resistant sunflower cultivar [GH99PHRR3 (VII) (R)] infected with leaf rust using a differential display reverse transcription polymerase chain reaction (DDRT-PCR) (M. Bezuidenhout, results unpublished). The clone showed homology to various putative protein kinases that are all involved in other plant defence responses. Results indicated that although the gene was present in both the resistant and susceptible cultivars, it was only inducibly expressed in the resistant cultivar. The aim of this study was therefore to further characterize the isolated cDNA fragment.

3.2 Materials and Methods

3.2.1 Cloning of the full length *D15* gene.

3.2.1.1 5'-Rapid amplification of cDNA ends (RACE) of clone *D15*.

The cloned *D15* gene fragment from *H. annuus* represented only the 3' portion of the complete gene. In order to clone the full length *D15* gene, 5'-RACE was employed (Fig. 3.1). The 5'/3' RACE 2nd generation kit (Roche) was used. First strand cDNA synthesis was done using 2 µg total RNA in the presence of 1× cDNA synthesis buffer, 1 mM deoxynucleotidetriphosphates (dNTPs), 25 U reverse transcriptase and 12.5 µM of the Bovis 73 primer (Table 3.1) (Fig. 3.1b). The reaction was incubated at 54°C for 1 h followed by another 5 min at 85°C. The synthesized cDNA was purified using the Favorgen PCR purification kit according to the manufacturer's specifications. A 3' oligo-dA tail was added to the cDNA by using 19 µl of the purified cDNA, 1× reaction buffer and 0.2 mM deoxyadenosine triphosphate (dATP) (Fig. 3.1c). The reaction was incubated at 95°C for 3 min and after cooling on ice, 80 U terminal transferase was added. The reaction was incubated at 37°C for 20 min and then at 70°C for 10 min.

The first PCR reaction (Fig. 3.1d) was performed using 5 µl of the poly-adenylated cDNA as template with 10 µM of Bovis 73 and the oligo-dT anchor primer respectively (Table 3.1). To this was added 1× reaction buffer (Kapa Biosystems), 0.2 mM dNTPs and 1 U *Taq* DNA-polymerase (Kapa Biosystems). The amplification regime was as follows, 94°C for 1 min followed by 10 cycles of 94°C for 15 sec, 60°C for 30 sec and 72°C for 3 min. An additional 25 cycles of 94°C for 15 sec, 60°C for 30 sec and 72°C for 5 min followed. This was followed by a final cycle of 72°C for 5 min. Of each reaction, 5 µl DNA was mixed with DNA loading buffer to a final concentration of 0.015% (w/v) bromophenol blue (BFB), 2.5% (w/v) ficoll. The reactions were separated on a 1% (w/v) agarose

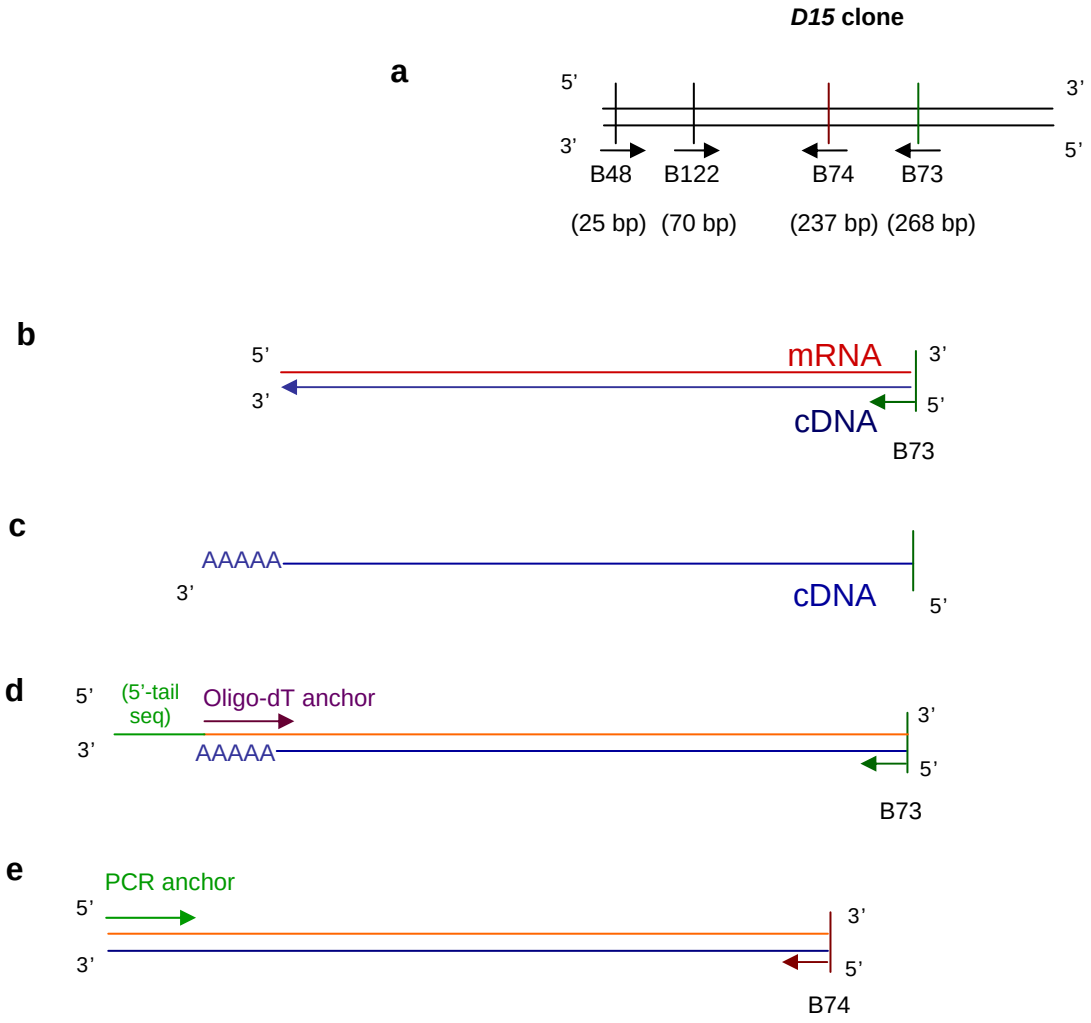


Figure 3.1. 5'-RACE scheme of clone D15. In a), clone D15 together with primers homologous to its sequence are indicated. Arrows indicate the orientation of the primers. In b), first strand cDNA synthesis with Bovis 73 is shown. In c), poly-adenylated first strand cDNA string is indicated. In d), the first PCR reaction done with the oligo-dT anchor primer and Bovis 73 is indicated. In e), a nested PCR reaction done with the PCR anchor primer and Bovis 74 is shown.

Table 3.1. Nucleotide sequences of the primers used in this study.

Primer	Sequence
Bovis 26	5'-CAA CTT TCG ATG GTA GGA TAG-3'
Bovis 27	5'-CTC GTT AAG GGA TTT AGA TTG-3'
Bovis 32	5'-GAA GAA TTC TCG AGC GGC CGC TTT TTT TTT TTT TTT TTT TVN-3'
Bovis 39	5'-GAA GAA TTC TCG AGC GGC-3'
Bovis 48	5'-TCG CCG GAG AAT GTG ATT T-3'
Bovis 73	5'-GTC GTT GTT CAA CCA ACT CC-3'
Bovis 74	5'-ATC ATT CCA CCA CTC ATC GT-3'
Bovis 122	5'-GCA AAC GCT AGA ATG CAC AA-3'
Bovis 261	5'-TTC CGA TCA ACC CGA TCT CT-3'
Bovis 262	5'-ACG TAC ATA CAC CCG ACA CGT-3'
Bovis 263	5'-CGT TTA TTC GAT GCC AGA GGT-3'
Bovis 264	5'-TTC ACT AAC TCT CCG TTT CCG-3'
Oligo-dT anchor primer	5'-GAC CAC GCG TAT CGA TGT CGA CTT TTT TTT TTT TTT TTV-3'
PCR anchor primer	5'-GAC CAC GCG TAT CGA TGT CGA C-3'
Sp6	5'-TAT TTA GGT GAC ACT ATA G-3'
T7	5'-TAA TAC GAC TCA CTA TAG GG-3'
	Where **Y** = C or T, **H** = A, T or C, **R** = A or G, **N** = A, T, C or G, **V** = A, C or G

gel containing 0.5 μg.ml^{-1} ethidium bromide prepared in 0.5× TAE [20 mM Tris (hydroxymethyl)aminomethane (Tris-HCl) pH 8, 0.5 mM ethylenedinitrilo-tetraacetic acid (EDTA), 0.01% (v/v) acetic acid] (Sambrook *et al.,* 1989) and separated at 12 V.cm^{-1} using 0.5× TAE as running buffer.

A nested PCR reaction (Fig. 3.1e) was then performed using 5 μl of the first PCR reaction as template, 25 μM of Bovis 74 and PCR anchor primer respectively (Table 3.1), 1× reaction buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$ and 0.5 U *Taq* DNA-polymerase (Kapa Biosystems). The amplification regime was as follows, 30 cycles of 94°C for 15 sec, 55°C for 30 sec and 72°C for 1 min. A final cycle of 72°C for 7 min followed. Again 5 μl of the reaction was separated on a 1% (w/v) agarose gel as described.

3.2.1.2 Cloning of *D15* RACE fragments.

The main amplified DNA fragment was cut from the gel, purified using the PCR and Gelband purification Kit (Favorgen Biotech Corp.) according to the manufacturer's specifications and re-amplified as described using 25 μM of Bovis 74 and the PCR anchor primers respectively (3.2.1.1). The re-amplified DNA fragments were cloned using the pGEM-T Easy vector system (Promega) according to the manufacturer's specifications. To the 10 μl ligation mixture, 20 μl *Escherichia coli* (JM 109) competent cells were added and mixed. The tube was incubated on ice for 20 min, heat shocked at 42°C for 50 sec and placed on ice for 2 min. After adding 980 μl Luria Bertani (LB) medium [1% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl], the tube was incubated at 37°C for 90 min. The cells were then plated on LB-plates [LB with 3.5% (w/v) bacterial agar] containing 50 μg.ml^{-1} ampicillin, 250 μg.ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and 250 μg.ml^{-1} isopropyl-β-D-thiogalactopyranoside (IPTG) (Sambrook *et al.,* 1989). The plates were incubated overnight at 37°C.

3.2.1.3 Southern blot analysis of the cloned *D15* RACE fragments.

The obtained white colonies were inoculated in 5 ml LB-medium containing 50 $\mu g.ml^{-1}$ ampicillin and incubated at 37°C overnight. Plasmid DNA was extracted using the Fast Plasmid mini kit (Eppendorf) according to the manufacturer's specifications. The presence of inserts was confirmed with PCR using SP6 and T7 primers (Table 3.1). Each reaction consisted of 1 µl purified plasmid DNA, 1× reaction buffer (Kapa Biosystems), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 25 µM SP6 and T7 primers respectively and 0.5 U *Taq* DNA-polymerase (Kapa Biosystems). The amplification regime was as follows, 30 cycles of 94°C for 15 sec, 50°C for 30 sec and 72°C for 45 sec. A final cycle at 72°C for 7 min followed. The amplified fragments were separated on an agarose gel as described (3.2.1.1).

To confirm that the recombinant plasmids contained RACE generated *D15* fragments, a Southern blot was performed on the amplified inserts. The separated DNA was denatured in 3 M NaCl, 0.4 M NaOH for 45 min. The DNA was then transferred to a Hybond-XL nylon membrane (GE Healthcare) for 4 h using 1.5 M NaCl, 0.4 M NaOH as transfer buffer (Chomczynski, 1992). Following transfer, the membrane was neutralized in 0.2 M sodium phosphate buffer pH 6.8 for 10 min and dried at 70°C for 15 min. The membrane was pre-hybridized in 10 ml Ultrahyb hybridization buffer (Ambion) for 30 min at 42°C.

The original *D15* cDNA fragment was denatured at 95°C for 5 min and cooled on ice for 5 min. The DNA fragment was labelled radio-active with the Rediprime II random prime labelling system (Amersham) according to the manufacturer's specification using 40 µCi [α-^{32}P]-deoxycytidine triphosphate (dCTP). The labelled probe was purified using a Sephadex G75 column according to Sambrook *et al.* (1989).

After denaturing the labelled probe for 5 min at 95°C, it was chilled on ice for 5 min and then added to the hybridization solution. Hybridization was done

overnight at 42°C. The following morning, the membrane was washed twice at room temperature with wash buffer I [2× SSC (0.3 M NaCl, 0.03 M sodium citrate), 0.1% (w/v) sodium dodecyl sulphate (SDS)] for 5 min and twice with wash buffer II [0.5× SSC (0.075 M NaCl, 0.0075 M sodium citrate), 0.1% (w/v) SDS] for 15 min at 50°C.

3.2.1.4 Sequencing of cloned *D15* RACE inserts.

The identified cDNA fragments were sequenced using the T7 primer (Table 3.1) and the BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems) according to the manufacturer's specifications. The sequenced cDNA fragments were purified by adding 5 µl of 125 mM EDTA and 2 volumes 100% (v/v) ethanol to each reaction and then incubating it at room temperature for 15 min. The tubes were centrifuged at 13 000 *g* for 30 min, the supernatant discarded, the pellet washed with 70% (v/v) ethanol, re-centrifuged and the pellet dried. The sequences were separated on a 6% (w/v) denaturing polyacrylamide gel at the Department of Botany at the University of Johannesburg.

3.2.2 The compilation of the *D15* contig.

3.2.2.1 Bioinformatic analysis of *D15*.

Research at the Arizona Genomics Institute includes the generation of Expressed Sequence Tags (ESTs) expressed in different *Helianthus* species that were treated with a variety of chemical and environmental factors (http://genome.arizona.edu). The *D15* cDNA fragment first showed homology to an EST published by the group. Using various ESTs, a contig was then assembled for *D15*. The following ESTs were used, namely accession numbers DY927062, EL476164, EE657247, EE649676, EE631180 and EL481607. The sequences were separately aligned with each other using ClustalW (www.ebi.ac.uk/clustalw). The contig was then assembled using CromasPro

(v1.32) and the resulting nucleotide sequence translated (www.au.expasy.org/tools/dna.html). The obtained amino acid sequence was used to search for homologous sequences on NCBI using the basic local alignment search tool (BLAST) tool (www.ncbi.nlm.nih.gov). The contig was further analysed by searching for conserved regions (http://elm.eu.org).

3.2.2.2 Genetic confirmation of the *D15* contig.

Based on the contig sequence, two primer pairs were developed in order to amplify the complete *D15* gene from *Helianthus.* The first pair (Bovis 261 and Bovis 262) were specific for the 5' and 3' untranslated regions (UTR) respectively, while the second pair (Bovis 263 and Bovis 264) were specific for the 5' and 3' ends of the gene itself (Table 3.1).

In order to test both primer pairs, genomic DNA was extracted from leaves of the resistant cultivar [GH99PHRR3 (VII) (R)]. Leaves were harvested, quick frozen in liquid nitrogen, ground to a fine powder, transferred to 1.5 ml micro-centrifuge tubes and stored at -80°C. Genomic DNA was extracted according to the cetyltrimethylammoniumbromide (CTAB) method (Sambrook *et al.,* 1989). The DNA concentration was determined and expressed as $\mu g.ml^{-1}$. The full length *D15* gene was PCR amplified from genomic DNA using 10 ng genomic DNA as template, a 1× concentration of Ready mix (Kapa Biosystems) and 10 µM of two primer pairs respectively. The reaction was placed at 94°C for 2 min, followed by thirty cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 2 min and a final cycle of 72°C for 7 min.

3.2.3 Cultivation and infection of sunflower plants.

Seed of eight different sunflower cultivars [Hysun 346 (P1), Hysun 350 (P7), Agsun 8251 (P3), MonoSun 150 (P12), Hysun 334 (P2), Hysun 333 (P16), GH99PHRR3 (VII) (R) and GH99S37-388RR (S)] were planted in a 2:1 soil

mixture of peat moss and potting soil in seedling trays and germinated at 22 - 26°C in a greenhouse at normal day/night cycles. The plants were fertilized once a week with 3 $g.l^{-1}$ Multifeed fertilizer.

Sunflower seedlings were inoculated with *Puccinia helianthi* spores when the plants were four weeks old. An equal amount of urediospores from the 'Hennenman 2004' and 'Letsitele 2004' isolates were used. Rust spores were resuspended in kerosene oil containing a drop of polyoxyethylenesorbitanmonolaurat (Tween 20) to a final concentration of approximately 150 000 $spores.ml^{-1}$ and sprayed under high pressure onto the dorsal and ventral sides of the leaves. The plants were left to dry for 30 min at room temperature and were then placed at 22 - 24°C in a dark Dew-simulation-chamber for 16 h to allow the fungus to germinate. The plants were then moved to the greenhouse. After two weeks, rust pustules were visible on the leaves of the susceptible cultivar. The plants were scored according to the severity of the infection and divided in groups of susceptible, intermediate resistant and resistant to leaf rust infection.

3.2.4 Southern blot analysis of *D15* in different cultivars.

3.2.4.1 Genomic DNA extraction.

Leaves from the eight different sunflower cultivars were harvested, quick frozen in liquid nitrogen and stored at -80°C. Genomic DNA was extracted and concentration determined as described (3.2.2.2).

3.2.4.2 DNA transfer and hybridization.

Isolated genomic DNA (25 µg) from the eight respective sunflower cultivars were digested with 80 U *Eco*RI in the presence of 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithioerythritol (DTE) for 36 h at 37°C. The samples

were lyophilized and dissolved in 60 µl distilled water containing loading buffer (3.2.1.1). The DNA was separated on a 0.8% (w/v) agarose gel (3.2.1.1), transferred to a Hybond-XL nylon membrane and hybridized with a radio-labelled *D15* probe (3.2.1.3). The membrane was exposed to an Imaging-screen (Kodak) for 1 day. After capturing the image on the BioRad Personal Molecular Imager FX (BioRad), the approximate sizes of the hybridized signals were calculated.

3.2.5 Expression analysis of *D15.*

Seed from five selected cultivars (P1, P16, P12, R and S) were planted and the plants infected as described (3.2.3). Taking time 0 h as the time when the plants were placed in the Dew-simulation-chamber, leaf samples were collected every 3 h for 30 h. The infected leaves were randomly harvested, quick frozen and ground as described (3.2.2.2).

3.2.5.1 Total RNA extraction from sunflower tissue.

Distilled water was treated with 0.1% (v/v) dimethylpyrocarbonate (DMPC), left overnight at room temperature and then autoclaved at 121°C for 20 min. All pestles, mortars and spatulas were washed with soap and 10% (w/v) SDS and then baked at 260°C for 3 h. The pestles, mortars and spatulas were finally sprayed with 100% (v/v) ethanol and set alight just before use.

Total RNA was extracted from 0.1 g ground sunflower tissue using the Tripure isolation reagent (Roche) according to the manufacturer's specifications. The total RNA was dissolved in 50 µl DMPC treated water, the concentration determined and expressed as $\mu g.ml^{-1}$ (Sambrook *et al.,* 1989).

3.2.5.2 RT-PCR analysis of gene expression.

The Titan One tube RT-PCR system (Roche) was used to test *D15* gene expression. Each reaction contained 10 ng total RNA, 0.2 µM dNTPs and a 1× concentration of the reaction buffer, 6 mM dithiothreitol (DTT), 2.5 mM $MgCl_2$ and 1 µl Expand high fidelity enzyme mix (Roche). The RT-step was initiated by using 25 µM Bovis 32 (Table 3.1). PCR amplification of the *D15* fragment was then done using 25 µM Bovis 48 and Bovis 39 (Table 3.1). The amplification regime was one cycle at 42°C for 30 min and 94°C for 2 min. Thirty cycles then followed at 94°C for 10 sec, 50°C for 1 min and 68°C for 4 min. This was followed by a final elongation step at 68°C for 7 min. As a control, *18S rRNA* was amplified as described for *D15* using Bovis 26 and 27 as primers. The reactions were separated on a 1% (w/v) agarose gel as described (3.2.1.1).

3.2.5.3 Testing *D15* gene expression in chemically treated sunflower.

Seed of the resistant cultivar [GH99PHRR3 (VII) (R)] was planted as described (3.2.3). After four weeks, five sets of plants were sprayed with 5 mM SA, 100 µM methyl jasmonate (MeJA), 20 mM H_2O_2, 200 µM menadione and water respectively. Taking time 0 h as the time when the plants were sprayed, leaf samples were collected at 0 h, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h and 48 h. Leaf samples were quick frozen in liquid nitrogen, ground into a fine powder and RNA extracted as described (3.2.5.1). mRNA was captured from 20 µg total RNA with the mRNA capture kit (Roche) according to the manufacturer's specifications and resuspended in 50 µl DMPC water.

Expression of the *D15* fragment was followed using the RobusT II RT-PCR kit (Finnzymes). Each reaction contained 4.5 µl purified mRNA, 25 µM of Bovis 48 and Bovis 73 respectively, 0.2 µM dNTPs, 1× reaction buffer, 2.5 mM $MgCl_2$ and 0.4 µl of the M-MulV reverse transcriptase enzyme and DNA polymerase respectively. The amplification regime for the reactions was one cycle at 48°C for

30 min and 94°C for 2 min. Thirty cycles followed at 94°C for 15 sec, 52°C for 30 sec and 68°C for 45 sec. This was followed by a final elongation step at 68°C for 7 min. As a control, *18S rRNA* were amplified as described (3.2.5.2). The reactions were separated on a 1% (w/v) agarose gel as described (3.2.1.1).

3.2.5.4 Expression analysis of *D15* contig.

The expression of the composed *D15* contig was tested in the R cultivar on RNA level by first synthesizing first strand cDNA from 1 µg total RNA using ImProm-II™ Reverse Transcriptase (Promega) according to the manufacturer's specifications. First strand cDNA synthesis was performed with the oligo-dT primer Bovis 32 (Table 3.1). The synthesized cDNA was diluted to a final volume of 100 µl. The PCR reactions consisted of 1 µl cDNA, 1× Ready Mix (Kapa Biosystems) and 10 µM of Bovis 261, 262 and Bovis 263, 264 respectively. The reactions were placed at 94°C for 2 min, followed by thirty cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 2 min and a final cycle of 72°C for 7 min. As a control, the *18S rRNA* was amplified. The PCR conditions for this reaction were as described (3.2.5.2).

3.3 Results

3.3.1 Background information of clone *D15.*

The aim of a previous M.Sc. project was to clone and identify genes encoding protein kinases that are involved in the defence response of sunflower after infection with *P. helianthi*. The *D15* cDNA clone was isolated from an infected resistant sunflower cultivar [GH99PHRR3 (VII) (R)] using DDRT-PCR (M. Bezuidenhout; results unpublished). On amino acid level the clone showed homology to various putative protein kinases with the best being an Avr9/Cf9 rapidly elicited protein from tobacco (Table 3.2). Results indicated that although the gene was present in the genomes of both resistant and susceptible cultivars, it was only inducibly expressed in the resistant cultivar following infection.

3.3.2 Cloning of the full length *D15* gene.

Since the previous study indicated a possible important role for the *D15* gene in the defence response of sunflower following *P. helianthi* infection, an attempt was made to clone the full length gene by using 5'-RACE. The 5'/3'-RACE kit (Roche) was used to synthesize first strand cDNA from 2 µg total RNA. After the 3'-poly-A tail was added to the first strand cDNA, a PCR was performed using the supplied oligo-dT anchor primer and Bovis 73 (Fig. 3.1). The result was a smear on the agarose gel (Fig. 3.2a). A second nested PCR reaction was done using the PCR anchor primer and Bovis 74 with 5 µl of purified DNA from the first PCR reaction as template (Fig. 3.1). While a smear was still evident, a band 1.4 kb in length was visible (Fig. 3.2a). In an attempt to increase the specificity of the PCR, the PCR reaction was repeated using only 0.5 µl purified cDNA from the first PCR reaction, but the same result was obtained (Fig. 3.2a).

A further attempt was made to increase the PCR specificity by performing a gradient PCR using the PCR anchor and Bovis 74 primers. The gradient included 12 individual reactions, each with a different annealing temperature which ranged

Table 3.2. BLAST analysis of clone D15 on amino acid level.

Clone	Genbank accession nr.	Homologous polypeptide sequences	E-value
D15	AAP03879	*Avr9/Cf9* rapidly elicited protein (*Nicotiana tabacum*)	$3e^{-15}$
	AAP31926	Protein kinase At2g30360 (*Arabidopsis thaliana*)	$6e^{-11}$
	AAC27394	Putative protein kinase (*Arabidopsis thaliana*)	$7e^{-05}$
	AAU03103	Protein kinase (OsPK4) (*Oryza sativa*)	$8e^{-04}$

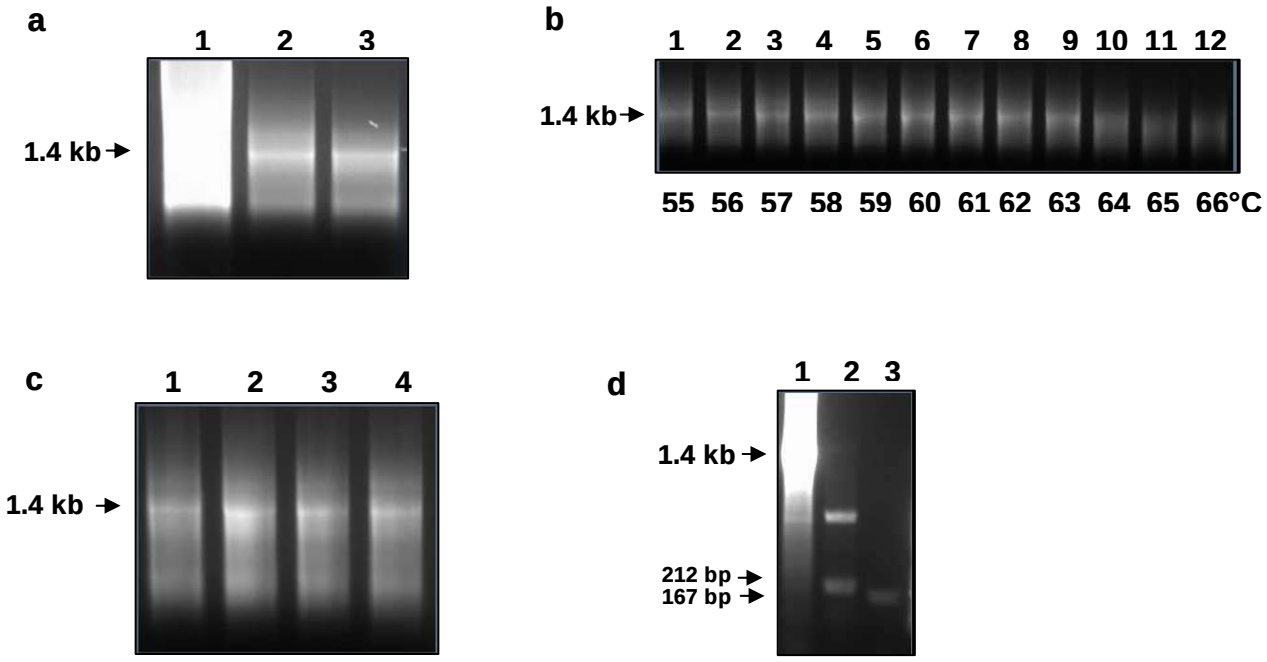


Figure 3.2. 5'-RACE analysis of clone *D15*. In a), the initial PCR reactions are indicated. The first lane represents the 1st PCR which was done using single stranded cDNA as template with the Oligo-dT anchor and Bovis 73 primers. In lane 2, the 2nd nested PCR reaction performed using the PCR anchor primer and Bovis 74 is indicated. In lane 3, the 2nd PCR is repeated but with $^{1}/_{10}$ the volume of cDNA template. In b), a gradient PCR to improve the PCR specificity is shown. Annealing temperatures were as indicated. In c), an attempt to increase the PCR specificity at an annealing temperature of 62°C is indicated. In lanes 1 and 2 the PCR reaction's template was diluted 1:20 and lanes 3 and 4 1:50. In lanes 2 and 4, 2 mM TMAC was added in an attempt to enhance primer specificity. In d), the amplification of different portions of *D15* from the purified 1.4 kb fragment is shown. In lane 1 the PCR was done with the PCR anchor primer and Bovis 74, in lane 2 Bovis 48 and 74 were used and Bovis 74 and 122 in lane 3.

from 55°C to 67°C (Fig. 3.2b). While an increase in temperature led to a decrease in the extent of the smear, it also caused a decrease in intensity of the 1.4 kb fragment. It was impossible to eliminate the smear without also losing the 1.4 kb fragment.

Even the addition of tetramethylammonium chloride (TMAC), a reagent that improves PCR specificity, did not eliminate the smear (Fig. 3.2c). Finally, the 1.4 kb fragment was cut and purified from the agarose gel and re-amplified. In order to verify that this fragment did represent the extended *D15* gene, three different PCR reactions using different gene-specific primer combinations were done (Fig. 3.2d). The first was performed with the PCR anchor primer and Bovis 74, the second with Bovis 48 and Bovis 74 and the third with Bovis 122 and Bovis 74 (Fig. 3.1). As expected, the first reaction produced the 1.4 kb fragment, the second the expected 212 bp fragment and the third the 167 bp fragment (Fig. 3.2d). The second larger fragment produced in the second reaction probably represent a non-specific amplified product. Since fragments with the expected sizes were amplified from the purified 1.4 kb fragment, indicates that it did indeed contain the extended *D15* gene fragment.

This RACE fragment was then cloned into pGEM-T and transferred to *E. coli* cells. When the amplified inserts of recombinant plasmids were probed using the *D15* cDNA clone as probe (Fig. 3.3), the majority of inserts hybridized with the probe, indicating homology to *D15.* However, when sequenced, no homology was found between the different clones and *D15* when the fragments were aligned. This was despite the fact that both forward and reverse primer sequences were found in the obtained sequences. This indicated that the primers were not specific enough for the *D15* template and that unrelated RACE products were produced. Due to this, an alternative means for the identification of the full length *D15* gene was followed.

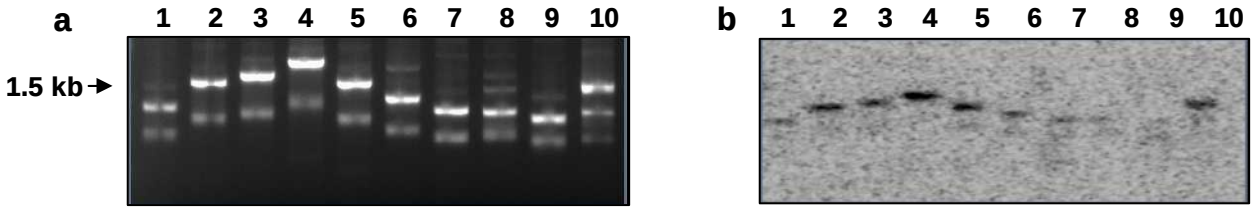


Figure 3.3. Southern blot analysis of *D15* RACE fragments. In a), PCR amplified cDNA inserts from ten recombinant RACE plasmids are indicated. In b), the hybridization results of the ten PCR amplified inserts probed with a *D15* labelled probe is indicated.

3.3.3 The construction and analysis of the *D15* contig.

The unsuccessful attempt to clone the full length *D15* gene sequence using RACE led to an alternative identification strategy. When *D15* was used in a BLAST analysis of ESTs, the fragment showed homology to an EST published by the Arizona Genomics Institute (http://genome.arizona.edu). Different sunflower ESTs from plants treated with a variety of chemical inducers or exposed to different environmental conditions, were then used to construct a 1 760 bp contig for clone *D15*.

In order to build the contig, *D15* was first aligned with the sequence of EST1 from *H. exilis* (Genbank accession nr EE657247) (Fig. 3.4). The alignment was significant with an e-value of 0. EST1 was 122 bp longer than *D15* on the 5'-end. This EST was then used in another BLAST search and it showed homology with a second EST from *H. paradoxus* (Genbank accession nr EL481607, e-value 0) (Fig. 3.5). In a similar way EST2 showed homology with EST3 from *H. paradoxus* (Genbank accession nr EL476164, e-value 0) (Fig. 3.6), EST3 with EST4 from *H. exilis* (Genbank accession nr EE649676, e-value 0) (Fig. 3.7), EST4 with EST5 from *H. annuus* (Genbank accession nr DY906292, e-value 0) (Fig. 3.8) and EST5 with EST6 from *H. exilis* (Genbank accession nr EE631180, e-value 0) (Fig. 3.9). Because of the extensive homology indicated by the alignments, it was concluded that the obtained fragments could be used to construct a complete contig representative of *D15* using ChromasPro v1.32.

The obtained EST sequences were used to assemble the *D15* contig of 1 760 bp using each of the overlapping ESTs (Fig. 3.10a). Within the nucleotide sequence, the 3'-UTR was 309 bp in length. The translated region included a start and two in-frame stop-codons and was 1 301 bp in length. The 5'-UTR consisted of 155 bp. Included within the 3'-UTR was a poly-A tail. The fact that two in-frame stop codons were found directly after each other, indicated that they most probably represent the end of the gene. A total of 34 single base pair differences were found within the coding region between the different ESTs.

```
D15             ------------------------------------------------------------
EE657247        CGTCAAGATAACAATAACAAATCAACGGCGTTAAACGCNGTTTGATATAATCTCGTTTTC 60

D15             ------------------------------------------------------------
EE657247        CTCCGGTTTAAACCTAACGCCGTTGTTTGACGGTGCAAGTAGCTCAACTTCGCCGGTGAA 120

D15             --TCGAGCGGCTGGTGGTGGAGGAGTCGCCGGAGAATGTGATTTCGAAAGTTGAGGAGAT 58
EE657247        AAACGAGCGGCTGGTGGTGGAGGAGTCGCCGGAGAATGTGATTTCGAAAGTTGAGGAGAT 180
                   ************************************************************

D15             TGTGAAGGATGCAAACGCTAGAATGCACAAACGGAAGGATTACGGTGTGGATTTGGTAGC 118
EE657247        TGTGAAGGATGCAAACGCTAGAATACACAAACGGAAGGATTACGGTGTGGATTTGGTAGC 240
                ********************** ***********************************

D15             GAAAAACGGTAAAGATGTGATTGAAATAGAGGTTTTCCGGTTGACGGAAAAGTTAGTTGT 178
EE657247        GAAAAACGGTAAAGATGTGATTGAAATAGAGGTTTTCCGGTTGACGGAAAAGTTAGTTGT 300
                ************************************************************

D15             GGTGGAGGTTCAGTGTTACGGTGGAGGTACAGAGTTCTACGATGAGTTGTGGAATGATAA 238
EE657247        GGTGGAGGTTCAGTGTTACGGTGGAGGTACAGAGTTCTACGATGAGTTGTGGAATGATAA 360
                ************************************************************

D15             AATTAAATCGGAGTTGGTTGAACAACGACGGGATGTACCGGAAACGGAGAGTTAGTGAAT 298
EE657247        AATTAAATCGGAGTTGGTTGAACAACGACGGGATGTACCGGAAACGGAGAGTTAGTGAAT 420
                ************************************************************

D15             AGTGACTCGCCGGCGGGGCACGTGTCGGGTGTATGTACGTAATTATTCAGATACATATTT 358
EE657247        AGTGACTCGCCGGCGGGGCACGTGTCGGGTGTATGTACGTAATTATTCAGATACATATTT 480
                ************************************************************

D15             TTTT--GACATGTTTGTTTTATTTTTTTACACGATTTTTTTTGTTGATAGAGAAAAATGA 416
EE657247        TTTTTTGACATGTTTGTTTTATTTTTTTACACGATTTTTTTTGTTGATAGAGAANAATGA 540
                ****  ********************************************** *****

D15             GTGAGGGAAAAATGAACCAAGGGTGGTCAAAATACGAGTGTGTGAATTTGTACATAACAT 476
EE657247        GTGAGGGAAAAATGAACCAAGGGTGGTCAAAATACGAGTGTGTGAATTTGTACATAACAT 600
                ************************************************************

D15             GTAACTTGCCTTGTAAATCATTGGGGCAATTGTTTGTTTGGTTTTGTTGTAAAATAATAC 536
EE657247        GTAACTTGCCTTGNTAATCATTGGGGCAACTGTTTGTTTGGTTTTGT-GTAAAATA-TAC 658
                *************  ************** ***************** ******** ***

D15             AAGTTTTATTTATTGTTTGAGAATAAAAAAAAAAAAAAAAAAAAGCGGCCGCTCGAGAATT 596
EE657247        AAGTTT-ATTTAT------------AAAAAAAAAAAAAAAAAAGGCCGCT---------- 694
                ****** ******            ***************** ** **

D15             C 597
EE657247        -
```

Figure 3.4. The nucleotide alignment of clone *D15* with EST1 (Genbank accession nr EE657247). Identical nucleotides are indicated with an *.

```
EE657247        ------------------------------------------------------------
EL481607        GAGCGTAAGGCGGACATCTGGTCATGCGGTATCATTTTGTTCGTGATGAATGCAGGTTTT 60


EE657247        ------------------------------------------------------------
EL481607        CTACCGTTTAACGATTCGAATCTCATGATGATGTATAAGAAGATCTACAAAGGTGAGTAC 120


EE657247        ------------------------------------------------------------
EL481607        CGGTGCCCTAAGTGGATGTCGCCGGAGCTCCGGCGGTTGTTATCTCGGTTACTTGACACA 180


EE657247        ------------------------------------------------------------
EL481607        AAACCTGACACTCGGATCACCGTCGACCAGATCAAATCCGATCCGTGGTTTAAAAAAGGA 240


EE657247        ----------------------------------------------------CGTCAA 6
EL481607        TACAAAGAGTCATCAAATCCAAATCTGGATGTCGATCTAGAAGCCGTTATGAAACGTGAA 300
                                                                   *** **

EE657247        GATAACAATAACAAATCAACGGCGTTAAACGCNGTTTGATATAATCTCGTTTTCCTCCGG 66
EL481607        GATAACAATAAAAAATCAACGGCGTTAAACGCG-TTTGATATAATCTCGTTTTCCTCCGG 359
                ********** ******************  *************************

EE657247        TTTAAACCTAACGCCGTTGTTTGACGGTGCAAGTAGCTCAACTTCGCCGGTGAAAAACGA 126
EL481607        TTTAAACCTAACGCCGTTGTTTGACGGCGCTTCTAGTTCAACTTCGCCGGTGAAAAACGA 419
                *************************** **   *** ***********************

EE657247        GCGGCTGGTGGTGGAGGAGTCGCCGGAGAATGTGATTTCGAAAGTTGAGGAGATTGTGAA 186
EL481607        GCGGCTGGTGGTGGATGAGTCGCCGGAGAATGTGATTTCGAAAGTTGAGGAGATTGTGAA 479
                *************** ********************************************

EE657247        GGATGCAAACGCTAGAATACACAAACGGAAGGATTACGGTGTGGATTTGGTAGCGAAAAA 246
EL481607        GGATGCAAACGCTAGAATGCACAGACGGAAGGATTATGGTGTGGATTTGGTAGCGAAAAA 539
                ****************** **** ************ **********************

EE657247        CGGTAAAGATGTGATTGAAATAGAGGTTTTCCGGTTGACGGAAAAGTTAGTTGTGGTGGA 306
EL481607        CGGTAAAGATGTGATTGAAATAGAGGTTTTCCGGTTGACGGANAAGTTAGTTGTGGTGGA 599
                ****************************************** *****************

EE657247        GGTTCAGTGTTACGGTGGAGGTACAGAGTTCTACGATGAGTTGTGGAATGATAAAATTAA 366
EL481607        GGTTCAGTGTCACGGTGGAGGTACAGAGTTCTACGATGAGTTGTGGAATGATAAAATTAG 659
                ********** ************************************************

EE657247        ATCGGAGTTGGTTGAACAACGACGGGATGTACCGGAAACGGAGAGTTAGTGAATAGTGAC 426
EL481607        ATCGGAGTTGGTTGAACAACGACGGGATGTACCGGAAACGGAGAGTTAGT------TGAC 713
                ************************************************      ****

EE657247        TCGCCGGCGGGGCACGTGTCGGGTGTATGTACGTAATTATTCAGATACATATTTTTTTTT 486
EL481607        TCGCCGGCGGGGCACGTGTCGGGTGTATGTACGTAA---------------------- 749
                ***********************************

EE657247        GACATGTTTGTTTTATTTTTTTACACGATTTTTTTTGTTGATAGAGAANAATGAGTGAGG 546
EL481607        ------------------------------------------------------------


EE657247        GAAAAATGAACCAAGGGTGGTCAAAATACGAGTGTGTGAATTTGTACATAACATGTAACT 606
EL481607        ------------------------------------------------------------


EE657247        TGCCTTGNTAATCATTGGGGCAACTGTTTGTTTGGTTTTGTGTAAAATATACAAGTTTAT 666
EL481607        ------------------------------------------------------------


EE657247        TTATAAAAAAAAAAAAAAAAAGGCCGCT 694
EL481607        ----------------------------
```

Figure 3.5. The nucleotide alignment of EST1 with EST2 (Genbank accession nr EL481607). Identical nucleotides are indicated with an *.

```
EL481607        ------------------------------------------------------------
EL476164        TGTGAAATTGTTTGAAGTTATGGCGACGAAAACGAAGATCTATGTCGTTATGGAGTGTGA 60


EL481607        ------------------------------------------------------------
EL476164        AAGGAGGTGAGTTGTTCGCGAAGGTAGCGAAAGGGCGGTTATCGGAATCGAATAGTCGGA 120

EL481607        ------------------------------------------------------------
EL476164        AGTATTTTCAACAGTTGATTTCCGCGGTCGGATATTGTCATTCGAAAGGTGTGTTTCATC 180

EL481607        ------------------------------------------------------------
EL476164        GAGACCTGAAACCGGAGAATCTGTTAATCGACGAGAACGGAGATCTGAAGGTTTCCGATT 240

EL481607        ------------------------------------------------------------
EL476164        TCGGTTTGAGTGCGTTGACGGATCAGATCCGAGTTGACGGATTGTTGCACACGTTATGCG 300


EL481607        ----------------------------------------------------GAGCGTAAG 9
EL476164        GTACGCCGTCGTATGTCTCGCCGGAGATTTTAACGAAGAGAGGCTACGACGGAGCG-AAG 359
                                                                   ***** *** 

EL481607        GCGGACATCTGGTCATGCGGTATCATTTTGTTCGTGATGAATGCAGGTTTTCTACCGTTT 69
EL476164        GCGGACATCTGGTCATGCGGTATCATTTTGTTCGTGATGAATGCAGGTTTTCTACCGTTT 419
                ************************************************************

EL481607        AACGATTCGAATCTCATGATGATGTATAAGAAGATCTACAAAGGTGAGTACCGGTGCCCT 129
EL476164        AACGATTCGAATCTCATGATGATGTATAAGAAGATCTACAAAGGTGAGTACCGGTGCCCT 479
                ************************************************************

EL481607        AAGTGGATGTCGCCGGAGCTCCGGCGGTTGTTATCTCGGTTACTTGACACAAAACCTGAC 189
EL476164        AAGTGGATGTCGCCGGAGCTCCGGCGGTTGTTATCTCGGTTACTTGACACAAAACCTGAC 539
                ************************************************************

EL481607        ACTCGGATCACCGTCGACCAGATCAAATCCGATCCGTGGTTTAAAAAAGGATACAAAGAG 249
EL476164        ACTCGGATCACCGTCGACCAGATCAAATCCGATCCGTGGTTTAAAAAAGGATACAAAGAG 599
                ************************************************************

EL481607        TCATCAAATCCAAATCTGGATGTCGATCTAGAAGCCGTTATGAAACGTGAAGATAACAAT 309
EL476164        TCATCAAATCCAGATCTGGATGTCGATCTAGAAACCGTTATGAAACGTGAAGATAACAAT 659
                ************ ******************* *************************

EL481607        AAAAAATCAACGGCGTTAAACGCGTTTGATATAATCTCGTTTTCCTCCGGTTTAAACCTA 369
EL476164        AAAAAATCAACGGCGTTAAACGCGTTTGATATAATCTCGTTTTCCTCCGGTTTAAACCTA 719
                ************************************************************

EL481607        ACGCCGTTGTTTGACGGCGCTTCTAGTTCAACTTCGCCGGTGAAAAACGAGCGGCTGGTG 429
EL476164        ACGCCGTTGTTTGACGGCGCTTCTAGTTCAACTTCGCCGGTGAAAAACGAGCGGCTGGTG 779
                ************************************************************

EL481607        GTGGATGAGTCGCCGGAGAATGTGATTTCGAAAGTTGAGGAGATTGTGAAGGATGCAAAC 489
EL476164        GTGGATGAGTCGCCGGAGAATGTGATTTTCGAAGTTGAGGAGATTGTGAAGGATGCAAAC 839
                ***************************   ****************************

EL481607        GCTAGAATGCACAGACGGAAGGATTATGGTGTGGATTTGGTAGCGAAAAACGGTAAAGAT 549
EL476164        GCTAGAATGCACAAACGGAAGGATTATGGTG----------------------------- 870
                ************* *****************

EL481607        GTGATTGAAATAGAGGTTTTCCGGTTGACGGANAAGTTAGTTGTGGTGGAGGTTCAGTGT 609
EL476164        ------------------------------------------------------------


EL481607        CACGGTGGAGGTACAGAGTTCTACGATGAGTTGTGGAATGATAAAATTAGATCGGAGTTG 669
EL476164        ------------------------------------------------------------


EL481607        GTTGAACAACGACGGGATGTACCGGAAACGGAGAGTTAGTTGACTCGCCGGCGGGGCACG 729
EL476164        ------------------------------------------------------------


EL481607        TGTCGGGTGTATGTACGTAA 749
EL476164        --------------------
```

Figure 3.6. The nucleotide alignment of EST2 with EST3 (Genbank accession nr EL476164). Identical nucleotides are indicated with an *.

```
EL476164        TGTGAAATTGTTTGAAGTTATGGCGACGAAAACGAAGATCTATGTCGTTATGGAGTGTGA 60
EE649676        ------------------------------------------------------------G 1


EL476164        AAGGAGGTGAGTTGTTCGCGAAGGTAGCGAAAGGGCGGTTATCGGAATCGAATAGTCGGA 120
EE649676        AAGGAGGTGAGTTGTTCGCGAAGGTAGCGAAAGGACGGTTATCGGAATCGAATAGTCGGA 61
                ********************************* ************************


EL476164        AGTATTTTCAACAGTTGATTTCCGCGGTCGGATATTGTCATTCGAAAGGTGTGTTTCATC 180
EE649676        AGTATTTTCAACAGTTGATTTCCGCAATCGGATATTGTCATTCGAAAGGTGTGTTTCATC 121
                ************************  ********************************


EL476164        GAGACCTGAAACCGGAGAATCTGTTAATCGACGAGAACGGAGATCTGAAGGTTTCCGATT 240
EE649676        GAGATCTGAAACCGGAGAATCTGTTAATCGACGAGAACGGAGATCTGAAGGTTTCCGATT 181
                **** ************************************************************


EL476164        TCGGTTTGAGTGCGTTGACGGATCAGATCCGAGTTGACGGATTGTTGCACACGTTATGCG 300
EE649676        TCGGTTTGAGTGCGTTGACGGATCAGATCCGAGTTGACGGATTGTTGCACACGTTATGCG 241
                ************************************************************


EL476164        GTACGCCGTCGTATGTCTCGCCGGAGATTTTAACGAAGAGAGGCTACGACGGAGCGAAGG 360
EE649676        GTACGCCGTCGTATGTCTCGCCGGAGATTTTAACGAAGAGAGGCTACGACGGAGCGAAGG 301
                ************************************************************


EL476164        CGGACATCTGGTCATGCGGTATCATTTTGTTCGTGATGAATGCAGGTTTTCTACCGTTTA 420
EE649676        CGGACATCTGGTCATGCGGTATCATTTTGTTCGTGATGAATGCAGGTTTTCTACCGTTTA 361
                ************************************************************


EL476164        ACGATTCGAATCTCATGATGATGTATAAGAAGATCTACAAAGGTGAGTACCGGTGCCCTA 480
EE649676        ACGATTCGAATCTCATGATGATGTATAAGAAGATCTACAAAGGTGGGTACCGGTGCCCTA 421
                ******************************************* **************


EL476164        AGTGGATGTCGCCGGAGCTCCGGCGGTTGTTATCTCGGTTACTTGACACAAAACCTGACA 540
EE649676        AGTGGATGTCGCCGGATCTCCGGCGGTTGTTATCGCGGTTACTTGACACAAAACCTGACA 481
                *************** ***************** ************************


EL476164        CTCGGATCACCGTCGACCAGATCAAATCCGATCCGTGGTTTAAAAAAGGATACAAAGAGT 600
EE649676        CTCGGATCACCGTCGACGAGATCAAATCCGATCCGTGGTTTAAAAAAGGATACAAAGAGT 541
                **************** ****************************************


EL476164        CATCAAATCCAGATCTGGATGTCGATCTAGAAACCGTTATGAAACGTGAAGATAACAATA 660
EE649676        CATCAAATCCAGATCTGGATCTAGATCTAGAAACCGTTATGAAACGTCAAGATAACAATA 601
                ******************* * ********************** ************


EL476164        AAAAATCAACGGCGTTAAACGCGTTTGATATAATCTCGTTTTCCTCCGGTTTAAACCTAA 720
EE649676        ACAAATCAACGGCGTTAAACGCGTTTGATATAATCTCGTTTTCCTCCGGTTTAAACCTAA 661
                * ************************************************************


EL476164        CGCCGTTGTTTGACGGCGCTTCTAGTTCAACTTCGCCGGTGAAAAACGAGCGGCTGGTGG 780
EE649676        CGCCGTTGTTTGACGGTGCAAGTAG---------------------------------- 686
                *************** **   ***


EL476164        TGGATGAGTCGCCGGAGAATGTGATTTTCGAAGTTGAGGAGATTGTGAAGGATGCAAACG 840
EE649676        ------------------------------------------------------------


EL476164        CTAGAATGCACAAACGGAAGGATTATGGTG 870
EE649676        ------------------------------
```

Figure 3.7. The nucleotide alignment of EST3 with EST4 (Genbank accession nr EE649676). Identical nucleotides are indicated with an *.

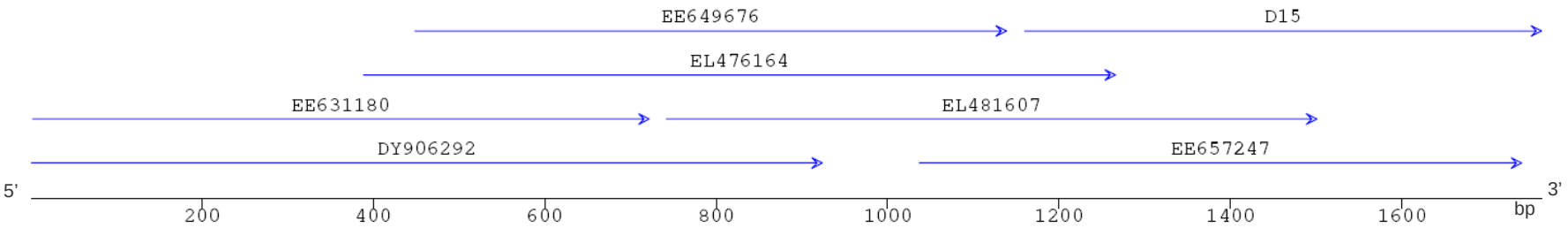
```
EE649676        ------------------------------------------------------------
DY906292        AATCAAATCTTTTTCACCTAAATTCTCAATTTTGCCTTCCCAACAAACATCCCCCAAACC 60

EE649676        ------------------------------------------------------------
DY906292        TTCTCCGATCATCTTCAACAATCTTCCCGTACACAAAAGAAACACCACCAATTCCGATCA 120

EE649676        ------------------------------------------------------------
DY906292        ACCCGATCTCTGGACTCGTTTATTCGATGCCAGAGGTCGCCAACATCATGTCGTCAACAA 180

EE649676        ------------------------------------------------------------
DY906292        CAAACACATTATTCGGTAAATACGAGATCGGAAGACTTCTCGGCTGCGGTGCATTCGCCA 240


EE649676        ------------------------------------------------------------
DY906292        AAGTCTATTACGCCAGAGACATCAACACCGGCCACAGCGTCGCCATTAAAGTCATCAACA 300


EE649676        ------------------------------------------------------------
DY906292        AGCTCAAAATCGCTCACAACAATCATTTAATTAACAACGTGAAACGCGAGATCGATATCA 360


EE649676        ------------------------------------------------------------
DY906292        TGAGGCGGTTACGGCATCCGAATATTGTGAAATTGTTTGAAGTTATGGCGACGAAAACGA 420


EE649676        --------------------------GAAGGAGGTGAGTTGTTCGCGAAGGTAGCGAAAG 34
DY906292        AGATCTATGTCGTTATGGAGTTTGTGAAAGGAGGTGAGTTGTTCGCGAAGGTAGCGAAAG 480
                                           **********************************

EE649676        GACGGTTATCGGAATCGAATAGTCGGAAGTATTTTCAACAGTTGATTTCCGCAATCGGAT 94
DY906292        GACGGTTATCGGAATCGAATAGTCGGAAGTATTTTCAACAGTTGATTTCCGCAATCGGAT 540
                ************************************************************

EE649676        ATTGTCATTCGAAAGGTGTGTTTCATCGAGATCTGAAACCGGAGAATCTGTTAATCGACG 154
DY906292        ATTGTCATTCGAAAGGTGTGTTTCATCGAGATCTGAAACCGGAGAATCTGTTAATCGACG 600
                ************************************************************

EE649676        AGAACGGAGATCTGAAGGTTTCCGATTTCGGTTTGAGTGCGTTGACGGATCAGATCCGAG 214
DY906292        AGAACGGAGATCTGAAGGTTTCAGATTTCGGTTTGAGTGCGTTGACGGATCAGATACGAG 660
                ********************** ******************************** ****

EE649676        TTGACGGATTGTTGCACACGTTATGCGGTACGCCGTCGTATGTCTCGCCGGAGATTTTAA 274
DY906292        TTGATGGATTGTTGCACACGTTATGCGGTACGCCGTCGTATGTCTCGCCGGAGATTTTAA 720
                **** ********************************************************

EE649676        CGAAGAGAGGCTACGACGGAGCGAAGGCGGACATCTGGTCATGCGGTATCATTTTGTTCG 334
DY906292        CGAAGAGAGGCTACGACGGAGCGAAGGCGGACATCTGGTCATGCGGTATCATTTTGTTCG 780
                ************************************************************

EE649676        TGATGAATGCAGGTTTTCTACCGTTTAACGATTCGAATCTCATGATGATGTATAAGAAGA 394
DY906292        TGATGAATGCAGGTTTTCTACCGTTTAACGATTCGAATCTCATGATGATGTATAAGAAGA 840
                ************************************************************

EE649676        TCTACAAAGGTGGGTACCGGTGCCCTAAGTGGATGTCGCCGGATCTCCGGCGGTTGTTAT 454
DY906292        TCTACAAAGGTGAGTACCGGTGCCCCTAGTGGATGTCGCCGGAGCTCCGGCGGTTGTTAT 900
                ************ ************  ***************** ****************

EE649676        CGCGGTTACTTGACACAAAACCTGACACTCGGATCACCGTCGACGAGATCAAATCCGATC 514
DY906292        CGCGGTTACTTGACACAA---------------------------------------- 918
                ******************

EE649676        CGTGGTTTAAAAAAGGATACAAAGAGTCATCAAATCCAGATCTGGATCTAGATCTAGAAA 574
DY906292        ------------------------------------------------------------


EE649676        CCGTTATGAAACGTCAAGATAACAATAACAAATCAACGGCGTTAAACGCGTTTGATATAA 634
DY906292        ------------------------------------------------------------


EE649676        TCTCGTTTTCCTCCGGTTTAAACCTAACGCCGTTGTTTGACGGTGCAAGTAG 686
DY906292        --------------------------------------------------
```

Figure 3.8. The nucleotide alignment of EST4 with EST5 (Genbank accession nr DY906292). Identical nucleotides are indicated with an *.

```
DY906292        AATCAAATCTTTTTCACCTAAATTCTCAATTTTGCCTTCCCAACAAACATCCCCCAAACC 60
EE631180        ---TCAATCATTTTCACCTAAATTCTCAATTTTGCCATCCCAACAAACATCCCCCGAACC 57
                     **** ************************* ****************** ****

DY906292        TTCTCCGATCATCTTCAACAATC--TTCCCGTACACAAAAGAAACACCACCAATTCCGAT 118
EE631180        TTCTCCGATCATCTTCAATCTTCCCTTCCCGTACACAAAAGAAACACCACCAATTCCGAT 117
                ******************   **  **********************************

DY906292        CAACCCGATCTCTGGACTCGTTTATTCGATGCCAGAGGTCGCCAACATCATGTCGTCAAC 178
EE631180        CAATCCGATCTCTGGACTCGTTTATTCGATGCCAGAGGTCGCCAACATCATGTCGTCGTC 177
                *** ****************************************************  *

DY906292        AACAAACACATTATTCGGTAAATACGAGATCGGAAGACTTCTCGGCTGCGGTGCATTCGC 238
EE631180        AACAAACACATTATTCGGTAAATACGAGATCGGAAGACTTCTCGGCTGCGGTGCATTCGC 237
                ************************************************************

DY906292        CAAAGTCTATTACGCCAGAGACATCAACACCGGCCACAGCGTCGCCATTAAAGTCATCAA 298
EE631180        CAAAGTCTATTACGCCAGAGACATCAACACCGGCCACAGCGTCGCCATTAAAGTCATCAA 297
                ************************************************************

DY906292        CAAGCTCAAAATCGCTCACAACAATCATTTAATTAACAACGTGAAACGCGAGATCGATAT 358
EE631180        CAAGCTTAAAATCGCTCACAACAATCATTTAGTTAACAACGTGAAACGCGAGATCGATAT 357
                ****** ********************** ****************************

DY906292        CATGAGGCGGTTACGGCATCCGAATATTGTGAAATTGTTTGAAGTTATGGCGACGAAAAC 418
EE631180        CATGAGGCGGTTACGGCATCCGAATATTGTGAAATTGTTTGAAGTTATGGCGACGAAAAC 417
                ************************************************************

DY906292        GAAGATCTATGTCGTTATGGAGTTTGTGAAAGGAGGTGAGTTGTTCGCGAAGGTAGCGAA 478
EE631180        GAAGATCTATGTCGTTATGGAGTTTGTGAAAGGAGGTGAGTTGTTCGCGAAGGTAGCGAA 477
                ************************************************************

DY906292        AGGACGGTTATCGGAATCGAATAGTCGGAAGTATTTTCAACAGTTGATTTCCGCAATCGG 538
EE631180        AGGACGGTTATCGGAATCGAATAGTCGGAAGTATTTTCAACAGTTGATTTCCGCAATCGG 537
                ************************************************************

DY906292        ATATTGTCATTCGAAAGGTGTGTTTCATCGAGATCTGAAACCGGAGAATCTGTTAATCGA 598
EE631180        ATACTGTCATTCGAAAGGTGTGTTTCATCGAGATCTGAAACCGGAGAATCTGTTAATCGA 597
                *** ********************************************************

DY906292        CGAGAACGGAGATCTGAAGGTTTCAGATTTCGGTTTGAGTGCGTTGACGGATCAGATACG 658
EE631180        CGAGAACGGAGATCTGAAGGTTTCCGATTTCGGTTTGAGTGCGTTGACGGATCAGATCCG 657
                ********************** ****************************** **

DY906292        AGTTGATGGATTGTTGCACACGTTATGCGGTACGCCGTCGTATGTCTCGCCGGAGATTTT 718
EE631180        AGTTGACGGATTGTTGCACACGTTATGCGGTACGCCGTCGTATGTCTCGCCGGAGATTTT 717
                ****** ******************************************************

DY906292        AACGAAGAGAGGCTACGACGGAGCGAAGGCGGACATCTGGTCATGCGGTATCATTTTGTT 778
EE631180        ------------------------------------------------------------

DY906292        CGTGATGAATGCAGGTTTTCTACCGTTTAACGATTCGAATCTCATGATGATGTATAAGAA 838
EE631180        ------------------------------------------------------------

DY906292        GATCTACAAAGGTGAGTACCGGTGCCCCTAGTGGATGTCGCCGGAGCTCCGGCGGTTGTT 898
EE631180        ------------------------------------------------------------

DY906292        ATCGCGGTTACTTGACACAA 918
EE631180        --------------------
```

Figure 3.9. The nucleotide alignment of EST5 with EST6 (Genbank accession nr EE631180). Identical nucleotides are indicated with an *.

a



b 5'CGGGACCCACAAATCAAATCTTTTTCACCTAAATTCTCAATTTTGCCTTCCCAACAAACATCCCCCAAACCTTCTCCGATCATCTTCAATCTTCCCGTACACAAAAGAAACACCA
CCAATTCCGATCAACCCGATCTCTGGACTCGTTTATTCG**ATG**CCAGAGGTCGCCAACATCATGTCGTCAACAACAAACACATTATTCGGTAAATACGAGATCGGAAGACTTCTC
GACTGCGGTGCATTCGCCAAAGTCTATTACGCCAGAGACATCAACACCGGCCACAGCGTCGCCATTAAAGTCATCAACAAGCTCAAAATCGCTCACAACAATCATTTAATTA
ACAACGTCAAACGCGAGATCGATATCACGAGGCGGTTACGGCATCCGAATATTGTGAAATTGTTTGAAGTTATGGCGACGAAAACGAAGATCTATGTCGTTATGGAGTTTGTGA
AAGGAGGTGAGTTGTTCGCGAAGGTAGCCAAAGGACGGTTATCGGAATCGAATAGTCGGAAGTATTTTCAACAGTTGATTTCCGCAATCGGATATTGTCATTCGAAAGGTGTGTTT
CATCGAGATCTGAAACCGGAGAATCTGTTAATCGACGAGAACGGAGATCTGAAGGTTTCCGATTTCGGTTTGAGTGCGTTGACGGATCAGATCCGAGTTGACGGATTGTTGCAC
ACGTTATGCGGTACGCCGTCGTATGTCTCGCCGGAGATTTTAACGAAGAGAGGCTACGACGGAGCGAAGGCGGACATCTGGTCATGCGGTATCATTTTGTTCGTGATGAATGC
AGGTTTTCTACCGTTTAACGATTCGAATCTCATGATGATGTATAAGAAGATCTACAAAGGTGAGTACCGGTGCCCTAAGTGGATGTCGCCGGAGCTCCGGCGGTTGTTATCGCG
GTTACTTGACACAAAACCTGACACTCGGATCACCGTCGACCAGATCAAATCCGATCCGTGGTTTAAAAAAGGATACAAAGAGTCATCAAATCCAGATCTGGATGTCGATCTAG
AAACCGTTATGAAACGTGAAGATAACAATAAAAAATCAACGGCGTTAAACGCGTTTGATATAATCTCGTTTTCCTCCGGTTTAAACCTAACGCCGTTGTTTGACGGCGCTTCTAGTT
CAACTTCGCCGGTGAAAAACGAGCGGCTGGTGGTGGAGGAGTCGCCGGAGAATGTGATTTCGAAAGTTGGGAGATTGTGAAGGATGCAAACGCTAGAATGCACAAACGG
AAGGATTACGGTGTGGATTTGGTAGCGAAAAACGGTAAAGATGTGATTGAAATAGAGGTTTTCCGGTTGACGGAAAAGTTAGTTGTGGTGGAGGTTCAGTGTTACGGTGGAGGTA
CAGAGTTCTACGATGAGTTGTGGAATGATAAAATTAAATCGGAGTTGGTTGAACAACGACGGGATGTACCGGAAACGGAGAGT**TAGTGA**ATAGTGACTCGCCGGCGGGGCA
CGTGTCGGGTGTATGTACGTAATTATTCAGATACATATTTTTTTTGACATGTTTGTTTTATTTTTTTACACGATTTTTTTTGTTGATAGAGAAAAATGAGTGAGGGAAAAATGAACCAAGGGTG
GTCAAAATACGAGTGTGTGAATTTGTACATAACATGTAACTTGCCTTGTAAATCATTGGGGCAACTGTTTGTTTGGTTTTGTTGTAAAATAATACAAGTTTTATTTATTGTTTGAGAAT**AAAAA**
AAAAAAAAAAAAAAGCGGCCGCTCGAGAATTC 3'

Figure 3.10. Construction of the D15 contig using different *Helianthus* ESTs. In (a), the overlapping regions of the ESTs and D15 are indicated. In (b), the nucleotide sequence of the *D15* contig is presented. The 5' and 3' untranslated regions are underlined and the start, and stop codons as well as the poly-A tail are indicated in bold red.

The nucleotide sequence was translated into an open reading frame of 418 amino acids (Fig. 3.11a). Analysis of the polypeptide sequence led to the identification of several conserved domains (Table 3.3). Included was a catalytic domain of a serine/threonine protein kinase which stretched from amino acids 19 – 274. A NAF domain was also identified and it stretched from amino acid 302 – 365. The NAF domain is known to define a group of heterologous kinases which is implicated in different signalling processes. Other regions included phosphorylation sites such as the casein kinase 1 (CK1), forkhead-associated phosphopeptide ligand (FHA) and glycogen synthase kinase-3 (GSK3) phosphorylation sites. A docking site for molecules involved in the MAPK cascade has also been noted. These molecules include substrates and phosphatases indicating the potential involvement of the protein in a MAPK signalling cascade.

The encoded polypeptide sequence was then used to search for homologous sequences using BLAST (Table 3.4). Several polypeptides showed homology with D15 including the calcineurin B-like (CBL) interacting protein kinase 16 (CIPK16) from *Populus trichocarpa* which showed the highest homology. Other protein kinases included CIPK11, a putative protein kinase (*Phaseolus vulgaris*), a putative protein serine/threonine kinase from *Gossypium hirsutum* and the *Avr9/Cf9* rapidly elicited protein from tobacco. As D15 showed high homology to the CIPK, the amino acid sequences were aligned and it showed significant homology between the two polypeptides (Fig. 3.11b).

Using the *D15* contig nucleotide sequence, two primer pairs were developed, one specific for the 5'- and 3'-UTR and one pair where the two primers straddle the start and stop codons respectively. Firstly, success of the primers to amplify the gene from genomic DNA was tested, using genomic DNA isolated from the resistant cultivar. Both primer sets amplified the expected sized fragments with the first primer set producing a single 1318 bp fragment which was much more

a

```
5' MPEVANIMSSTTNTLFGKYEIGRLLDCGAFAKVYYARDINTGHSVAIK
   VINKLKIAHNNHLINNVKREIDITRRLRHPNIVKLFEVMATKTKIYVVME
   FVKGGELFAKVAKGRLSESNSRKYFQQLISAIGYCHSKGVFHRDLKPE
   NLLIDENGDLKVSDFGLSALTDQIRVDGLLHTLCGTPSYVSPEILTKRG
   YDGAKADIWSCGIILFVMNAGFLPFNDSNLMMMYKKIYKGEYRCPK
   WMSPELRRLLSRLLDTKPDTRITVDQIKSDPWFKKGYKESSNPDLDVDL
   ETVMKREDNNKKSTALNAFDIISFSSGLNLTPLFDGASSSTSPVKNERLV
   VEESPENVISKVEEIVKDANARMHKRKDYGVDLVAKNGKDVIEIEVFR
   LTEKLVVVEVQCYGGGTEFYDELWNDKIKSELVEQRRDVPETES 3'
```

b

```
D15             MPEVANIMSSTTNTLFGKYEIGRLLDCGAFAKVYYARDINTGHSVAIKVINKLKIAHNNH 60
ABJ91223        MPEIESVS---ENALFGKYEVGKLLGCGAFAKVYHARDVQTGKSVAIKIINKKKIS-NPS 56
                ***: .:     *:******:*:**.********:***::**:*****:*** **: *

D15             LINNVKREIDITRRLRHPNIVKLFEVMATKTKIYVVMEFVKGGELFAKVAKGRLSESNSR 120
ABJ91223        LMSNIKREISIMRRLNHPHIVKLIEVLATKTKIYFVIEYVKGGELFAKVAKGRFSEDLSR 116
                *:.*:****.* ***.**:****:**:*******.*:*:**************:**. **

D15             KYFQQLISAIGYCHSKGVFHRDLKPENLLIDENGDLKVSDFGLSALTDQIRVDGLLHTLC 180
ABJ91223        KYFHQLISAVGYCHSRGIFHRDLKPENLLLDENGSLKVSDFGLSAVTDQIRTDGLLHTLC 176
                ***:*****:*****:*:***********:****.**********:*****.********

D15             GTPSYVSPEILTKRGYDGAKADIWSCGIILFVMNAGFLPFNDSNLMMMYKKIYKGEYRCP 240
ABJ91223        GTPAYVAPEILAKKGYDGAKVDVWSCGVVLFVLTAGFLPFNDPNLMAMYKKIYKGEFRCP 236
                ***:**:****:*:******.*:****::***:.********.*** *********:***

D15             KWMSPELRRLLSRLLDTKPDTRITVDQIKSDPWFKKG-YKESSNPDLDVDLETVMKREDN 299
ABJ91223        KWMSTDLKRFLSRLLDTNPETRITIDEILKDPWFKKGKHKEINFYDEELN-KAVDEKKDE 295
                ****.:*:*:*******:*:****:*:* .******* :** .  * ::: ::* :::*:

D15             NKKSTALNAFDIISFSSGLNLTPLFDGASSSTSPVKNERLVVEESPENVISKVEEIVKDA 359
ABJ91223        ELASTSLNAFDIISFSSGLNLSGLFDDSYNVVD--DGERFVSTESPENLMKKVEEFAKEE 353
                :  **:***************: ***.: . ..  ..**:*  *****::.****:.*:

D15             NARMHKRKDYGVDLVAKNGKDVIEIEVFRLTEKLVVVEVQCYGGGTEFYDELWNDKIKSE 419
ABJ91223        RLRVKRRKEWGLEMEGQNGNLMIAVEVHRLTDTLFVVEAKRSGGDAGCFKETWKNKLKPP 413
                . *:::**::*::: .:**: :* :**.***:.*.***.:  **.:  :.* *::*:*.

D15             LVEQRRDVPETES 432
ABJ91223        NYLLG-------- 418
```

Figure 3.11. Sequence analysis of the D15 contig. In a), the virtual amino acid sequence of the contig is presented. In b), the sequence is significantly aligned with the CBL-interacting protein kinase (CIPK16) from *Populus trichocarpa* [Genbank accession nr ABJ91223 (e-value 0)].

Table 3.3. Conserved domains present in the D15 polypeptide.

Domain	Description	Positions
Catalytic domain	Serine / Threonine protein kinase	19 - 274
NAF domain	Serine / Threonine protein kinase	302 - 365
MAPKK docking site	Interacting molecules in the MAPK cascade (eg. substrates, phosphotases)	366 - 386
Casein Kinase 1	CK1 phosphorylation site	9 - 15
FHA phosphopeptide ligand binding site	Motifs for binding phosphothreonine modules in FHA domains	10 -16, 388 -394
GSK3 phosphorylation site	Site recognised by GSK3 for Ser/Thr Phosphorylation	7 - 14

Table 3.4. BLAST analysis of the translated D15 contig on amino acid level.

Genbank accession nr.	Homologous polypeptides	E-value
ABJ91223	CBL-interacting protein kinase 16 (*Populus trichocarpa*)	0.0
NP180595	CIPK11 (SNF1-Related protein kinase) (*Arabidopsis thaliana*)	2e $^{-145}$
ABJ91222	CBL-interacting protein kinase 15 (*Populus trichocarpa*)	7e $^{-144}$
AAP03879	Avr9/Cf-9 rapidly elicited protein 216 (*Nicotiana tabacum*)	7e $^{-141}$
CAN83248	Hypothetical protein (*Vitis vinifera*)	6e $^{-139}$
BAG06674	Protein kinase (*Phaseolus vulgaris*)	6e $^{-133}$
ABJ91227	CBL-interacting protein kinase 21 (*Populus trichocarpa*)	3e $^{-133}$
AAT64036	Putative serine-threonine kinase (*Gossypium hirsutum*)	8e $^{-127}$
NP193605	CIPK12 kinase (*Arabidopsis thaliana*)	5e $^{-123}$
NP195802	ATSR1 Serine/threonine protein kinase (*Arabidopsis thaliana*)	1e $^{-122}$
AAO17040	Calcineurin B-like-interacting protein kinase (*Pisum sativum*)	4e $^{-119}$

intense than that of the second 1312 bp fragment (Fig. 3.12a). In addition to being fainter, the amplified fragment using Bovis 263 and 264 was also predictably smaller than the first.

Amplification of the contig using both sets of primers was similarly tested on cDNA that was prepared from total RNA isolated from the resistant cultivar [(GH99RRPHRR3 (VII) (R)]. As expected, the correct size fragments were again amplified, but to much greater intensity than from the genomic DNA (Fig. 3.12b). This confirmed the expression of the *D15* gene.

Since the *D15* gene plays a potential role in the defence response of sunflower to *P. helianthi* infection, it was decided to rename it to *Helianthus annuus* leaf rust *D15* (*HaLRD15*).

3.3.4 Infection phenotype of different sunflower cultivars infected with *P. helianthi.*

Eight different sunflower cultivars were screened for their resistance level against infection with *P. helianthi*. They were divided into resistant, intermediate resistant and susceptible to leaf rust. The infected susceptible plants showed severe signs of infection with the formation of many rust pustules over at least 20% of the dorsal and ventral leaf surfaces 10 days post infection (Fig. 3.13). The cinnamon-brown pustules were surrounded by a chlorotic border which indicated that the chloroplasts had degenerated in the infected and neighbouring cells. The susceptible cultivars were Hysun 333 (P16) and GH99S37-388RR (S).

In contrast to the susceptible plants, the resistant [Hysun 346 (P1) and GH99PHRR3 (VII) (R)] plants showed almost no signs of infection and the effects of the disease were limited (Fig. 3.13). Chlorotic flecks were visible on the resistant plants, indicating a resistance response to the infection. Chlorotic flecks were also visible on the plant leaves of the intermediate resistance cultivar [MonoSun 150 (P12)], but less rust pustules were formed (Fig. 3.13).

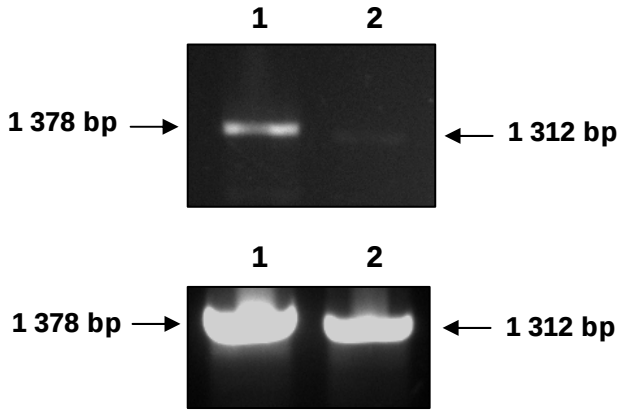


Figure 3.12. The amplification of the *HaLRD15* gene from sunflower. a), In lane 1, the gene was amplified with Bovis 261 and Bovis 262 and in lane 2, with Bovis 263 and Bovis 264 from genomic sunflower DNA. b), In lane 1, the contig is amplified with the same primer combination as in a) but using cDNA as template.

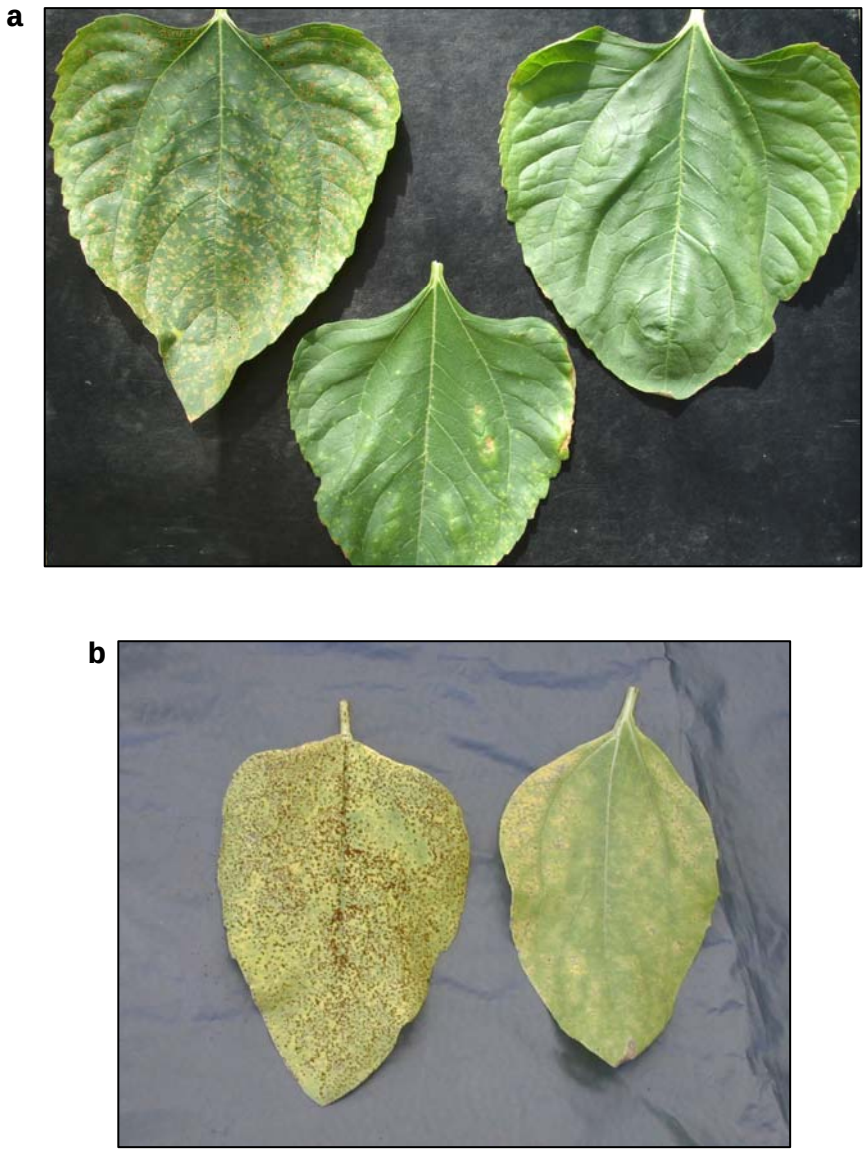


Figure 3.13. *P. helianthi* infection of different sunflower cultivars. In (a), leaves from infected susceptible [Hysun 333 (P16)] (left), intermediate resistant [MonoSun 150 (P12)] (middle) and resistant cultivars [Hysun 346 (P1)] (right) are presented ten days after infection. In (b), leaves from infected susceptible [GH99S37-388RR (S)] (left) and infected resistant [GH99PHRR3 (VII) (R)] (right) plants are shown.

3.3.5 Southern blot analysis of *HaLRD15* in different sunflower cultivars.

Eight sunflower cultivars showing different levels of resistance against leaf rust were screened for the presence of the *HaLRD15* gene using a Southern blot. After hybridization at high stringency, cross hybridizing DNA fragments were detected in all of the cultivars (Fig. 3.14). The sizes of the two fragments were approximately 11.5 and 5.5 kb respectively. Two of the resistant cultivars (P1 and P7) displayed both bands, while the third resistant cultivar (R) only had the smaller band. Of the intermediate resistant cultivars, P3 showed both bands while P12 only had the larger fragment. The larger fragment was also present in the P16 susceptible cultivar, while P2 and the susceptible (S) cultivar again had two bands present. Thus, the number of cross hybridizing fragments did not correlate with either the resistance or susceptible phenotypes.

3.3.6 Expression of *HaLRD15* in cultivars with differences in resistance.

To evaluate the role of *HaLRD15* in sunflower cultivars that exhibit different resistance phenotypes upon infection with *P. helianthi*, the expression of the gene was tested using *HaLRD15* specific primers (Fig. 3.15 – 3.19). The quality and quantity of the extracted RNA was confirmed by amplifying the *18S rRNA*. The *rRNA* were at all times constitutively expressed and is an indication that the amount of RNA used for each RT-PCR reaction was indeed equal.

The expression of *HaLRD15* in two cultivars that showed complete resistance, exhibited very different profiles (Fig. 3.15, 3.16). In the R cultivar, the gene was up regulated at 3 hpi followed by a second up regulation from 9 to 21 hpi with a sharp down regulation for the remainder for the time trial (Fig. 3.15). In contrast, expression in the P1 cultivar was only slightly induced at 9, 15 and 24 hpi. In between, a sharp decrease in expression was found (Fig. 3.16). No significant induction of expression followed for the rest of the time trial. It must be noted that the basal expression levels of *HaLRD15* at 0 hpi in the P1 cultivar was higher than that in the R cultivar.

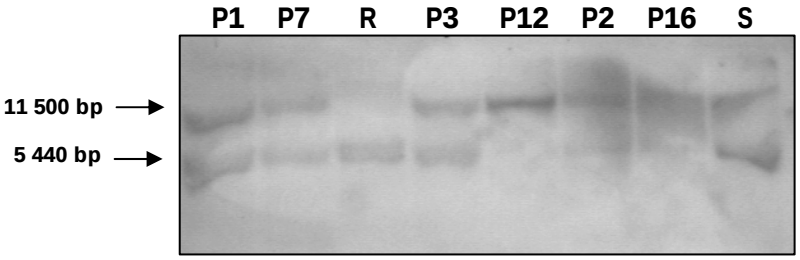
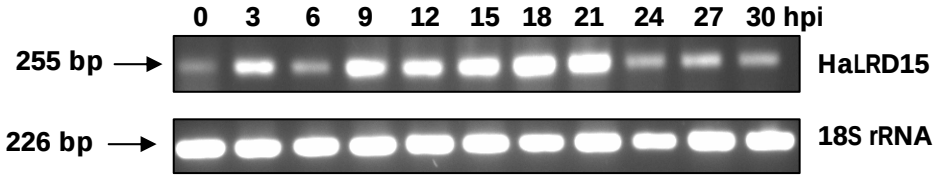


Figure 3.14. Southern blot analysis of *HaLRD15* in eight different sunflower cultivars. The approximate sizes of cross hybridizing fragments are indicated by arrows. The names of the different cultivars are as indicated. The cultivars were indicated from highly resistant (left) to highly susceptible (right).

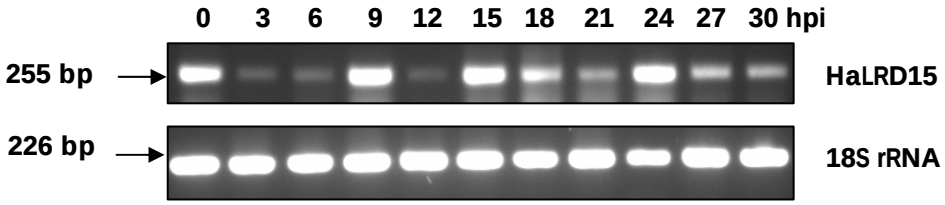
a



b

Figure 3.15. Expression analysis of *HaLRD15* in resistant (R) sunflower infected with *P. helianthi*. In a), the expression of both *HaLRD15* and the *18S rRNA* genes are indicated. Fragment sizes and time intervals are as indicated. In b), a leaf of the infected R cultivar is indicated.

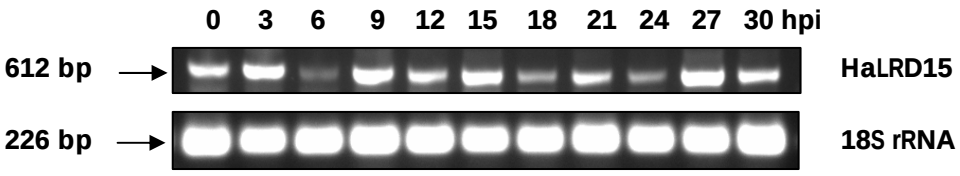
a



b

Figure 3.16. Expression analysis of *HaLRD15* in resistant (P1) sunflower infected with *P. helianthi*. In a), the expression of both *HaLRD15* and the *18S rRNA* genes are indicated. Fragment sizes and time intervals are as indicated. In b), a leaf of the infected P1 cultivar is indicated.

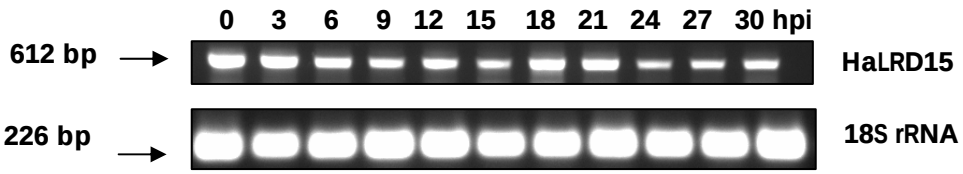
a



b

Figure 3.17. Expression analysis of *HaLRD15* in intermediate resistant (P12) sunflower infected with *P. helianthi*. In a), the expression of both *HaLRD15* and the *18S rRNA* genes are indicated. Fragment sizes and time intervals are as indicated. In b), a leaf of the infected P12 cultivar is indicated.

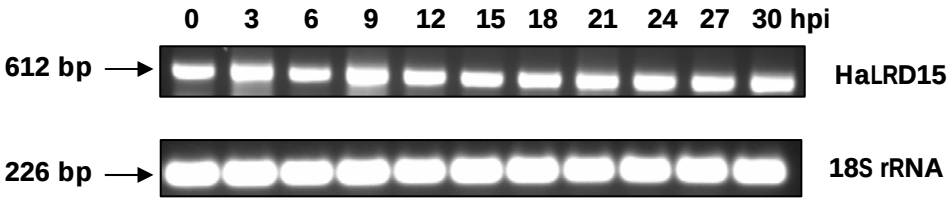
a



b

Figure 3.18. Expression analysis of *HaLRD15* in susceptible (P16) sunflower infected with *P. helianthi*. In a), the expression of both *HaLRD15* and the *18S rRNA* genes are indicated. Fragment sizes and time intervals are as indicated. In b), a leaf of the infected P16 cultivar is indicated.

a



b

Figure 3.19. Expression analysis of *HaLRD15* in susceptible (S) sunflower infected with *P. helianthi*. In a), the expression of both *HaLRD15* and the *18S rRNA* genes are indicated. Fragment sizes and time intervals are as indicated. In b), a leaf of the infected S cultivar is indicated.

Gene expression in the intermediate P12 cultivar, displayed a slight increase at 3 and 9 hpi, but the levels were not much higher than 0 hpi (Fig. 3.17). No change in gene expression was visible throughout the time trial in the susceptible P16 and S cultivars (Fig. 3.18, 3.19). The basal expression values were however again higher at 0 hpi than that of the R cultivar. It is however clear that these higher levels of expression could be due to more total RNA used, as is evident by the much stronger expression levels of *18S rRNA.*

3.3.7 *HaLRD15* gene expression following chemical treatments.

Resistant sunflower [(GH99PHRR3 (VII) (R)] was treated with defence related compounds to confirm the involvement of *HaLRD15* in the plant defence response. When treated with MeJA, *HaLRD15* showed induced expression from 2 to 12 hpi followed by a down regulation until 48 hpi (Fig. 3.20a). In menadione, H_2O_2 and SA treated sunflower, gene expression showed very little changes throughout the time trial (Fig. 3.20b). As a control, sunflower plants were treated with distilled water and harvested simultaneously (Fig. 3.20e). *HaLRD15* expression showed an increase at 2 hpi within the water treatment, but it did not correlate with all the other treatments, implicating that the induced expression in the MeJA treated sunflower was due to the MeJA itself.

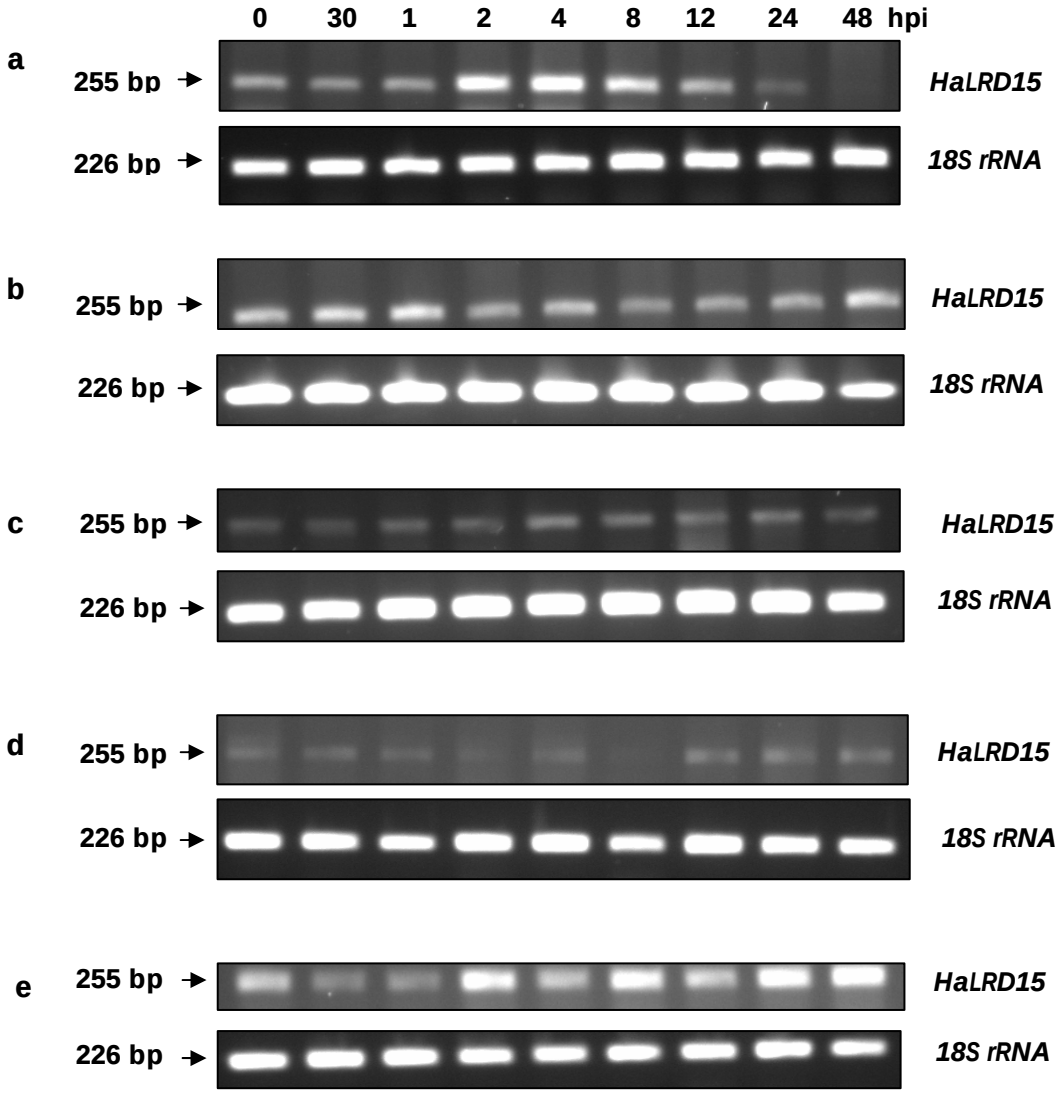


Figure 3.20. Expression analysis of *HaLRD15* in the resistant (R) cultivar after treatment with different chemicals. In a), the expression of *HaLRD15* in plants treated with MeJA is shown, in b), plants treated with menadione, in c), plants treated with H_2O_2, in d), plants treated with SA and in e), plants treated with H_2O. Fragment sizes and time-intervals are as indicated.

3.4 Discussion

Many environmental factors influence the well being of plants. One such factor is pathogenic organisms. In order for plants to survive infection, inducible plant defence responses have evolved. These responses depend on effective signalling events. This enables the plant to firstly recognize the potential pathogen at the cell wall and then to amplify the initial alarm signal which leads to the arrest of infection. Protein kinases play a central role in these signalling cascades when a stress signal is conveyed through the plant cell (Romeis, 2001).

A partial cDNA sequence isolated from *P. helianthi* infected resistant sunflower, showed homology to protein kinase genes from other plant species that were placed under stress related conditions (Table 3.2). A 1760 bp contig for the *HaLRD15* gene was assembled using ESTs which were expressed in sunflower in response to different stress stimuli (Fig. 3.10). The alignment between the ESTs was significant with only 34 single base pair changes that were detected within the coding region represented between the six contigs. Within the nucleotide sequence of the contig, a start as well as two in-frame stop codons were found, indicative of a complete gene.

The derived polypeptide of the *HaLRD15* gene showed homology to various protein kinases, including the previously found rapidly elicited protein involved in the *Avr9/Cf9* interaction (Table 3.4). The highest homology however was with the CBL-interacting protein kinase 16 (CIPK16) from *Populus trichocarpa* with an e-value of $7e^{-151}$. This alignment was significant with only a few differences evident (Fig. 3.11). HaLRD15 also showed significant homology to other CIPKs from *Arabidopsis thaliana* and *Pisum sativum* with e-values of $5e^{-169}$ and $3e^{-129}$ respectively. Based on these significant e-values, it was concluded that HaLRD15 was a CIP kinase that is involved in the defence response of sunflower following *P. helianthi* infection.

In plant defence signalling, calcium levels play an important role in the regulation of gene expression (McAinsh and Hetherington, 1998). External stimuli such as light and various other stress factors cause changes in cellular Ca^{2+} levels which can affect plant growth and development (Rudd and Franklin-Tong, 2001). Calcium serves as a second messenger and its intracellular concentration is precisely balanced by calcium which is readily available in the vacuoles, endoplasmic reticulum and mitochondria (Zielinski, 1998). Ca^{2+} signalling is very specific and it is detected by Ca^{2+} sensors (Luan *et al.,* 2002).

A recent addition to the group of calcium sensor proteins is the calcineurin B-like proteins (CBLs) (Kudla *et al.,* 1999). The CBL proteins contain helix-turn-helix domains called EF hands that are able to bind Ca^{2+} (Sanchez-Barrena *et al.,* 2006). The function of CBLs is to interact and regulate the CIPK group of Ser/Thr kinases. The CIPKs are most likely the targets of calcium signals which are sensed and transduced by CBL proteins. CIPKs have a catalytic domain at the N terminus that is closely related to sucrose non-fermenting-like and cAMP-dependant protein kinases from various organisms (Shi *et al.,* 1999). This structural feature also assigns CIPKs to the SNF-like group of protein kinases (Hrabak *et al.,* 2003). CIPK also contains a regulatory domain at the C terminus which interacts with the catalytic domain at the N-terminus to keep the enzyme inactive through autoinhibition (Guo *et al.,* 2001).

CIPKs which have been discovered in *Arabidopsis* showed a conserved 24-amino acid region that is required and sufficient to mediate CBL-CIPK interaction (Albrecht *et al.,* 2001). This functional and important conserved amino acid motif, Asn-Ala-Phe, has been designated the NAF domain. CBL binds to the NAF domain of CIPK thereby disrupting the intramolecular domain interaction of CIPK which then activates the enzyme (Gong *et al.,* 2004).

The analysis of the 418 amino acid sequence of HALRD15 showed that a catalytic domain typical of serine/threonine protein kinases was present (Table 3.3). Furthermore, a NAF domain and two phosphorylation sites, namely Casein

kinase 1 (CK1) and glycogen synthase kinase-3 (GSK3), were also present. CK1 has been shown to be ubiquitously expressed in many cell types (Gietzen and Virshup, 1999). Together with CK1, GSK3 is involved in the phosphorylation of substrate proteins (Welsh and Proud, 1993). In mammalian cells, CK1 phosphorylates a transcription factor, called the nuclear factor of activated T cells (NFAT), within the SSR-1 domain which confines the enzyme in an inactive state. It has also been shown that GSK3, in collaboration with CK1, regulates NFAT activity by phosphorylation on the SP-2 motif within NFAT (Okamura *et al.,* 2004). The presence of the catalytic domain as well as the NAF domain and respective phosphorylation sites, is an indication that HaLRD15 could be involved in the perception of ligands in the form of Ca^{2+} via an interaction with CBL.

A docking site for molecules involved in a MAPK cascade was also found within the HaLRD15 amino acid sequence (Table 3.3). The docking site was specific for MAPKK proteins (Jacobs *et al.,* 1999). This leads to the possibility that HaLRD15, in association with the CBL protein, could form the start of a MAPK signalling cascade where the signal is transferred to a consecutive MAPKK. This proposal was further strengthened by the fact that HaLRD15 contained motifs that indicated specific protein-protein interactions.

These interactions were implicated by the presence of a forkhead-associated (FHA) phosphopeptide ligand binding site. This type of domain represents a class of phosphorylation-dependant protein-protein interaction domains with a specificity for phosphothreonine and phosphoserine residues (Ding *et al.,* 2007). Many FHA domains have been found in protein kinases, protein phosphatases and transcription factors (Li *et al.,* 2000). FHA domains have been shown to bind phosphothreonine which might act as a negative regulator in the kinase function (Ding *et al.,* 2007).

Several CBL-CIPK interacting proteins have been identified and analysed in *Arabidopsis* (Halfter *et al*., 2000). Two of these were the CBL sensor SOS3 and the CIPK SOS2 which appears to form part of the calcium-regulated signalling

pathway that specifically mediates signalling during salt stress. Halfter *et al.* (2000) found that SOS3 interacts with and activates a protein kinase encoded by SOS2. The binding between SOS2 and SOS3 is calcium dependant as the expression of *sos* mutants suggests that SOS2/SOS3 functions in the regulation of intracellular Na^+ and K^+ homeostasis (Halfter *et al.,* 2000).

Another set of these interacting proteins were identified in peas (*Pisum sativum*) (Mahajan *et al.,* 2006). Immunofluorescence labelling showed that PsCIPK is located in the outer membrane of the cell whereas PsCBL was present within the cytosol (Mahajan *et al.,* 2006). PsCIPK was a functional protein kinase that autophosphorylated in the presence of Mn^{2+} and Mg^{2+} and trans-phosphorylated casein in the presence of Mg^{2+} on the threonine residues (Mahajan *et al.,* 2006). PsCIPK also contained a NAF domain which is known to regulate kinase activity when the CBL-protein with its EF-hand binds to it. Mahajan *et al.* (2006) suggested that the interaction of CBL with CIPK may not be affected by the phosphorylation of CIPK, since it was shown that PsCBL interacts with both phosphorylated and non-phosphorylated forms of PsCIPK. It is therefore possible that these different phosphorylation events between the proteins could provide a means of cross-talk between various signalling pathways involving calcium.

The expression of both *PsCIPK* and *PsCBL* were up-regulated by Ca^{2+} in a dose-dependent manner (Mahajan *et al.,* 2006). Other factors which up-regulated gene expression for both *PsCIPK* and *PsCBL* were cold, salinity, wounding and SA (Mahajan *et al.,* 2006). This suggests that these genes play major roles in regulating stress responses due to different environmental signals. Since pathogen infection represents a stress condition, a possible role for HaLRD15 during the defence response can be envisaged.

With the *HaLRD15* gene resembling a *CIPK* gene potentially involved in the perception of stress related signals, the expression of the gene was analysed. It was important to determine in which sunflower cultivars the gene was present as well as to determine the number of gene copies within the genome. Since

HaLRD15 contained no internal *Eco*R1 restriction site within the coding region, *Eco*R1 digested genomic DNA was probed with *HaLRD15*.

Different sunflower cultivars which were resistant (P1, P7 and R), intermediate resistant (P3 and P12) and susceptible (P2, P16 and S) to *P. helianthi* infection, all contained the *HaLRD15* gene. The copy number however differed between the cultivars (Fig. 3.14). The first two resistant cultivars each had two copies of the gene but the R cultivar had only one. Two gene copies were present in the intermediate resistant (P3) cultivar with only one in the P12 cultivar. Within the susceptible cultivars, the gene copy number also differed with P2 and P16 having one and the S cultivar having two copies. From this it was quite clear that the resistant phenotype could not be linked to either the presence or the number of gene copies. This indicates that the gene is most probably a general defence response related gene that could facilitate signalling during different stress responses.

Five cultivars were next selected for the expression analysis of *HaLRD15*. The aim was to test whether the induction of *HaLRD15* expression differs between resistant and susceptible cultivars (Fig. 3.15-19). Even though the gene was expressed in all the resistant cultivars, the induction levels were much more evident in the R cultivar compared to the P1 and P12 cultivars. This led to the conclusion that the expression of *HaLRD15* is regulated differently in the R cultivar in comparison with the other two resistant cultivars. This could indicate that the presence of different resistance genes in the different cultivars activates different signal transduction pathways. The induced expression of *HaLRD15* in the R cultivar could thus lead to the activation of a unique resistance response. This unique expression of *HaLRD15* was further confirmed when the gene was constitutively expressed in the susceptible cultivars.

When the expression profile of *HaLRD15* was analysed respectively in the R and S cultivars, the basal expression level in the S cultivar was higher than that of the R cultivar. This was most probably due to the fact that more total RNA was used

for the RT-PCR reactions of the S cultivar as a similar difference was observed in the expression levels of the *18S rRNA* gene (Fig. 3.19).

The resistant R cultivar was also treated with various defence related compounds. The aim was to further link HaLRD15 with defence related signalling pathways. *HaLRD15* gene expression was only induced in MeJA treated plants which showed inducible expression from 2 to 12 hpi (Fig. 3.20). This was of great importance as MeJA is implicated to be involved in many pathways related to pathogen stress (Lorenzo *et al.,* 2003). JA in its volatile form has anti-inflammatory properties and is known to induce a group of defence genes (Wasternack and Hause, 2002). MeJA has been implicated in the defence response of plants against herbivorous attack (Maleck *et al.,* 2000).

To summarise, a putative CIPK was identified from sunflower. This protein is proposed to play a role during the defence response of sunflower following infection with *P. helianthi.* It is however clear that the role of HaLRD15 could be resistance gene specific because of the different expression profiles in the different resistant cultivars. Furthermore, the unique structural properties of the protein putatively place the enzyme at the start of a calcium-mediated MAPK cascade. These assumptions will however have to be confirmed in the future.

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Chapter Four:

Characterization of a putative DUF26 kinase from sunflower

4.1 Introduction.

Plants have evolved a sophisticated system which they use to recognize and guard against potential pathogens. The specificity of this system is determined by the interaction between a pathogenic *Avr*-gene and its corresponding plant *R*-gene (Hammond-Kosack and Jones, 1997). The specific genetic interaction between the *Avr* and *R*-genes leads to the activation of various host defence responses that finally results in resistance often characterized by the HR (Heath, 2000).

The largest class of plant *R*-genes encodes proteins with a nucleotide-binding site (NBS) (Meyers *et al.,* 2003). This protein family are related to the mammalian caspase recruitment domain (CARD)/nucleotide-binding oligomerization domain (Nod) family which functions in innate immunity (Inohara and Nunez, 2003). The NBS-LRR protein encodes a protein consisting of three domains. The first is a variable domain while the second contains a conserved NBS domain and the third contain LRRs. Specific residues located in the carboxy-terminal domain of the LRR region are responsible for bacterial recognition. Upon recognition the amino-terminal domain of the LRRs modulates activation. The conserved region on the NBS is then able to bind and hydrolyse ATP which relays the signal to a consecutive protein.

The plant NBS-LRR resistance genes can be divided into two major subfamilies based on the structures of the N-terminal domain (Meyers *et al.,* 1999). Members of the first subfamily have an additional N-terminal domain that shares similarity to the intracellular signalling domains of the *Drosophila* Toll or the mammalian interleukin-1 receptor (TIR). These proteins are called the TIR-NBS-LRR or TNL proteins (Meyers *et al.,* 1999). The TNL genes have been found mostly in dicotyledonous plants (Pan *et al.,* 2000). The second group of NBS-LRR resistance genes encodes a product that have a coiled-coil (CC) domain in the N-

terminal region. These are called the CC-NBS-LRR or CNL genes and are found in both pro- and eukaryotes (Meyers *et al.,* 1999; Pan *et al.,* 2000).

The presence of either a TIR or CC domain determines which pathway the signal will follow in the subsequent transduction thereof (Inohara *et al.* 2002) since the two subfamilies require different downstream factors for signalling (Pan *et al.,* 2000). The genes from the TNL group depend on the enhanced disease susceptibility (*EDS1*) signalling pathway while CNL-genes use the nonrace-specific disease resistance (*NDR1*) signalling pathway (Aarts *et al.,* 1998). To demonstrate this, a mutational analysis was performed in *Arabidopsis* (Aarts *et al.,* 1998). The *RPS2* gene, which mediates resistance to *Pseudomonas syringae*, encodes a protein of the CNL class. When a mutation occurred in *NDR1,* the resistance conferred by *RPS2* in response to the pathogenic *avrRpt2*, was compromised (Aarts *et al.,* 1998). This was similarly proven for the TNL class when a mutation in *Arabidopsis EDS1* led to susceptibility against *Peronospora parasitica*, despite the fact that *Arabidopsis* carried several 'resistance for *P. parasitica* (RPP) genes. This indicated that *EDS1* is essential for signalling via the TNL class of resistance proteins (Aarts *et al.,* 1998).

Plant NBS-LRR proteins are numerous and confer resistance against a wide variety of pathogens that include viruses, bacteria, fungi and insects (Milligan *et al.,* 1998; Rossi *et al.,* 1998). The 'resistance to powdery mildew 8' (*RPW8*) gene has been identified from *Arabidopsis.* The gene encodes a NBS-LRR protein with a transmembrane domain and a CC domain (Xiao *et al.,* 2001). When, a *RPW8* transgene plant that contained an *eds1* mutant background, was infected with *Erysiphe cruciferarum,* the resistance reaction were abolished. This confirmed that *RPW8* required *EDS1* for resistance (Xiao *et al.,* 2001). NBS-LRR proteins can also play a role as interacting proteins. The *Pto* resistance gene confers resistance to the bacterial pathogen *Pseudomonas syringae* in tomato and is able to specifically interact with the Avr-Pto avirulence protein (Zhou *et al.,* 1997). This interaction depends on the NBS-LRR containing Prf protein that detects the association between Pto and Avr-Pto (Tang *et al.,* 1999). In previous studies, the

conserved motifs in the NBS-LRR domain were used to design primers specific for this region. Several disease resistance gene homologues from wheat lines were cloned using these primers (Lacock *et al.,* 2003).

Thus, keeping in mind the importance of the NBS-LRR region and its role in the plant defence system, the aim of this study was to amplify and clone putative NBS-LRR genes involved in the defence response of sunflower infected with *P. helianthi* using these NBS-LRR region specific primers.

4.2 Materials and Methods.

4.2.1 Cultivation and infection of sunflower plants.

Seeds of the resistant [GH99PHRR3 (VII) (R)] and susceptible [GH99S37-388RR (S)] sunflower cultivars were planted in a 2:1 soil mixture of peat moss and potting soil in seedling trays and germinated at 22 - 26°C in a greenhouse at normal day/night cycles. The plants were fertilized once a week with 3 $g.l^{-1}$ Multifeed fertilizer.

Sunflower seedlings were inoculated with spores of the *P. helianthi* UVPhe2 pathotype when the plants were four weeks old. Rust spores were resuspended in kerosene oil to a final concentration of approximately 150 000 $spores.ml^{-1}$ and sprayed under high pressure onto the dorsal and ventral sides of the leaves. The plants were left to dry for 30 min at room temperature and were then placed at 22 - 24°C in a dark Dew-simulation-chamber for 16 h to allow the rust spores to germinate. The plants were then moved to the greenhouse. Taking time 0 when the plants were placed in the Dew-simulation-chamber, leaves were harvested every three hours until 30 hpi.

4.2.2 DDRT-PCR amplification of putatively differentially expressed cDNAs encoding NBS containing polypeptides.

All the solutions and apparatus needed for RNA extraction were prepared and treated as described (3.2.5.1). Total RNA was extracted from sunflower tissue and the concentration determined (3.2.5.1).

The RobusT II RT-PCR kit (Finnzymes) was used to amplify differentially expressed cDNA fragments that putatively encoded polypeptides that contained NBS domains. In order to amplify these genes, degenerate primers specific for the NBS domains were designed (Lacock *et al.,* 2003). Two sets of RT-PCR

reactions were done, where the first was performed with primers NBS1 and NBS2 (Table 4.1). The second set was done with NBS1 in combination with Bovis 39 that was identical to the 5'-tail of the anchored oligo-dT primer (Bovis 32) used for first strand cDNA synthesis. Each RT-PCR reaction consisted of 10 ng total RNA, 0.2 mM dNTPs, 1× reaction buffer, 2 µCi [α-^{32}P]-dCTP, 2.5 mM $MgCl_2$, 0.4 µl of the enzyme mix and 50 µM of the respective primers.

The amplification regime was as follows, one cycle at 45°C for 30 min, one cycle at 94°C for 2 min and ten cycles at 94°C for 10 sec, 55°C for 1 min and 68°C for 1 min. Twenty cycles of 94°C for 10 sec, 55°C for 1 min and 68°C for 1 min then followed with an additional 1 min added to the extension steps. A final cycle at 68°C for 7 min followed.

The amplified cDNA samples were dissolved in 0.25% (w/v) BFB, 0.25% (w/v) xylene cyanol, 15% (w/v) ficoll, 50% (v/v) formamide and resolved on a 8% (w/v) denaturing poly-acrylamide gel. The gel contained 8 M urea and 1× TBE (0.45 M Tris-HCl pH 8, 0.12 M boric acid, 0.01 M EDTA pH 8). The gel was pre-run at 40 W for 30 min where after the samples were loaded and separated at 40 W for 90 min. The running buffer used was 1× TBE. The gel was dried for 2 h at 65°C and exposed to an imaging-screen (Kodak) for 24 h (3.2.4.2). The gel was then exposed to an X-ray film for six days.

4.2.3 Cloning of differentially expressed cDNA fragments.

After development, the X-ray film was aligned with the dried acrylamide gel and the selected bands cut from the gel. To each gel slice, 100 µl of 10 mM Tris (pH 8) was added, the tubes incubated at room temperature for 10 min and then boiled for 10 min. After centrifuging the tubes for 15 min at 12 000 *g*, the supernatant was transferred to a clean tube and stored at -20°C.

Table 4.1 Nucleotide sequence of all primers used in this study.

Primer	Sequence
Bovis 26	5'-CAA CTT TCG ATG GTA GGA TAG-3'
Bovis 27	5'-CTC GTT AAG GGA TTT AGA TTG-3'
Bovis 32	5'-GAA GAA TTC TCG AGC GGC CGC TTT TTT TTT TTT TTT TTT TVN-3'
Bovis 39	5'-GAA GAA TTC TCG AGC GGC-3'
Bovis 220	5'-GAA ACA ATC GTG TTG CCC GTA GCA-3'
Bovis 221	5'-GCA ATC GTT ACA CAG CTG ATC CGA-3'
Bovis 222	5'-AAT TCA CTA CCG CCT GGA ATG GGT-3'
Bovis 281	5'-CCC CAA AAT GCA ACC GAT AT-3'
Bovis 282	5'-AAC TCT TCC TCC AAC TTT CCC-3'
NBS1	5’-GGA ATG GGN GGN GTN GGN AAR AC-3'
NBS2	5'-YCT AGT TGT RAY DAT DAY YYT RC-3’
Oligo-dT anchor primer	5'-GAC CAC GCG TAT CGA TGT CGA CTT TTT TTT TTT TTT TTV-3'
PCR anchor primer	5’-GAC CAC GCG TAT CGA TGT CGA C-3’
SP6	5’-TAT TTA GGT GAC ACT ATA G-3’
T7	5’-TAA TAC GAC TCA CTA TAG GG-3’
	Where **Y** = C / T, **H** = A, T / C, **R** = A / G, **N** = A, T, C / G, **V** = A, C / G, **D =** A, T / G

The individual cDNA fragments were re-amplified using the same primer-combination as used for the DDRT-PCR. Each reaction consisted of 1 µl recovered cDNA, 1× reaction buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 25 µM of the respective primers and 1 U *Taq* DNA-polymerase (Kapa Biosystems). The amplification regime was as follows, one cycle of 94°C for 2 min, 30 cycles at 94°C for 15 sec, 50°C for 30 sec and 72°C for 45 sec. A final cycle of 7 min at 72°C followed.

The PCR products were resolved on a 1% (w/v) agarose gel as described (3.2.1.1). The amplified fragments were cut from the gel and purified using the FavorPrep Gel/PCR purification kit (Favorgen Biotech) according to the manufacturer's specifications. The purified fragments were cloned into pGEM-T Easy (Promega), transferred to *E. coli* and the colonies plated on LB-plates containing amplicillin (3.2.1.2). Selection for recombinant plasmids was done using α-complementation (3.2.1.2).

White colonies were inoculated in 5 ml LB containing 50 $\mu g.ml^{-1}$ ampicillin and grown overnight at 37°C. Plasmid DNA was extracted using the Fast Plasmid mini kit (Eppendorf). The presence of an insert in each plasmid was confirmed with PCR by using primers homologous to the SP6 and T7 promoter regions of the plasmid (Table 4.1). The PCR reaction and amplification regime were as described (4.2.3). The bands were cut from the gel and purified as described above.

The amplified fragments were sequenced (3.2.1.4). The nucleotide sequences were translated (www.au.expasy.org/tools/dna.html) and homologous polypeptides for each of the amino acid sequences were identified using the BLAST tool (www.ncbi.nlm.nih.gov). To confirm the homology between the sequences, both were aligned with each other (www.ebi.ac.uk/clustalw). The NBS6 clone was also further analyzed (http://elm.eu.org).

4.2.4. 5'-RACE of DUF26.

The cloned NBS6 fragment was only a partial sequence. In order to amplify the full length gene, 5'-RACE was performed as previously described (3.2.1.1). First strand cDNA synthesis was done by using 2 µg total RNA and the gene specific primer Bovis 222 (Table 4.1) (Fig. 4.1a). The reaction was incubated at 55°C for 1 h followed by another 5 min at 85°C to denature the mRNA/cDNA hybrid (Fig. 4.1b). The synthesized cDNA was purified and the 3'-poly-A tail was added to the cDNA (Fig. 4.1c). The first PCR reaction was performed using 5 µl of the poly-adenylated cDNA as template with 10 µM Bovis 222 and the Oligo-dT anchor primer respectively (Table 4.1) (Fig. 4.1d). The amplification regime was as described in 3.2.1.1.

Of each PCR reaction, 5 µl DNA was separated on a 1% (w/v) agarose gel (3.2.1.1). The first nested PCR reaction was then performed using 5 µl of the first PCR reaction as template, with 25 µM of Bovis 221 and PCR anchor primer, respectively (Table 4.1) (Fig. 4.1e). A second nested PCR was performed with 5 µl of the first nested PCR as template, using 25 µM Bovis 220 and the PCR anchor primer as primers respectively (Fig. 4.1f). The PCR reactions were performed as described (3.2.1.1).

The amplified RACE fragments from the second nested PCR were purified with a PCR and Gelband purification Kit (Favorgen Biotech). The DNA fragments were then cloned into pGEM-T and reombinant plasmids selected as described (3.2.1.3).

The white colonies were transferred to four new LB-plates containing ampicillin. After culturing the colonies overnight at 37°C, they were transferred to a Hybond-XL nylon membrane (GE Healthcare) (Sambrook *et al.,* 1989). The colonies on the membranes were lysed and the plasmid DNA denatured by placing the membranes sequentially on filter papers saturated with 1% (w/v) SDS for 5 min, 3

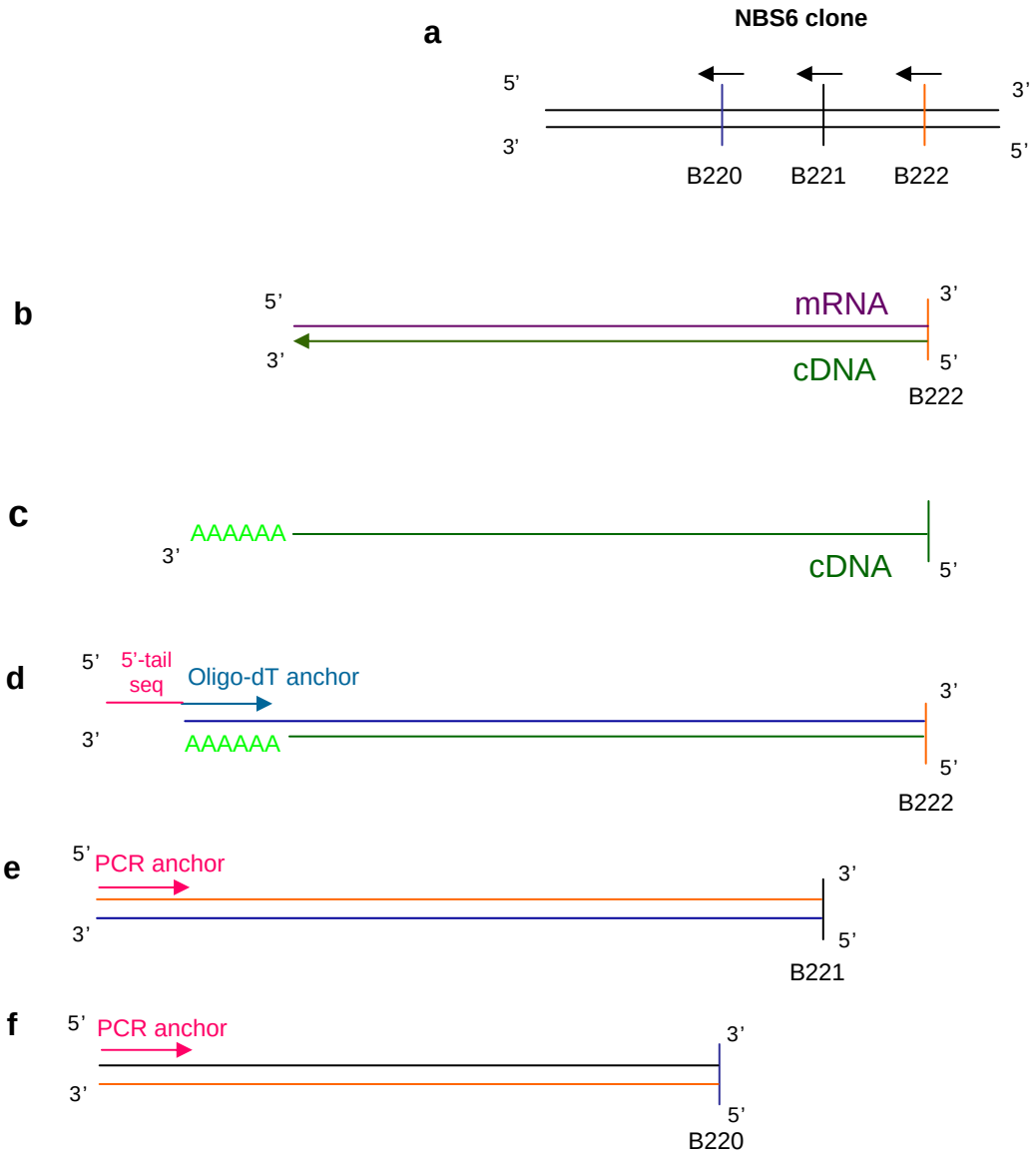

Figure 4.1. 5'-RACE scheme for clone DUF26. In a), clone DUF26 is indicated with the primers homologous to its sequence. In b), first strand cDNA synthesis with Bovis 222 is shown, in c), the poly-adenylated cDNA and in d), the first PCR reaction done with the oligo-dT primer and Bovis 222. In e), the nested PCR reaction done with the PCR anchor primer and Bovis 221 is shown, while in f), the second nested PCR reaction done with Bovis 220 and the PCR anchor primer is shown.

M NaCl, 0.4 M NaOH for 5 min and 0.2 M sodium phosphate buffer pH 6.8 for 5 min (Sambrook *et al.,* 1989). After drying the membranes at 70°C for 15 min, they were pre-hybridized in 20 ml hybridization solution [50% (v/v) formamide, 3 M NaCl, 0.2 M $NaH_2PO_4.H_2O$, 0.02 M EDTA, 0.5% (w/v) SDS, 1% (w/v) polyvinylpyrrolidone (PVP), 1% (w/v) ficoll, 1% (w/v) bovine serum albumine (BSA), 0.1 $\mu g.ml^{-1}$ salmon sperm DNA] for 30 min at 42°C.

The NBS6 cDNA fragment was labelled radio-active and added to the hybridization solution (3.2.1.3). After hybridization the membranes were washed and exposed to an Imaging-screen (Kodak) for 1 day. The inserts of recombinant plasmids showing the strongest hybridization signals were then sequenced (3.2.1.4).

4.2.5 Analysis of DUF26.

4.2.5.1 The presence of the DUF26 gene fragment in sunflower cultivars.

The presence of the *DUF26* gene in sunflower cultivars was tested by amplifying the gene fragment from genomic DNA extracted from infected and uninfected resistant and susceptible cultivars (3.2.4.1). Each reaction consisted of 10 ng genomic DNA, 10 µM of the gene specific primers Bovis 281 and 282 (Table 4.1) and 1× Kapa *Taq* Ready mix (Kapa Biosystems). The amplification regime was as follows, 94°C for 2 min and 30 cycles at 94°C for 15 sec, 60°C for 30 sec and 72°C for 45 sec. A final cycle of 72°C for 7 min followed. The fragments were separated on a 1% (w/v) agarose gel (3.2.1.1).

4.2.5.2 Analysing NBS6 expression in *P. helianthi* infected and chemically treated sunflower.

Resistant [GH99PHRR3 (VII) (R)] and susceptible [GH99S37-388RR (S)] sunflowers were cultivated and infected with the leaf rust fungus (4.2.1) and

harvested at six hour time intervals for 96 h. Another set of resistant sunflower cultivars were cultivated as described (4.2.1). After four weeks, five sets of plants were sprayed with 5 mM SA, 100 µM MeJA, 20 mM H_2O_2, 200 µM menadione and water respectively. Taking time 0 h as the time when the plants were sprayed, leaf samples were collected at 0 h, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h and 48 h.

Leaf samples for both sets of plants were quick frozen in liquid nitrogen, ground into a fine powder and RNA extracted as described (3.2.5.1). The mRNA capture kit (Roche) was used to first isolate mRNA from 5 µg total RNA according to the manufacturer's specifications. The RobusT II RT-PCR kit (Finnzymes) was used to determine the expression of the *DUF26* fragment. Each reaction contained 2 µl purified mRNA and 10 µM Bovis 281 and 282 respectively. The RT-PCR conditions were as described (3.2.5.3). As a control, *18S rRNA* was amplified using Bovis 26 and 27 (Table 4.1). The amplification regime was as described (3.2.5.2). The fragments were separated on a 1% (w/v) agarose gel (3.2.1.1).

4.3 Results

4.3.1 Isolation of putative NBS domain encoding genes from infected sunflower using DDRT-PCR.

DDRT-PCR was used to amplify and identify putative genes encoding proteins with an NBS-domain which are involved in the interaction between the leaf rust fungus and sunflower. The DDRT-PCR was performed with RNA isolated from infected resistant [GH99PHRR3 (VII) (R)] plants. A total of 30 putatively differentially expressed cDNAs fragments were isolated from the gel (Fig. 4.2a). The first primer combination (NBS1 and NBS2) led to the isolation of 23 cDNA fragments, while the second (Bovis 32 and 39) resulted in 7 isolated cDNAs. All of the fragments were visibly differentially regulated at different time-intervals from as early as 3 hpi.

After the fragments were purified from the gel, they were re-amplified using the same primer combination as was used for the DDRT-PCR. The second set however was re-amplified using only NBS1 and Bovis 39. The fragments were cloned into pGEM-T and the presence of the inserts confirmed with PCR using the SP6 and T7 primers (Fig. 4.2b). All the DDRT-PCR fragments were successfully re-amplified and cloned.

4.3.2 Sequence analysis of DDRT-PCR clones.

Sequence analysis of the cloned cDNAs indicated that the greater majority of the sequences were of insignificant value as they showed homology to ribosomal RNA, cloning vectors and bacterial proteins (Table 4.2). However, five of the cDNA clones were deemed to be of significant value. The first clone that was analysed was NBS5. This clone had a nucleotide sequence of 463 bp that was translated to a 146 amino acid sequence (Fig. 4.3). When the polypeptide was

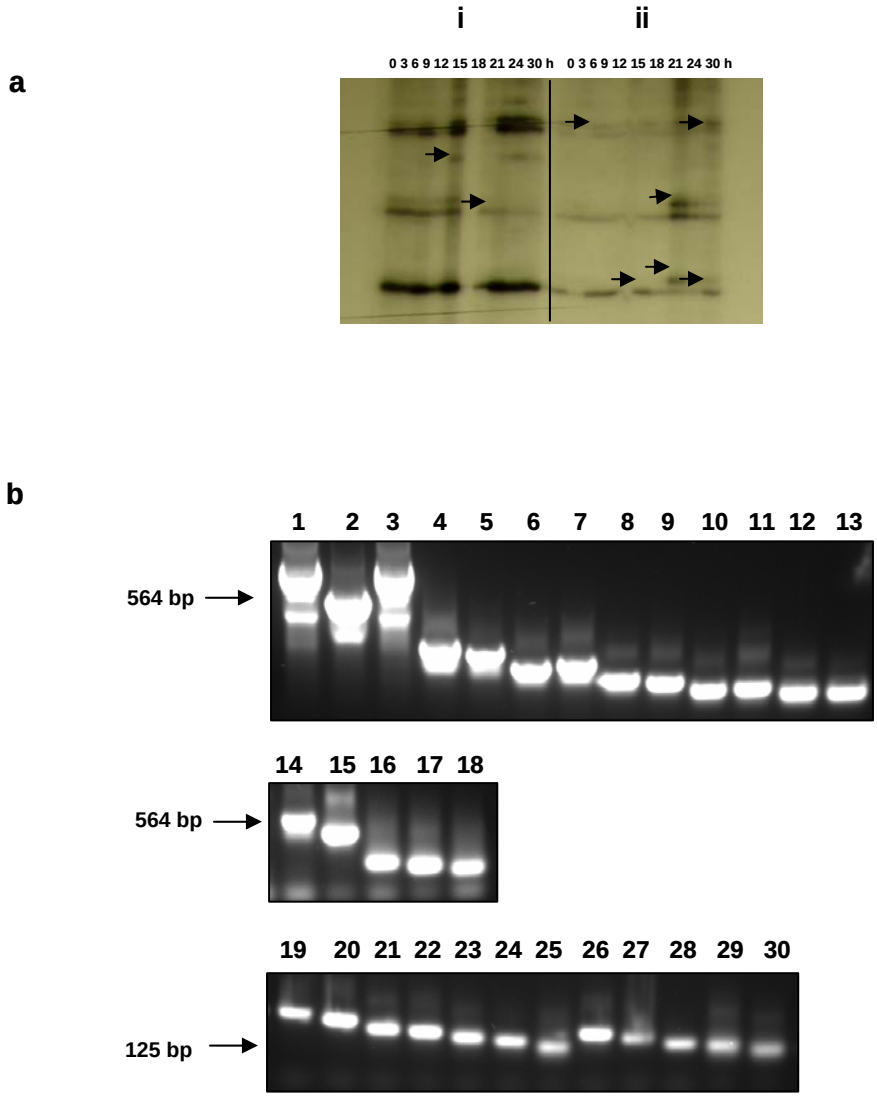

Figure 4.2. The amplification of cDNA fragments obtained with DDRT-PCR. In a) an example is shown of the DDRT-PCR fragments after separation on the gel. The differentially expressed bands are indicated by the arrows. In (i), the fragments amplified by the Bovis 32, 39 and NBS1 are indicated, and in (ii) fragments amplified with NBS1 and NBS2 are shown. In b), the re-amplified cDNA clones after extraction from the DDRT-PCR gel are shown. The fragment sizes are as indicated by the arrows.

Table 4.2. BLAST analysis of the DDRT-PCR clones on amino acid level.

Clone	BLAST homologies	Accession nr.	E-values
NBS1/7/8/11/14/16/18/19/34/37/31/20/22	16S ribosomal RNA		n/a
NBS10/12/13/15/17/24/25	Cloning vector		n/a
NBS3	Bacterial protein		n/a
NBS4	Mitochondrial cDNA		n/a
NBS5	ATPase with conserved domain (*Lactococcus lactis*)	YP-796551	$5e^{-75}$
NBS6	Hypothetical protein [containing DUF26 putative domain protein kinase (*Vitis vinifera*)]	CAN74851	$1e^{-23}$
NBS9	Cytochrome (*Beta vulgaris*)	ABD36062	$5e^{-33}$
NBS21	RNA polymerase promotor		n/a
NBS32	Glyceraldehyde-3-phosphate-dehydrogenase B (*Arabidopsis*)	AAD10210	$4e^{-66}$
NBS35/36	Hypothetical protein		n/a
NBS38	D1 protein of photosystem II (psbA) (*Prunus persica*)	AAL01428	$1e^{-17}$

a 5' **GGAATGGGGGGGGTGGGAAAAAC**TACAATGGCTTTCCAATTTGCAAAATATTTGCAAGA
AAATAATAAAAGTACATTATTAATTGATTTGGATTCACAAAAAAGTTTAACTGGTACAT
TTGAGACAAAAGATTTTAATTTTAAAGATAAACACAATATTTCAGAAATTCACAGCAAT
CCCCGAATTGGAATCATTGCTACTACGGTTGAAAAAAATATTGATGTAATTCCTTCAAC
TAGTAATTTAGAAGAAATCGCAGATAGCTTGGCAACTAAACCTAATAAAGAATTATTGT
TATTTATGTGGTTTGTAAAAAATTCAAAAGAACTAAATCAAAAATATGATTATATTATT
TTAGACTTACCGCCTGCATGGAATTTATTAACCAAAAATGGTGTGGCAGTTGCGGATAA
GGTAATATCACCAATGGAACCAAGTCGTCTTGCCCACCCCACCCATTCCA 3'

b

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

c

```
NBS5            ----------------TMAFQFAKYLQENNKSTLLIDLDSQKSLTGTFETKDFNFKDKHN 44
YP-796551       MQVISFMAIKGGVGKTTMAFQFAKYLQENNKSTLLIDLDSQKSLTGTFETKDFNFKDKHN 60
                            ****************************************

NBS5            ISEIHSNPRIGIIATTVEKNIDVIPSTSNLEEIADSLATKPNKELLLFMWFVKNSKELNQ 104
YP-796551       ISEILSNPQIGIIATTVEKNIDVIPSTSNLEEIADNLATKPNKELLLFMWFVKNSKELNQ 120
                **** ***:************************.********************** 

NBS5            KYDYIILDLPPAWNLLTKNGVAVADKVISPMEPSRLAHPTHS----------------- 146
YP-796551       KYDYIILDLPPAWNLLTKNGVAVADKVISPMEPSRFGYESHTKVLQSVSTLKNEVVDPVS 180
                **********************************:.: :*:

NBS5            ------------------------------------------------------------
YP-796551       GKSYVSAKIYFLGNRVKHNTNSSKEFLEALKNINDVIGIVPEKEAINTSMLLKKGIFDYL 240


NBS5            ----------------------------
YP-796551       EETGQTHTQNKFIASLKKVFNQIETKGIE 269
```

Figure 4.3. Sequence analysis of clone NBS5. In a), the nucleotide sequence of clone NBS5 is presented with the sequence of the NBS1 primer indicated in bold. In b), the amino acid sequence is presented. In c), the alignment of clone NBS5 with the ATPase polypeptide (Accession nr. YP-796551) is presented. The asterisks (*) indicate amino acids which are identical in all sequences, the (:) means that conserved substitutions have been observed while the (.) is indicative of semi-conserved substitutions.

used to search for homologous sequences using BLAST, it showed homology to an ATPase (Genbank Accession nr. YP-796551, e-value $5e^{-75}$) identified in *Lactococcus lactis*. To confirm the homology, the amino acid sequence of clone NBS5 was significantly aligned with an ATPase polypeptide (Fig. 4.3). ATPase generates the motive force for the transport of nutrients and the regulation of intracellular pH.

The 390 bp nucleotide sequence of the second clone, NBS6, was translated to a 122 amino acid sequence (Fig. 4.4). When used to search for homologous sequences, the polypeptide showed good homology to a hypothetical protein identified in *Vitis vinifera* that contained the domain of unknown function 26 (DUF26) (Genbank Accession nr. CAN74851, e-value $1e^{-23}$) (Table 4.2). To confirm the homology, the amino acid sequences of clone NBS6 and the hypothetical protein were aligned which showed good homology (Fig. 4.4c). When the NBS6 polypeptide was further analysed, several domains were found (Table 4.3). The first was a class IV WW domain which partially stretched from amino acids 55-63 and 113-118. The defining feature of a WW domain, from whence it derives its name, are two tryptophan residues (single letter code W). It functions in the complexity of interactions in linking cell signalling to the membrane cytoskeleton. Several phosphorylation sites such as the proline-directed kinase, CK1, FHA and GSK were also present on the amino acid sequence.

The third clone NBS9, had a nucleotide sequence of 296 bp and was translated to a 90 amino acid sequence. The polypeptide sequence showed highest homology to a *Cytochrome c* gene identified in *Beta vulgaris* (Genbank accession nr. ABD36062, e-value $5e^{-33}$) (Table 4.2) (Fig. 4.5).

Clone NBS32 had a nucleotide sequence of 679 bp and was translated to a 162 amino acid sequence (Fig. 4.6). The polypeptide was used to search for

a 5' **GGAATGGGGGGAGTTGGTAAAAC**GTTTTATGTGCATAGGGAGAACCCCCAAAATGCAACCGATATTGATCGGTTCAATGGTGCTTTGGG
GCCACTAATGAAGAAGTTGAGAAGTGATGCGGCTGCTGGCGGCCCCTTAAGGAAGTTTGCTACGGGCAACACGATTGTTTCAAACTCCA
CGACGATCTACGGGCTTGTGCAGTGTACTCCAGACTTATCGGATCAGCTGTGTAACGATTGCTTAGATGGTGCAATCAATCAAATTTCA
GTTTCGTTTAATGGGAAAGTTGGAGGAAGAGTTTATGCACCTATGTGTAATATTAGGTATGAGACATATTCTTTTACTTCAGCGAATGA
TCAGGTTTTACCGACGCCACCCATTCCAGGCGGT 3'

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

c

```
NBS6            ------------------------------------------------------------
CAN74851        MEVPSTPPIVPLVASSPPSIEATLVDEELVHIANDDQQGQKTYRGRGRGRRRGXGAHGGL 60

NBS6            ------------------------------------------------------------
CAN74851        HVSPIKPIVEHTHHRXPLRKRKAXSCXSLLIDERMMISASIFFRRKRPKATTVNGKLSSR 120

NBS6            ------------------------------------------------------------
CAN74851        DIQKAAHLVVNCRAWWSILEVMYLLPLSFLFPSKSLTESQAEKRMESPRLLFFLYPILIL 180

NBS6            ------------------------------------------------------------
CAN74851        HLLAVTNAQPRFLSYWCSNGVGNYTNNSTYKANLNTLLTSLSSNNEIDYGFYNFSAGQNS 240

NBS6            ------------------------------------------------------------
CAN74851        DKVNAIALCRGDVMPTACRSCINDSRIQLTQLCPNQKEAIGWYDNCMLRYSNDSIFGTQQ 300

NBS6            --FYVHRENPQNATDIDRFNGALGPLMKKLRSDAAAGGPLRKFATGNTIVSNSTTIYGLV 58
CAN74851        SSPYFYMRNSKNASDVEEFNQVLGNMMASLRSKAASGDWRRKFATGEANVTSFQSIYGLM 360
                   *.: .*.:**:*::.** .** :* .***.**:*.  ******:: *:.  :****:

NBS6            QCTPDLSDQLCNDCLDGAINQISVSFNGKVGGRVYAPMCNIRYETY---SFTSAN----- 110
CAN74851        QCTPDLSELSCSNCLEGATNEIPTCCDSRKGGRVVKPSCNLRYETYRFYDFTAANAPPPS 420
                *******:  *.:**:** *:*... :.: ****  * **:*****   .**:**

NBS6            --DQVLPTPPIPGG---------------------------------------------- 122
CAN74851        PPSADLSPPPLANTTSTQETRSNSSRTIILIIVPSVIILIIILVGFICFFSRKRSSMEKL 480
                  .  *..**:..

NBS6            ------------------------------------------------------------
CAN74851        ETHDEDEITNVESLHFDFDTIRVATNNFSDSNKLGQGGFGPVYKGKLSNGQYVAVKRLSS 540

NBS6            ------------------------------------------------------------
CAN74851        GSAQGELEFKNEAVLVAKLQHRNLVRLLGFCLDGAERLLIYEFVPNTSLDHFIFDLIRRA 600

NBS6            ------------------------------------------------------------
CAN74851        QLDWERRYKIIGGIARGLLYLHEDSRLRIIHRDLKASNILLDAEMNPKISDFGMARLFLV 660

NBS6            ------------------------------------------------------------
CAN74851        DQTQGSTSRIVGTYGYMAPEYAMHGHFSVKTDVYSFGVLVLELVSGQRNNCFRVSENIEH 720

NBS6            ------------------------------------------------------------
CAN74851        LLSYAWKNWREGTATNLIDPTMRISSISEIMRCIHIGLLCVQENEADRPTMASIALMLNS 780

NBS6            ------------------------------------------------------------
CAN74851        YSLSLPVPSHPAFFMNTSMNRDMSLELEDNSRVAQSNYLPSRSSHFSVNEASITDPYPR 839
```

Figure 4.4. The sequence analysis of clone NBS6. In a), the nucleotide sequence of clone NBS6 is presented with the sequence of the NBS1 primer indicated in bold. In b), the amino acid sequence is presented. In c), the alignment of clone NBS6 with a hypothetical protein containing a DUF26 domain (Accession nr. CAN74851) is presented. The asterisks (*) indicate amino acids which are identical in all sequences, the (:) means that conserved substitutions have been observed while the (.) is indicative of semi-conserved substitutions.

Table 4.3. Conserved domains present in the NBS6 polypeptide.

Domain	Description	Position
DUF26 domain	Unknown function	55 - 123
Class IV WW domain	Phosphorylation dependent interaction	58 - 63, 113 - 118
Proline-directed kinase	Phosphorylation site	58 - 64
Casein Kinase 1	CK1 phosphorylation site	49 - 55, 105 - 111
FHA phosphopeptide ligands	Motifs for binding phosphothreonine modules in FHA domains	59 - 65, 114 - 120
GSK3 phosphorylation site	Site recognised by GSK3 for Ser/Thr Phosphorylation	46 - 53, 100 - 107

a 5' **TGGAATGGAAGGGGTGGGGAAAAC**CTATGTGGGGCACCTTTTGGGTGTGGGATGCTCGT
TTAACCTCTGTATTAATCTTGGTTTTTATTTACATGGGTGCACTGGGTTTTCAAAAGCT
TTCTATCGAACTGGCTCCTATTTTAATCTGTGTTGGACTGATCGATATACCCATAATCA
AGTTTTCAGTCAACTGGTGGAATACATTGCATCAACCTGGGAGCATTAGCCGATATGGT
ACATCAATACATGTTCCTATGCCCATTCCAATCTTGTCTTCCCTACGCCTCCCATTCCA
A 3'

b

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

c

```
NBS9            ------------------------------------------------------------
ABD36062        MSKTRNYAQILIGSWLFLTAMAIYLSLWVAPLDFQQGGNSRILYVHVPVAWMSILVYIVT 60

NBS9            -------------------------------------------MWGTFWVWDARLTSVLI 17
ABD36062        AINTFLFLLTKHPLFLRSSGTGIEMGAFFTLFTLVTGGFWGRPMWGTFWVWDARLTSVFI 120
                                                          ***************:*

NBS9            LVFIYMGALGFQKLSIELAPILICVGLIDIPIIKFSVNWWNTLHQPGSISRYGTSIHVPM 77
ABD36062        LLFIYLGALCFQKLSVELASILICVGLIDIPIIKFSVNWWNTLHQPGSISRFGTSIHVSM 180
                *:***:*** *****:***.*****************************:******.*

NBS9            PIPILSSLR-----------------LPFQ----------------- 90
ABD36062        LIPILSNFANFLFLTCLLFVIEIRLLILSFLESSLTEEIEAREGSLRA 228
                 *****.:                  *.*
```

Figure 4.5. The sequence analysis of clone NBS9. In a), the nucleotide sequence of clone NBS9 is presented with the sequence of the NBS1 primer indicated in bold. In b), the amino acid sequence is presented and in c), the alignment of clone NBS9 with Cytochrome c (Accession nr. ABD36062). The asterisks (*) indicate amino acids which are identical in all sequences, the (:) means that conserved substitutions have been observed while the (.) is indicative of semi-conserved substitutions.

a 5' GAAGAATTCTCGAGCGGCGGCATTGAACATAGTCCCAACGAGTACGGGTGCAGCCAAGG
CTGTGTCTTTGGTCCTGCCACAACTTAAGGGCAAGCTCAACGGCATAGCACTTCGTGTT
CCCACCCCTAATGTCTCGGTTGTTGACCTTGTGGTCAATGTTGCTAAGAAGGGACTAAC
GGCTGAAGATGTAAATGCTGCTTTCAGGAAAGCCGCCGACGGGCCATTGAATGGAATCT
TGTCGGTTTGTGACGAGCCACTTGTGTCAGTTGACTTTAGGTGCTCCGATGTTTCGTCC
ACTATTGACTCGTCGTTGACTATGGTCATGGGTGACGATATGGTTAAGGTTGTTGCTTG
TCAACGTGTGGTTGATCTGGCACATTTGGTGGCGGGCAAATGGCCAGGTGTTGCACCAG
TAGGAAGCGGAGATCCGTTGGAGGACTTTTGCAAGAAAAATCCCGGTGAAGAAGAGTGC
AAAGTTTACGAATTTTAATTATATCCTTTGTTAATACTATCTTCTTCTTCTTCTCT
TCGAGCTTTCTATAAAATGTCATAGTTATATATTATGTCTAAACTGTGTAAAGACT**TGA**
CACACGATA**TAG**ACATTTTTCACATCTATATATATCTTTAACTTTTATGTT**AAAAAAAA**
AAAAAAAAGCGCCCGCTCGAGAATTCTTCA 3'

b

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

c

```
NBS32           ------------------------------------------------------------
AAD10210        MSSIGGEASFFDAVAAQIIPKAVTTSTPVRGETVAKLKVAINGFGRIGRNFLRCWHGRKD 60

NBS32           ------------------------------------------------------------
AAD10210        SPLEVVVLNDSGGVKNASHLLKYDSMLGTFKAEVKIVDNETISVDGKLIKVVSNRDPLKL 120

NBS32           ------------------------------------------------------------
AAD10210        PWAELGIDIVIEGTGVFVDGPGAGKHIQAGASKVIITAPAKGADIPTYVMGVNEQDYGHD 180

NBS32           ----------------------------------------------------KNSRAAA 7
AAD10210        VANIISNASCTTNCLAPFAKVLDEEFGIVKGTMTTTHSYTGDQRLLDASHRDLRRARAAA 240
                                                                   :.:****

NBS32           LNIVPTSTGAAKAVSLVLPQLKGKLNGIALRVPTPNVSVVDLVVNVAKKGLTAEDVNAAF 67
AAD10210        LNIVPTSTGAAKAVSLVLPQLKGKLNGIALRVPTPNVSVVDLVINVEKKGLTAEDVNEAF 300
                ****************************************:** ********** **

NBS32           RKAADGPLNGILSVCDEPLVSVDFRCSDVSSTIDSSLTMVMGDDMVKVVAC--------Q 119
AAD10210        RKAANGPMKGILDVCDAPLVSVDFRCSDVSTTIDSSLTMVMGDDMVKVVAWYDNEWGYSQ 360
                ****:**::***.*** *************:******************        *

NBS32           RVVDLAHLVAGKWPGVAPVGSGDPLEDFCKKNPGEEECKVYEFS 163
AAD10210        RVVDLAHLVASKWPGAEAVGSGDPLEDFCKTNPADEECKVYD-- 402
                **********.****. .************.**.:******:
```

Figure 4.6. The sequence analysis of clone NBS32. In a), the nucleotide sequence of clone NBS32 is presented. Two stop codons are indicated in bold black and a poly-A tail in bold blue. In b), the amino acid sequence is shown. In c), the alignment of clone NBS32 with the glyceraldehyde 3-phosphate dehydrogenase B (Accession nr. AAD10210) is presented. The asterisks (*) indicate amino acids which are identical in all sequences, the (:) means that conserved substitutions have been observed while the (.) is indicative of semi-conserved substitutions.

homologous sequences and it showed homology to a glyceraldehyde 3-phosphate dehydrogenase B (GADPH) subunit (*Arabidopsis thaliana*) (Genbank Accession nr. AAD10210, e-value $4e^{-66}$). When aligned, the homology was clearly evident (Fig. 4.6c).

The last clone which was of significant value was NBS38 (Fig. 4.7). It had a 160 bp nucleotide sequence which was translated to 53 amino acids. It showed homology to the D1 protein of photosystem II (*psbA*) (Genbank Accession nr. AAL01428, e-value $1e^{-17}$) (Table 4.2). Within these last two clones, the NBS1 primer sequence was not observed.

4.3.3 Analysis of the putative NBS6 kinase.

The putative DUF26 protein kinase has a domain of unknown function which stretches from amino acid 55 – 123 (Table 4.4). It was decided to further analyse the clone in an attempt to gain information on its composition and function.

In an attempt to amplify and clone the full length gene, 5'-RACE was performed (Fig. 4.1). After adding the poly-A tail, a PCR reaction was performed using the first PCR reaction as template with Bovis 221 and the PCR anchor primer using a gradient temperature ranging from 56 – 60°C. When separated on a gel, a slight smear was visible in the background with a defined band 750 bp in size (Fig. 4.8a). When the second nested PCR was performed with Bovis 220 and the PCR anchor primer, a slightly smaller fragment was visible (Fig 4.8b). However the visibility of a clear band was compromised by the overloading of the sample. It was decided to clone the amplified PCR products of the second nested PCR reaction amplified at 58°C into pGEM-T vector.

Colonies containing recombinant plasmids were probed with the radio-labelled NBS6 fragment (Fig. 4.9). Strong hybridization signals were evident for some colonies, with others showing weak or no hybridization at all. The strong signals indicated clones which could be sequenced in the attempt to obtain the full length

a 5' GAAGAATTCTCGAGCGGCTCCCTATTTAGTGCTATGCATGGTTCCTTGGTAACCTCTAG
TTTGATCAGGGAAACCACAGAAAATGAATCTGCTAATGAAGGTTACAGATTCGGTCAAG
AGGAAGAAACTTATAATATCGTAGCCGCTCGAGAATTCTTCA 3'

b E E F S S G S L F S A M H G S L V T S S L I R E T T E N E S
A N E G Y R F G Q E E E T Y N I V A A R E F F

c

```
NBS38           ------------------------------------------------------------
AAL01428        MTAILERRESESLWGRFCNWITSTENRLYIGWFGVLMIPTLLTATSVFIMAFIAAPPVDI 60

NBS38           ------------------------------------------------------------
AAL01428        DGIREPVSGSLLYGNNMSSRAIIPTFAAIGLHFYPIWEAASVDEWLYNGGPYELIVLQFL 120

NBS38           ---------------------------------------------EEFSS---------- 5
AAL01428        LGVAWYMGRELELSFRLGMRPWIAVAYSAPVAAATAVFLIYPIGQASFSDGMPLGISGTI 180
                                                             .**.

NBS38           --------------------------GSLFSAMHGSLVTSSLIRETTENESANEGYRFG 38
AAL01428        NFMIVFQTDHNILMHPFHMLGVAGVFGGSLFSAMHGSLVTSSLIRETTENESANEGYRFG 240
                                         ********************************

NBS38           QEEETYNIVAAREFF---------------------------------------------- 53
AAL01428        QEEETYNIVAAHGYFGRLIFQYASFNNSRSLHFFLAAWPVVGIWFTALGISTMAFNLNGF 300
                ***********: :*

NBS38           ------------------------------------------------
AAL01428        NFNHPVVHTHSSVINTWADKVNRANLGMEVMHERNAHNFPLDLAAVEAPSTNG 353
```

Figure 4.7. The sequence analysis of clone NBS38. In a), the nucleotide sequence of clone NBS38 is presented and in b), the amino acid sequence is shown. In c), the alignment of clone NBS38 with the D1 protein of photosystem II (Accession nr. AAL01428) is presented. The asterisks (*) indicate amino acids which are identical in all sequences, the (:) means that conserved substitutions have been observed while the (.) is indicative of semi-conserved substitutions.

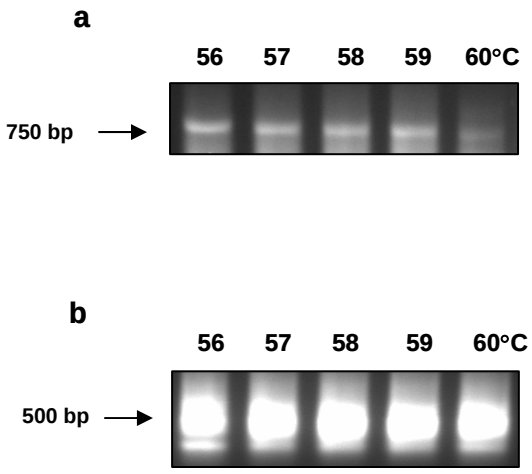

Figure 4.8. Gradient PCR amplification of the 5'-RACE NBS6 clone. In a), the first nested PCR reaction was performed by using Bovis 221 and the PCR anchor primer. In b), the second nested PCR reaction was performed with Bovis 220 and the PCR anchor primer. The annealing temperatures and fragment size are as indicated.

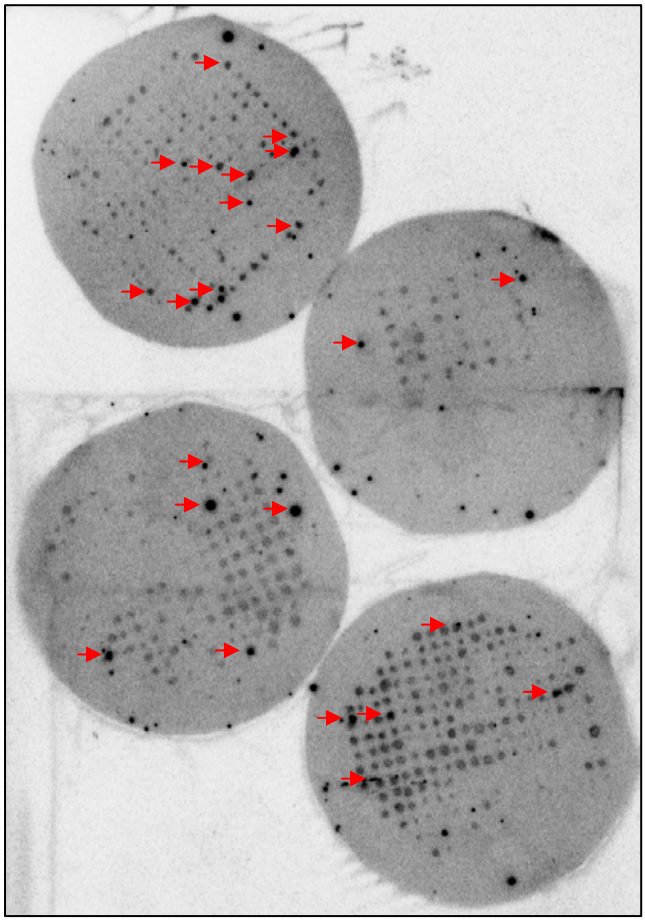

Figure 4.9. Screening of *E. coli* colonies containing recombinant plasmids for the presence of the 5'-RACE NBS6 fragment. The 23 colonies that were selected for sequencing are indicated by the arrows.

gene (Fig. 4.9). A total of 23 colonies were selected of which 16 were sequenced using the SP6 and T7 primers respectively.

Of the 16 clones which were sequenced only five gave usefull sequence information as the rest could not be successfully sequenced despite several attempts. Three of the 5'-RACE clones (1, 2 and 23), were identical (Fig. 4.10a). Present within the clone's sequences were the reverse primer Bovis 220 which was used in the second nested PCR. Upon analysing the clones 4 and 15, it showed significant homology to each other except for a 180 bp deletion in clone 15 (Fig. 4.10b). In both clones 4 and 15 the PCR anchor primer sequence and a poly-T tail were present. Based on the different primer sequences present in all the cloned sequences, an idea could be formed on how these clones could be aligned to obtain the full length RACE fragment for NBS6 (Fig. 4.10c). Contig sequences were assembled for the first three clones (1, 2 and 23) and for clones 4 and 15. But when the contig sequences were used for a BLAST search, it showed no homology to any DUF26 region nor to the hypothetical protein (Fig. 4.4). The first contig had homology to a chloroplast genome sequence from *H. annuus* (Accession nr. DQ383815, e-value 0). The second contig also showed homology to a chloroplast genome sequence isolated from *Guizotia abyssinica* (Accession nr. EU549769, e-value 0). Thus the assumption was made that the 5'-RACE amplification of NBS6 was unsuccessful. A search was done to obtain ESTs with possible homology to the NBS6 sequence with the intention to build a contig for the gene, but no similarities were found.

4.3.3.1 Determining the presence of NBS6 in sunflower.

The origin of the NBS6 gene fragment from the sunflower genome was tested by using PCR. The gene fragment was amplified from genomic DNA from both the resistant and susceptible cultivars which were both infected (IR, IS) and uninfected (CR, CS) with the leaf rust fungus. The gene fragment was amplified with gene specific primers Bovis 281 and 282. After the reactions were separated

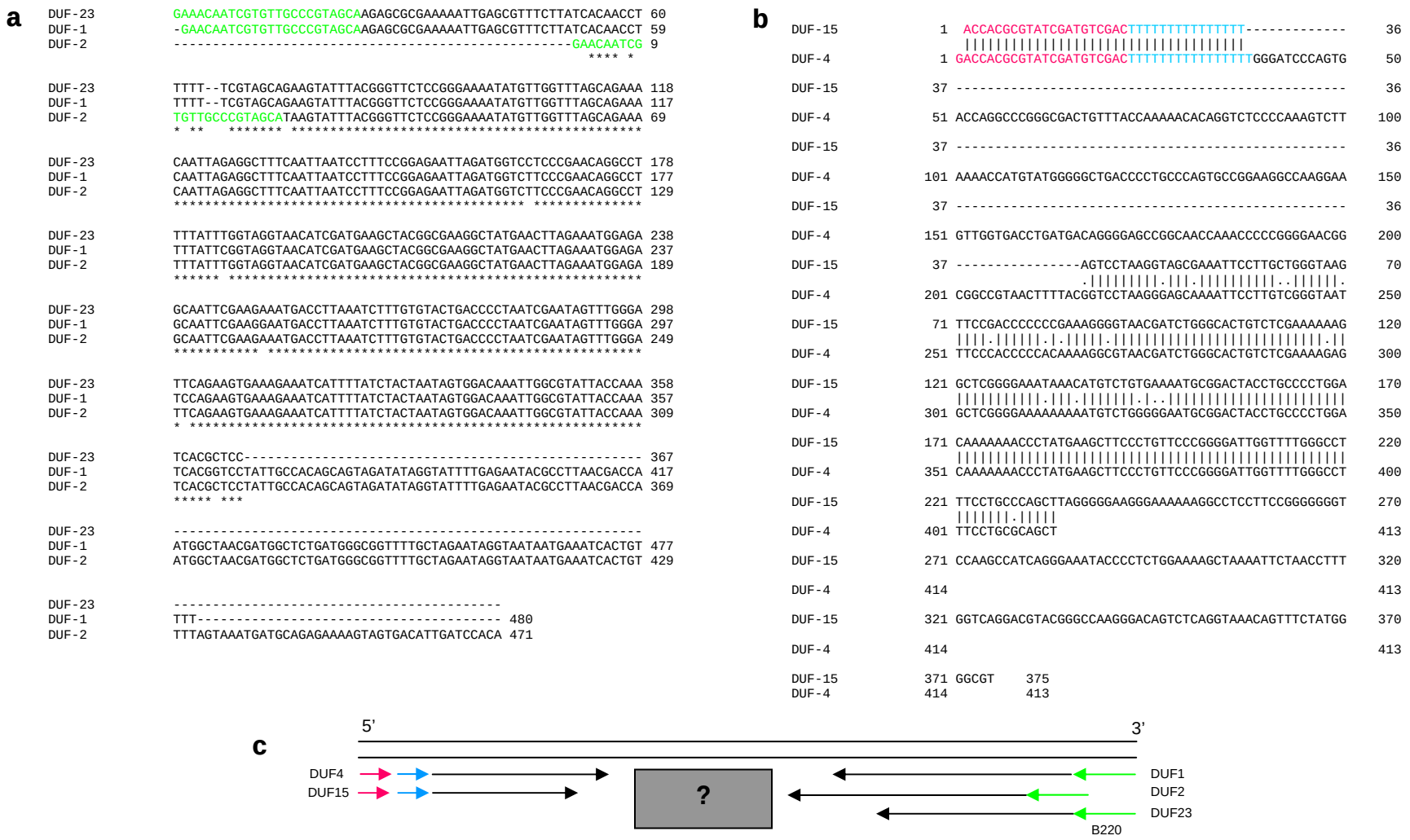

Figure 4.10. The alignment of 5'-RACE fragments for clone NBS6. In a), RACE clones 1, 2 and 23 were aligned. The reverse primer Bovis 220 is indicated in green. In b), the alignment for clones 4 and 15 is presented. The PCR anchor primer is indicated in pink while the oligo-dT attached by where the terminal transferase is indicated in blue. In c), the overlapping regions of the clones are aligned in an attempt to combine the RACE sequences to obtain the full length gene for the NBS6 clone. The ? represents an unknown region.

on an agarose gel, each lane contained a single amplified fragment of the expected 255 bp size, indicating that the amplified DUF26 fragment was of sunflower origin and not from the pathogen (Fig. 4.11a).

4.3.3.2 NBS6 expression in *P. helianthi* infected sunflower.

The expression of NBS6 in sunflower cultivars following infection with the leaf rust fungus was tested using RT-PCR (Fig. 4.12). To ensure that the amount of RNA used for all the RT-PCR reactions was equal, the *18S rRNA* gene was first amplified from all the samples. From the amplified profiles, it was clear that equal amounts of mRNA were indeed used since all time intervals produced a band with equal intensity.

The expression of NBS6 showed a low level of induction in the infected resistant cultivar at 12 and 48 hpi with lower expression levels at the other time intervals (Fig. 4.12a). Gene expression in the infected susceptible plants was also at a constant level with an up-regulation of expression which occurred at 36 and 45 hpi (Fig. 4.12c). Expression in both CR and CS plants was at a relative constant level except at 24 – 30 hpi in the CR plants where a small increase was seen (Fig. 4.12b and d). The basal expression levels in the CS plants were however higher than the rest.

4.3.3.3 NBS6 expression in chemically treated sunflower.

Resistant [(GH99PHRR3 (VII) R] sunflower seedlings were treated with different defence related compounds. A sharp induction of gene expression was seen 30 min after treatment with menadione (Fig. 4.13a) with a lower secondary induction at 24 h. A similar expression profile was seen in the MeJA (Fig. 4.13b) and H_2O_2 treated plants (Fig. 4.13d). The expression profiles did however differ regarding the time-intervals of induction. The SA treated plants did show an increase of expression at 4 h with a subsequent decrease. As a control, plants treated with distilled water showed constitutive expression levels (Fig. 4.13e).

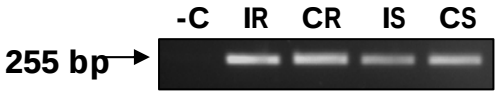

Figure 4.11. PCR analysis of NBS6 to determine the origin of the gene. The amplification of NBS6 in the IR, CR, IS and CS plants is shown. The negative control and fragment size are as indicated.

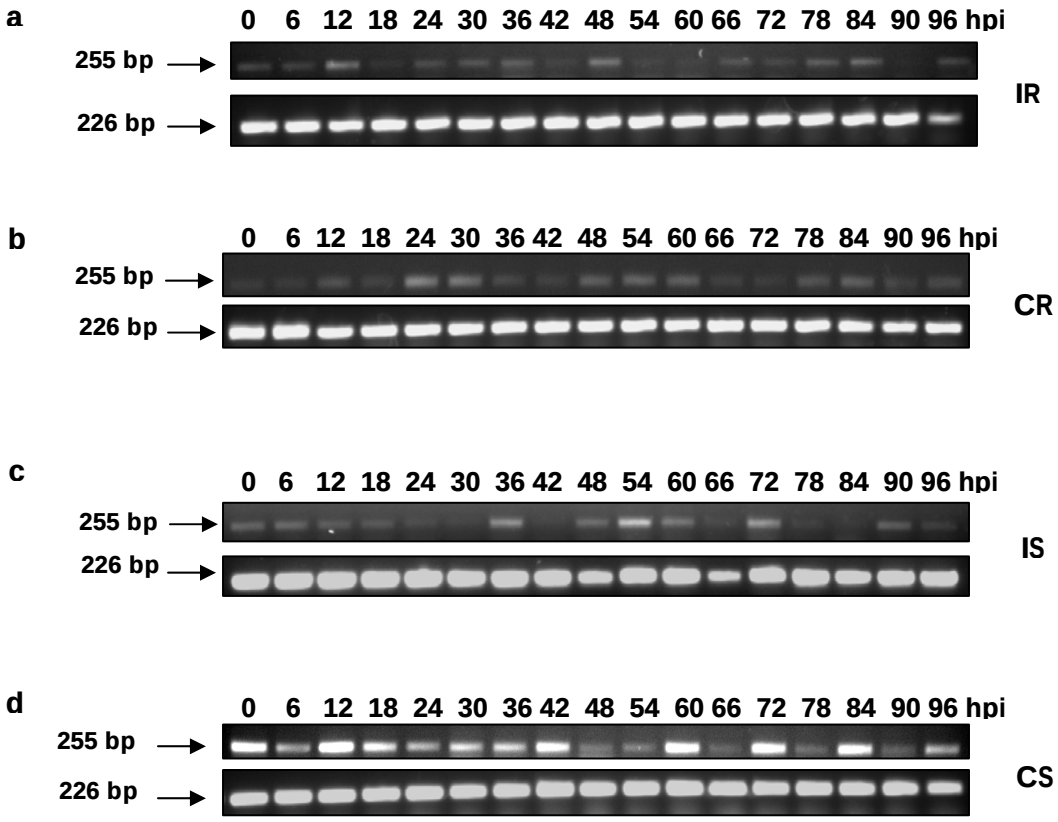

Figure 4.12. Expression analysis of NBS6 and the control *18S rRNA* gene in sunflower. Indicated is the expression in a) the IR plants, b) the CR plants, c) the IS plants and d) the CS plants is shown. The sizes of the fragments are as indicated.

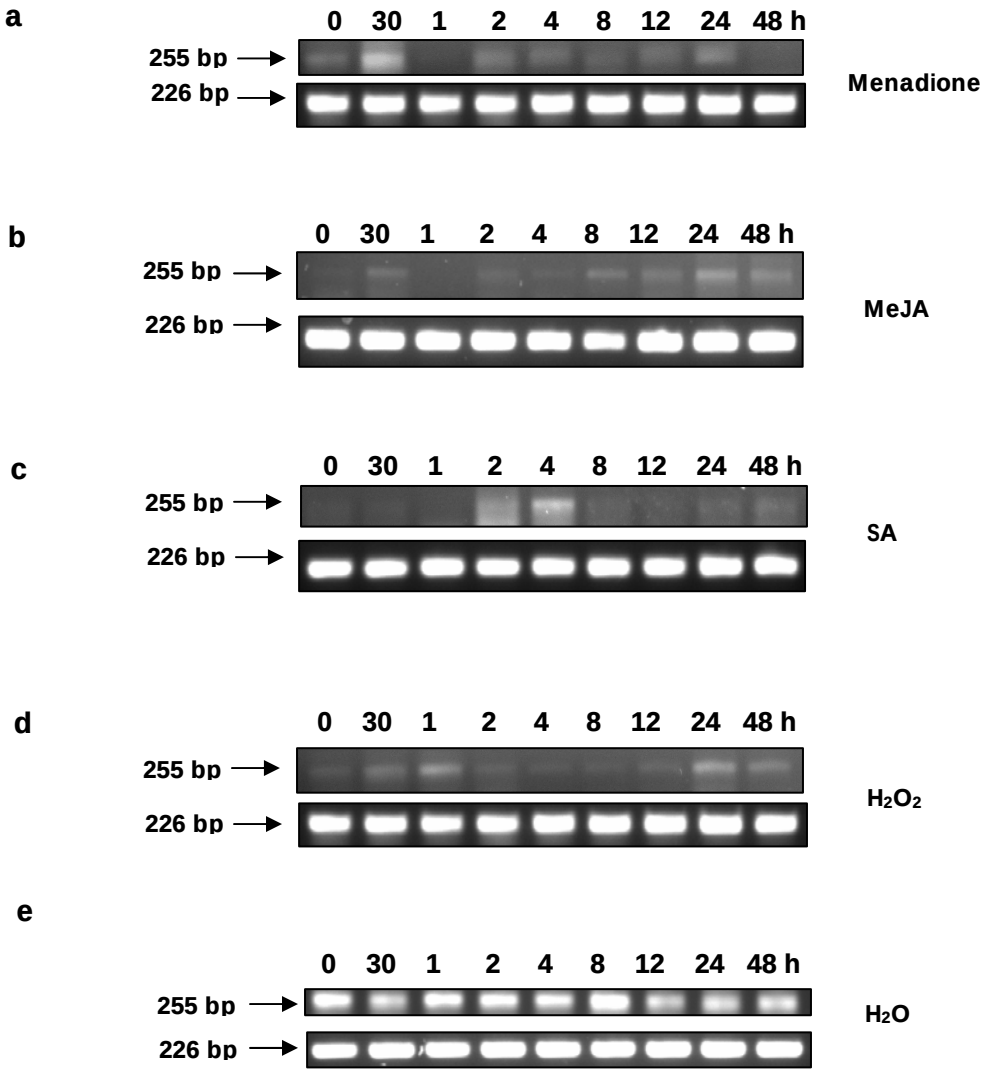

Figure 4.13. Expression analysis of *DUF26* in chemical treated resistant sunflower. In a) menadione, b) MeJA, c) SA, d) H_2O_2 and in d) H_2O treated plants. The sizes of the fragments are indicated with an arrow. As control *18S rRNA* gene is shown.

4.4 Discussion.

During a plant's defence response to invading pathogens, certain defence signalling mechanisms are required that will enable the plant to successfully contain the pathogen. Taking part in these signalling systems are resistance genes of which most contain a NBS domain and a LRR region (Belkhadir *et al.,* 2004). The aim of this study was to amplify genes which encode proteins that contain a NBS domain from sunflower plants which were infected with the leaf rust fungus.

In order to amplify such genes, a DDRT-PCR approach was followed using degenerate primers that were specific for the NBS domain. The successful cloning of several disease resistance gene homologues from wheat lines infested with *Diuraphis noxia* using these primers was previously reported (Lacock *et al.,* 2003). A greater number of resistance gene analogs were isolated from this study compared to other randomised studies. Kruger *et al.* (2002) on the other hand obtained ESTs of miscellaneous nature from wheat infected with *Fusarium graminearum* using these same primers. Included were sequences related to general plant metabolism and cell structure.

In this study, very little significant genes were however amplified and cloned (Table 4.2). This might be explained by the fact that the primers used for the amplification were originally designed for monocotyledonous plants (Lacock *et al.,* 2003) and was therefore possibly not specific enough to amplify similar genes from sunflower. Degenerate primers designed specifically for dicotyledonous plants as well as a greater number of amplified clones could result in a more successful attempt to isolate genes containing NBS-domains from sunflower.

Five of the isolated clones were deemed to be significant. Three (NBS 9, 32 and 38) showed homology to genes involved in plant photosynthetic systems (Table 4.2). The NBS9 clone showed homology to a *Cytochrome C* gene which encodes

a protein that transfers electrons between the Cytochrome b_6f complex and photosystem I of photosynthetic systems (Katoh and Takamiya, 1961). The NBS32 clone was homologous to a glyceraldehyde-3-phosphate dehydrogenase gene. This encoded polypeptide is involved in phosphate sugar metabolism (Frigge *et al.,* 1999). The third clone NBS38 was homologous to the D1 protein of photosystem II which is encoded by the *psbA* gene (Fig. 4.7). These proteins provide a protein backbone for the photosystem II reaction centre to which several co-factors attaches (Mattoo *et al.,* 1984). Since the identification of these genes have implicated photosynthesis to be influenced by the plant-pathogen interaction (Scharte *et al.,* 2005), the effect of the leaf rust pathogen on photosynthesis in sunflower would be analysed in chapter 5.

A fourth clone showed homology to an ATPase gene (Table 4.2). This enzyme plays a role in the plant plasmamembrane where it generates the driving force for the transport of nutrients and the regulation of intracellular pH (Serrano, 1989). The H^+-ATPase is an electron-pump which couples ATP hydrolysis to proton transport out of the cell. The electrochemical gradient is then used by secondary transporters to transport ions and metabolites across the membrane (Palmgren and Harper, 1999).

The fifth clone showed homology to a hypothetical protein kinase containing a DUF26 domain (Table 4.2). Several RLKs from *Arabidopsis* contain two copies of the DUF26 domain which consists of a C-X8-C-X2-C motif repeat in the extracellular domains of membrane bound kinases and secretory proteins (Bateman *et al.,* 2002). The DUF26 domain is 55 amino acids long and contain four conserved cysteine residues. The C-X8-C-X2-C repeat is however a novel motif distinct from the cysteine-rich sequences present in the extracellular domains from other RLKs such as the S-domain class and CRINKLY4 (Chen, 2001).

Analysis of the NBS6 clone on amino acid level indicated the presence of several phosphorylation regulated motifs (Table 4.3). The first two motifs, the class IV

WW domain and the proline-directed protein kinase site, play key roles to mediate the interaction of proteins containing these motifs with their cognate ligands. The latter site is targeted by proline-directed protein kinases such as cyclin-dependent protein kinases (CDKs) and MAPKs which phosphorylates the serine/threonine residues that precede the key proline amino acid (Lu *et al.,* 2002). Upon phosphorylation the WW domain is then able to bind to a variety of different proline containing motifs present on ligand proteins (Macias *et al.,* 1996). These interactions depend on a conserved tryptophane amino acid in the WW domain. Different amino acid side chains within the WW domain further play key roles in the varied specificity of the different interactions (Li *et al.,* 1999).

The other phosphorylation sites present on clone NBS6 was CK1, GSK3 and FHA. As showed in chapter 3, these sites are all again implicated to be involved in protein-protein interactions. Combined, the presence of a DUF26 domain and several phosphorylation dependent protein-protein interaction motifs within NBS6 indicates the involvement of the encoded protein in possible signal transduction events.

An unsuccessful attempt was made to clone the 5'-end of the *NBS6* gene using 5'-RACE. Despite promising results leading up to the sequencing of individual clones, the obtained sequences did not match the original sequence of clone *NBS6*. In addition, the contigs that were generated did not show any relationship towards each other. This clearly indicated that the attempt to use 5'-RACE to clone the full length gene was unsuccessful. There might be several possible causes for this. The most important reason could have been false priming that occurred during the RACE process. This was most probably due to the primers not being specific enough.

Again an attempt was made to obtain the full length gene using ESTs (Chapter 3), but BLAST analysis yielded no fragments that shared significant homology to NBS6. Therefore even at this stage the identity of the full length gene remains inconclusive.

As sequence analysis implicates the DUF26 kinase to be potentially involved in signal transduction events, the expression thereof was further analysed in the interaction between sunflower and the leaf rust fungus (Fig. 4.12). It was shown that the gene was initially isolated from the sunflower genome and not from the fungal genome. This was assumed, as the gene was amplified from both the resistant and susceptible cultivars whether it was infected with the leaf rust fungus or not (Fig. 4.11).

In order to confirm the involvement of the encoded polypeptide in the plant-pathogen interaction, the expression profiles thereof were determined in resistant and susceptible plants that were both infected and uninfected with *P. helianthi* (Fig. 4.12). Although the expression levels were very low, an induction of gene expression was seen in both the IR and IS plants. However, induced gene expression was seen at a much earlier time interval in the IR plants than in the IS plants. Gene expression in the CR plants was induced at a later stage than in the IR plants and constitutively in the CS plants. Several RLKs containing the DUF26 domain have been implicated to be involved in the resistance response (Ohtake *et al.,* 2000). This might be an indication of the involvement of NBS6 in the general defence response of sunflower.

To further implicate a role for clone NBS6 in the activated defence response pathways, resistant sunflower was treated with defence related compounds such as menadione and SA (Fig. 4.13). The expression profiles implicated this gene to be involved in a response where oxidative stress and SA respectively plays a role. The transcription levels of the C-X8-C-X2-C containing motif, *RKC1* homologue, were shown to accumulate at high levels upon SA treatment (Ohtake *et al.,* 2000). Similarly, the C-X8-C-X2-C motif is present within the *AtRLK3* gene identified in *Arabidopsis* (Czernic *et al.,* 1999). *AtRLK3* was highly expressed upon oxidative stress caused by the treatment with menadione and SA. ROS and SA are signalling molecules that play a key role in the plant's defence response. SA is instrumental in the activation of *PR*-gene expression (Klessig *et al.,* 2000)

and reactive oxygen species such as O^{2-} and H_2O_2 can cause cellular damage as well as leads to the induction of cellular protectant genes (Hu *et al.,* 2003).

Since DUF containing RLKs have been implicated to play a role during oxidative stress conditions (Ohtake *et al.,* 2000), it is therefore safe to speculate that *NBS6* could be similarly involved. This however has to be proven by first cloning the full length gene and then analyse the encoded protein.

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Chapter Five:

The effect of pathogen infection on photosynthetic capacity of sunflower.

5.1 Introduction.

Plants are constantly exposed to fluctuations in the environment as well as by pathogens. Plants have therefore evolved defence mechanisms to combat these changes. These defence responses or mechanisms are however very energy demanding. An effective photosynthesis capacity is thus required to keep the carbohydrate and energy availability in the cells at normal levels in order for the resistance response to be effective (Koch, 1996).

The process of photosynthesis enables a plant to generate carbohydrates, ATP and O_2 by using water and CO_2 in the presence of light energy (Mauseth, 1995). The conversion of light energy takes place in a two part process which is both light-dependent and light-independent. The light dependant reactions consist of photosystem II (PSII) and photosystem I (PSI) that are located in the thylakoid membranes of the chloroplasts. In PSII, a water molecule is split and the free electron excites the P680 electron donor. The excited electron is then transferred via several electron carriers such as phaeophytin plastoquinone (PQ) and plastocyanin (PC) to PSI where it is activated a second time by light with a wavelength of P700. The excited electrons are then accepted by the electron-end acceptor $NADP^+$ which is reduced to $NADPH_2$. The rate at which these electrons are transferred occur at a constant rate under normal photosynthetic conditions.

The second step of photosynthesis is the light-independent reactions (Mauseth, 1995). These reactions take place in the chloroplast stroma. Carbon-fixation takes place during the Calvin-Benson cycle. The carbon from CO_2 becomes attached to ribulose 1,5-biphosphate (RuBP) to from an unstable six-carbon intermediate. This is followed by a chain of reactions where the energy from the excited electrons in the form of ATP and NADPH in the presence of certain enzymes is used to produce sugar phosphate molecules. The enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) catalyses the formation of 3-phosphoglycerate via CO_2 fixation. Subsequent reactions that include a series of

other enzymes such as glyceraldehyde-3-phosphate-dehydrogenase use the ATP and NADPH to form six carbon-sugars (Mauseth, 1995).

The process of photosynthesis is affected when pathogens infect a plant (Scholes and Rolfe, 1996). Biotrophic pathogens need living tissue for growth and reproduction thereby disturbing the normal metabolism of the plant. A decrease of photosynthetic activity has been shown in incompatible plant-pathogen interactions (Swarbrick *et al.,* 2006). The reaction of *Arabidopsis* to both a virulent and avirulent strain of *P. syringae* showed that the major difference in photosynthesis during the two different interactions were the tempo of the responses. By measuring chlorophyll fluorescence, a decrease in photosynthesis was detected earlier in the compatible compared to incompatible interaction. In both cases this was accompanied by an increase of cell wall invertase activity and alterations of the sugar content in the cell. This enzyme is extracellular and it cleaves sucrose in the apoplast into glucose and fructose. The resulting hexoses are transported into the cell (Roitsch *et al.,* 2003). Higher invertase activity indicates that the effects of the pathogen on the primary and secondary metabolism were the same in both cases. But the reaction was earlier in the incompatible interactions. This was due to the resistant plants being able to recognise the pathogen via an elicitor-based signal where the susceptible plants depended on metabolic (sugar) signal (Swarbrick *et al.,* 2006). Furthermore, in an incompatible interaction between tobacco and *Phytophthora nicotianae,* chlorophyll fluorescence imaging showed that photosynthesis is a highly localized process that occurs in single mesophyll cells (Scharte *et al.,* 2005). Thus, it was proposed that plants decrease its photosynthetic capacity and other assimilatory metabolism upon pathogen invasion in order to initiate respiration and other processes needed for defence (Scharte *et al.,* 2005).

There are several reasons why the plant metabolism alters when it is invaded by a pathogen. Pathogens withdraw nutrients from the plant for its own need. Sugars are not only nutrients for the plant, but to the pathogen as well. Such evidence was found upon the infection of tobacco with *P. nicotianae* and wheat

with *Puccinia graminis*. Elevated levels of soluble sugars were noticed in the apoplast (Wright *et al.,* 1995; Herbers *et al.,* 2000). The status of the carbohydrate level directly affects the development of disease resistance. Sugars not only act as nutrient for the plant and the invading pathogen, but might also regulate signal transduction and gene regulation (Koch, 1996). The down regulation of photosynthetic genes has been attributed to the accumulation of hexose sugars (Pego *et al.,* 2000). The elevation of carbohydrate production seen in pathogen-invaded plants is therefore very cost-intensive for the plant as the demand for assimilates increases (Swarbrick *et al.,* 2006).

The reduction of photosynthesis has been shown to be a fast process whereas the down-regulation of photosynthetic gene expression is a slower process (Sinha *et al.,* 2002). Using photoautotrophic cell cultures of tomato and treating it with fungal elicitors resulted in a rapid decrease in photosynthesis, later followed by the down-regulation of photosynthetic gene expression (Sinha *et al.,* 2002).

The effect of invading pathogens on the photosynthetic systems can be monitored by *in vivo* chlorophyll a fluorescence. This non-invasive method is based on measuring the fluorescence of chlorophyll *a* in a dark adapted plant when exposed to saturating light pulses (Schreiber *et al.,* 1986). It is a very sensitive marker for the efficiency of photosynthesis since the energy absorbed from photons can either be used for electron transport or the energy will be dissipated as heat or chlorophyll fluorescence (Van Kooten and Snel, 1990). Thus, chlorophyll fluorescence is an indication of any changes in energy conversion taking place in the PSII reaction centres. It can also show any impairment that the photosystems might have in the second phase of the complex process of photosynthesis (Govindjee, 2004).

The aim of this study was to determine the effect that *P. helianthi* have on sunflower when providing resistance to the pathogen by using chlorophyll a fluorescence. Since photosynthetic related genes such as *psbA* and *glyceraldehyde-3-phosphate dehydrogenase* were obtained using DDRT-PCR

(Chapter 4), an attempt would be made to determine whether an impaired photosynthetic capacity due to the invading pathogen will have a negative influence on the resistance response of sunflower to the leaf rust pathogen.

5.2 Materials and Methods.

5.2.1 Cultivation and infection of sunflower plants.

Seed of the resistant [GH99PHRR3 (VII) (R)] and susceptible [GH99S37-388RR (S)] sunflower cultivars were planted in a 2:1 soil mixture of peat moss and potting soil in seedling trays and germinated at 22 – 26°C in a greenhouse at normal day/night cycles. The plants were fertilized once a week with 3 $g.l^{-1}$ Multifeed fertilizer.

When the sunflower seedlings were four weeks old it was inoculated with spores of the UVPhe2 strain of leaf rust (*Puccinia helianthi*). Rust spores were resuspended in kerosene oil containing a drop of Tween 20 to a final concentration of approximately 150 000 $spores.ml^{-1}$ and sprayed under high pressure onto the dorsal and ventral sides of the leaves. The control plants were sprayed with kerosene oil containing a drop of Tween 20. The plants were left to dry for 30 min at room temperature and were then placed at 22 - 24°C in a dark Dew-simulation-chamber for 16 h to allow the rust to germinate. The plants were then moved to the greenhouse. Taking time 0 when the plants were placed in the Dew-simulation-chamber, leaves were randomly harvested every six hours until 96 hpi. The leaves were quick-frozen in liquid nitrogen, ground to a fine powder and stored at -80°C.

5.2.2 Measuring chlorophyll fluorescence.

Chlorophyll a fluorescence of the infected and uninfected plants of both the resistant and susceptible cultivars was measured using a FMS-2 fluorometer from Hansatech. Taking time 0 h as the time just before infection, readings were taken every 24 h for 7 days after infection until visible signs of infection were seen on the leaves. Three readings were taken per time-interval for each of the resistant and susceptible plants which were both infected and uninfected. The

leaves were dark adapted for 5 min using the provided clips before each reading was taken. The measurement protocol was as follows: the minimal fluorescence of a dark-adapted sample (F_o) was first taken followed by a single saturating light flash (0.7 sec) that was applied after which the maximum fluorescence of a dark-adapted sample (F_m) was taken. The actinic light source was turned on 20 sec after at 70 µmol photons.m^{-2}.s^{-1} followed by a series of saturating flashes where the minimum steady state fluorescence (F_s) and the maximum steady state fluorescence ($F_{m'}$) were measured. From these data, the maximum quantum yield (F_v/F_m), the quantum yield of PSII (ϕPSII), the portion of open PSII (*q*P) reaction centres and non-photochemical quenching (NPQ) were calculated using the following equations:

$Fv/Fm = (Fm-Fo) / Fm$
$\Phi PSII = (Fm'-Fs) / Fm'$
$qP = (Fm'-Fs) / (Fm'-Fo')$
$NPQ = (Fm-Fm') / Fm'$

5.2.3 Total RNA extraction from sunflower plants.

All the solutions and apparatus needed for RNA extraction were prepared and treated as described (3.2.5.1). Total RNA was extracted from sunflower tissue and the concentration determined (3.2.5.1).

5.2.4 RT-PCR analysis of gene expression.

Gene fragments showing high homology to genes involved in the photosynthetic processes were obtained by using DDRT-PCR (Chapter 4). In order to test gene expression, specific primers were designed for *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*), *psbA* and three additional genes involved in the resistance response and photosynthesis namely *glutathione S-transferase* (*GST*)

and the large (*RbcL*) and small (*RbcS*) subunits *Rubisco* (Table 5.1). In order to test the expression of these genes, mRNA was first captured from 5 µg total RNA isolated for each time interval using the mRNA capture kit (Roche) according to the manufacturer's specifications. The Robust II RT-PCR kit (Finnzymes) was then used to determine the expression of the respective genes. Each reaction contained 2 µl purified mRNA, 10 µM of the respective primers (Table 5.1), 0.2 µM dNTPs, 1× reaction buffer, 2.5 mM $MgCl_2$ and 0.4 µl of the enzyme mix.

The amplification regime for all the reactions was one cycle at 48°C for 30 min and 94°C for 2 min. Thirty cycles of 94°C for 15 sec, X°C for 30 sec and 68°C for 45 sec followed. This was followed by a final elongation step at 68°C for 5 min. The annealing temperatures (X) for each gene were as indicated (Table 5.1). As a control, the *18S rRNA* gene was amplified using Bovis 26 and 27 (Table 5.1). The conditions were as described (3.2.5.2).

Table 5.1. Nucleotide sequences of the primers used in this study.

Primer name	Primer sequence	Gene name	Annealing temp.
Bovis 26	5'-CAA CTT TCG ATG GTA GGA TAG-3'		
Bovis 27	5'-CTC GTT AAG GGA TTT AGA TTG-3'	*18S rRNA*	50°C
Bovis 265	5'-TTT CTG ATG GTA TGC CTC TAG GA-3'		
Bovis 266	5'-GCA GCT AAG TCT AGA GGG AAA TT-3'	*psbA*	63°C
Bovis 269	5'-ATG GCG AAT GAG GTG AAG TT-3'		
Bovis 270	5'-TAC GCC TTA CCA AAC CTT GA-3'	*GST*	56°C
Bovis 271	5'-GGG AGG TAT TTA TGT CAC CAC A-3'		
Bovis 272	5'-GTT TTT AGC GGA TAA CCC CA-3'	*RbcL*	56°C
Bovis 273	5'-TGG CTT CGA TCT CCT CCT CA-3'		
Bovis 274	5'-CCT GGA AGC AAT GAA CAT GA-3'	*RbcS*	63°C
Bovis 283	5'- ATA GTC CCA ACG AGT ACG GGT-3'		
Bovis 284	5'-CCA TAG TCA ACG ACG AGT CAA-3'	*GAPDH*	56°C

5.3 Results

5.3.1 Sunflower cultivation and infection with leaf rust.

Resistant [GH99PHRR3 (VII) (R)] and susceptible [GH99S37-388RR (S)] sunflower cultivars were cultivated and infected with the leaf rust fungus. Seven days after infection, the infected susceptible plants showed severe signs of infection with rust pustules covering the ventral and dorsal sides of the leaves (Fig. 5.1b). In contrast to the susceptible plants, the resistant plants showed no signs of rust pustules being present (Fig. 5.1a). Instead, the presence of yellow chlorotic flecks was an indication of a resistance response to the leaf rust pathogen infection.

5.3.2 Chlorophyll fluorescence analysis.

As stated before chlorophyll a fluorescence was measured in order to determine the effect of *P. helianthi* infection on the sunflowers photosynthetic capability. The first parameter determined was the maximum quantum yield (F_v/F_m) for the plants. These values reflect the maximum quantum efficiency of PSII and are used as a sensitive indicator of plant photosynthetic performance. When the F_v/F_m values were analysed, it was found that the photosynthetic capability of the IR plants increased until 48 hpi and then gradually decreased for the rest of the trial (Fig. 5.2a). In comparison, the CR plants showed a slight increase in photosynthetic capability over the time period. In contrast, the susceptible plants showed a general increase in the maximum photosynthetic capability for both the infected and uninfected plants (Fig. 5.2a). In both cases however, infection of sunflower led to lower F_v/F_m values, indicating that the presence of the pathogen decrease the maximum quantum efficiency of sunflower. From the figures it was also quite clear that the levels in the resistant plants fluctuated a lot, while the susceptible plants showed more constant trends.

a

b

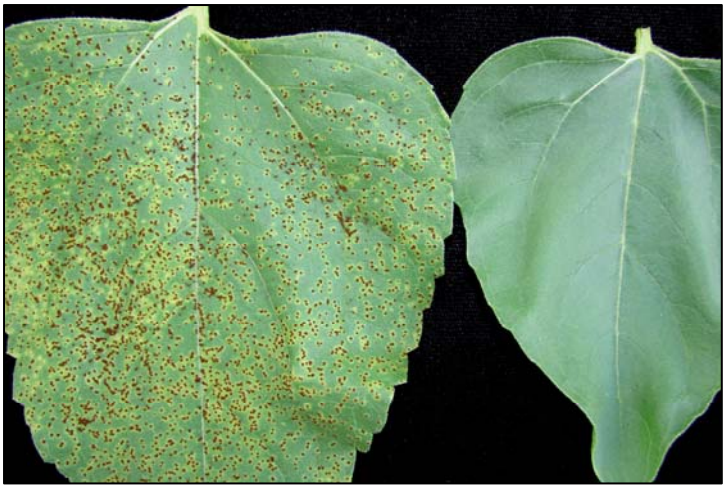
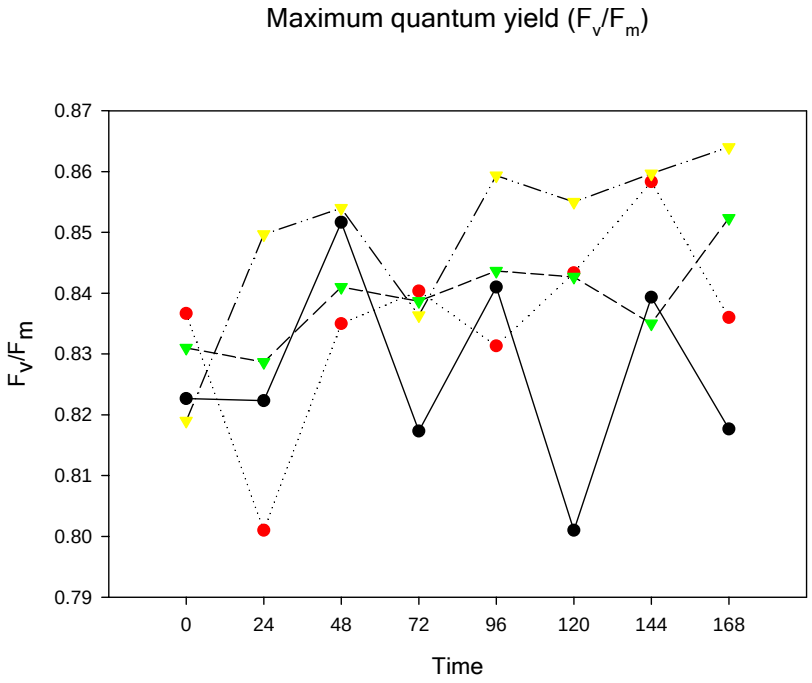


Figure 5.1. Resistant and susceptible sunflower cultivars infected with *P. helianthi*. In a), leaves of the infected resistant [GH99PHRR3 (VII) (R)] cultivar (on the left) and uninfected resistant cultivar are presented. In b), leaves of the infected susceptible [GH99S37-388RR (S)] cultivar (on the left) and uninfected susceptible cultivar are shown.

a



b

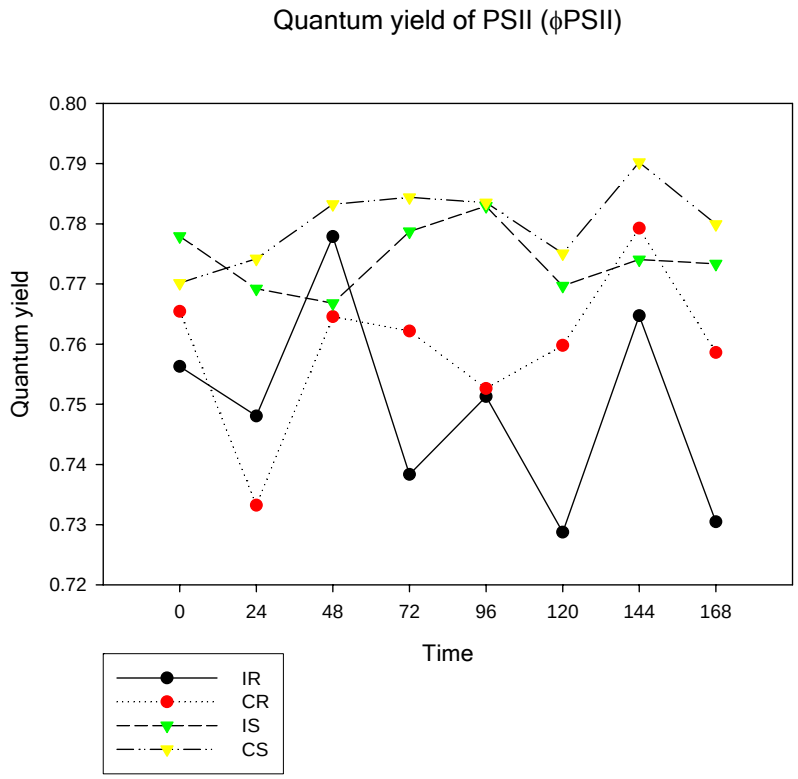


Figure 5.2. The analysis of chlorophyll a fluorescence (F_v/F_m and ϕPSII) in *P. helianthi* infected and uninfected sunflower cultivars. In a), the maximum quantum yield (F_v/F_m) is shown and in b), the quantum yield of PSII (ϕPSII). The different cultivars and treatments are as indicated. Note that the scale of the Y-axis is such that the variations occur much more pronounced than what they are.

The second parameter used regarding the fluorescence analysis was to calculate the quantum yield of PSII (ϕPSII). This parameter measures the efficiency whereby the absorbed light by chlorophyll associated with PSII is channelled in photochemistry. This provides information of the rate of electron transport which is also an indication of overall photosynthesis efficiency. Data analysis indicated that the resistant plants had an overall lower quantum efficiency than the susceptible plants for both infected and uninfected plants (Fig. 5.2b). The fluctuations within the resistant plants were again much higher than in the susceptible plants. While the levels for the CS, IS and IR plants either remained unchanged or increase slightly, ϕPSII for the IR plants showed a decreasing trend. This indicated that the IR plants were struggling with the infection load.

The third parameter, namely *q*P gives an indication of the proportion of PSII reaction centres that are open. A change in *q*P is due to the closure of the reaction centres which is the result of saturation of photosynthesis by light. When data were analysed regarding the portion open for PSII a gradual decrease for all the plants was observed (Fig. 5.3a). It was however clear that the infected plants had lower values than the uninfected plants for both the resistant and susceptible cultivars, with the IR plants again having the lowest values of all the cultivars and showing the biggest decline.

The last parameter analysed was the non-photochemical quenching (NPQ). The parameters measuring NPQ and *q*P are inverse proportional as NPQ gives an indication of the excess heat being dissipated while *q*P indicates the amount of light energy used by functional PSII reaction centres. When the data were analysed, it was found that both IR and CR plants showed a similar pattern for NPQ that reached a peak at 72 hpi (Fig. 5.3b). Over the entire time trial, levels were however constant in the susceptible plants, both IS and CS showed a significant increase in heat dissipation when the values of time 0 h were compared to the values of 168 hpi. A difference was seen between the IR and IS plants as the IR plants dissipated more heat earlier on compared to the IS plants, but during the latter stages the IR released less heat.

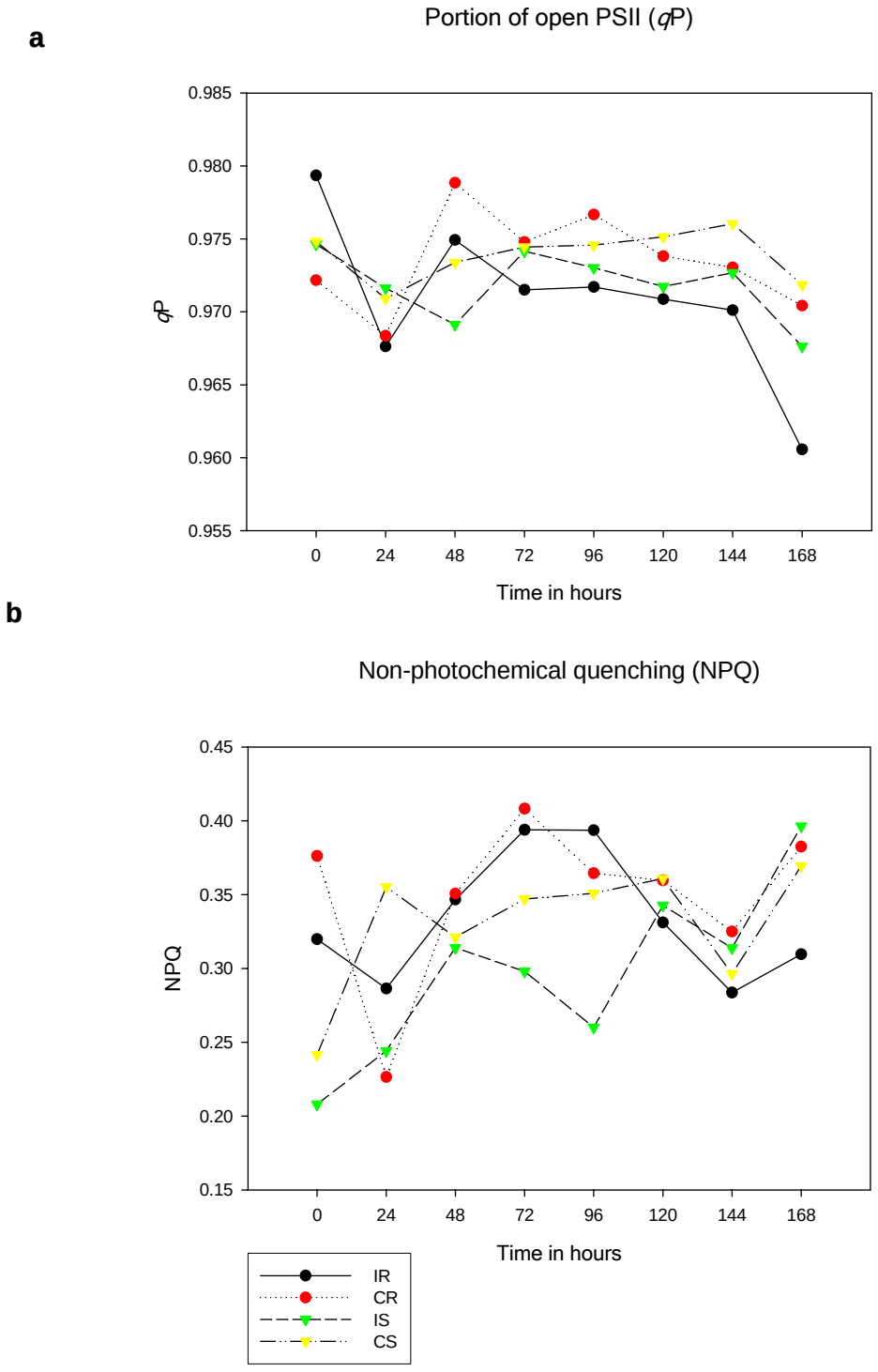


Figure 5.3. The analysis of chlorophyll a fluorescence (*q*P and NPQ) in *P. helianthi* infected and uninfected sunflower cultivars. In a), the portion of open PSII reaction centres (*q*P) is indicated and in b), the non-photochemical quenching (NPQ) of the plants. The different cultivars and treatments are as indicated. Note the scale of the Y-axis.

5.3.3. Photosynthesis related gene expression in *P. helianthi* infected sunflower.

In order to further analyse and determine the effect of infection on the photosynthetic system of the plant, the expression of some genes which take part in the photosynthetic system, was analysed. The expression of the *GAPDH* gene showed constitutive expression in the IR plants, except for a down-regulation early in the time trial from 6-24 hpi (Fig. 5.4a). A transient increase was found at 18 hpi that was at the same level of 0 hpi. A similar pattern of constitutive expression during the later stages of the time trial was shown in the IS plants, but the earlier down-regulation of the gene was over a longer time period (Fig. 5.5a). Even though the expression of *GADPH* is indicated as being lower at 0 hpi in the IS plants, it has to be kept in mind that the *18S rRNA* gene expression of time 0 hpi was at a much lower level compared to the rest. This implies that the 0 hpi expression of all the genes in the IS plants were actually higher as indicated in the photos.

Expression of the *RbcL* gene was constitutive in the IR plants with a down-regulation of the gene at 72 hpi (Fig. 5.4b). Gene expression in the IS plants was also constitutive, but with a slight down-regulation during the early time intervals when keeping the lower *18S rRNA* levels in mind (Fig. 5.5b). The *RbcS* gene was also down-regulated in the IR plants from 6 hpi followed by a strong up-regulation at 60 hpi (Fig. 5.4c). Low levels of *RbcS* gene expression were shown in the IS plants with a slight up-regulation at 54 hpi (Fig. 5.5c). The low gene expression of *18S rRNA* at 0 hpi indicates a high level of *RbcS* at the same time interval (Fig. 5.5f).

Expression of the *psbA* gene was more or less at constitutive levels throughout the trials in both IR and IS plants (Fig. 5.4d and 5.5d). A possibility does exist that smaller variations in gene expression could be missed due to the near saturation of the RT-PCRs. This would be best solved using qRT-PCR.

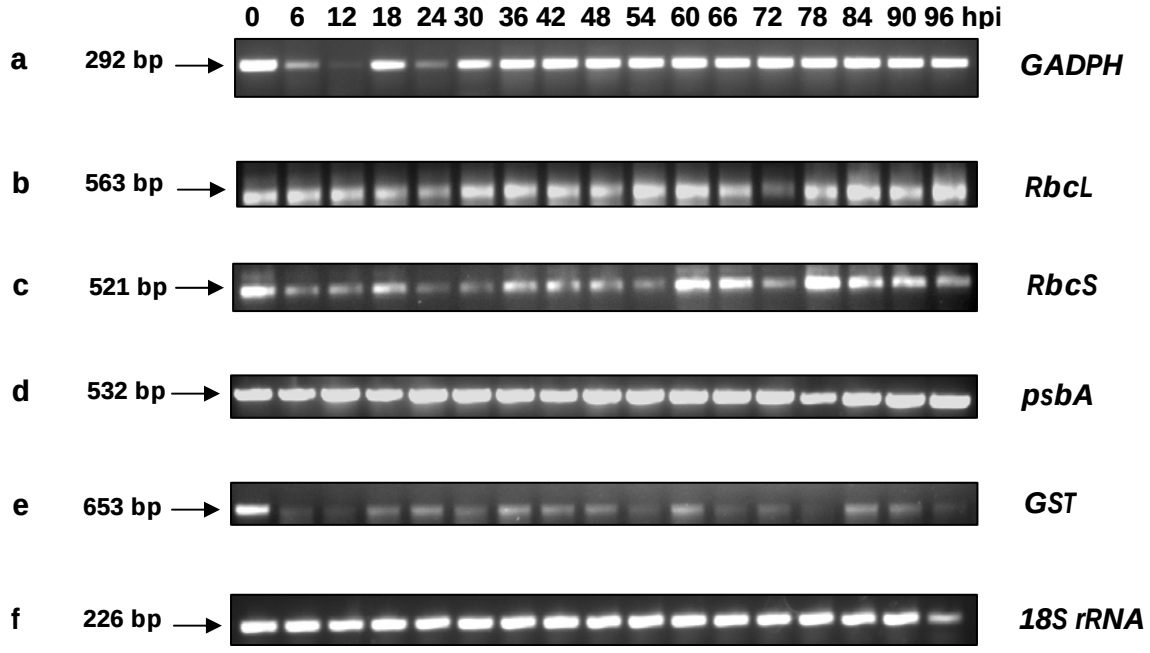


Figure 5.4. Expression analysis of photosynthesis related genes in the IR plants. Gene expression is shown in a) for *GADPH*, b) *RbcL*, c) *RbcS*, d) *psbA*, e) *GST* and f) *18S rRNA*. The fragment sizes and time intervals are indicated by arrows.

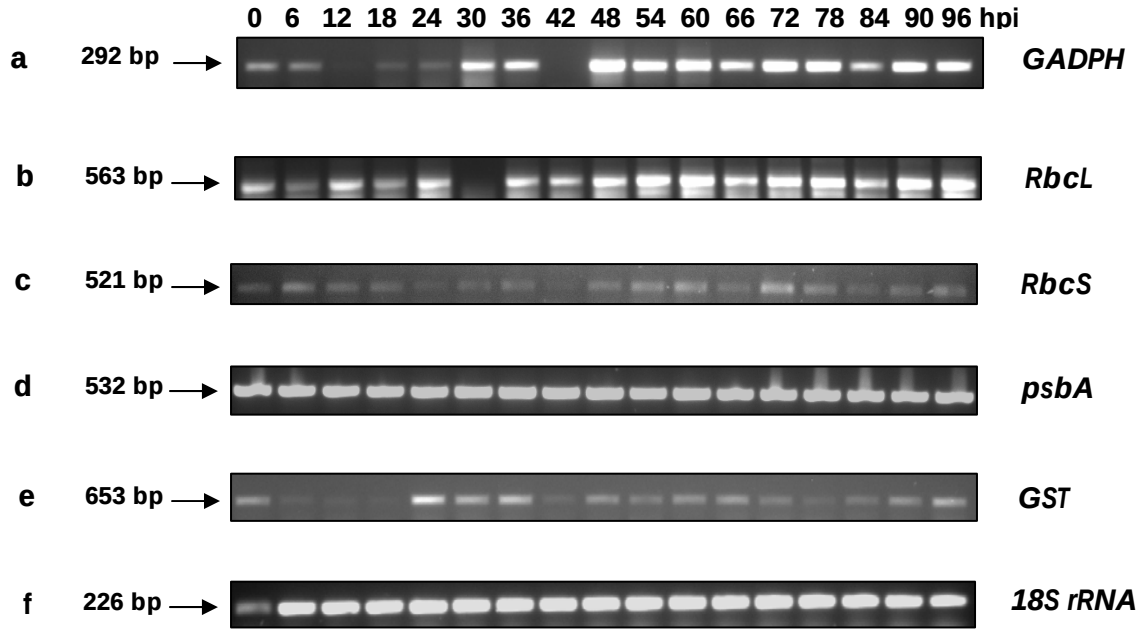


Figure 5.5. Expression analysis of photosynthesis related genes in the IS plants. Gene expression are shown in a) for *GADPH*, b) *RbcL*, c) *RbcS*, d) *psbA*, e) *GST* and f) *18S rRNA*. The fragment sizes and time intervals are as indicated by arrows.

The expression of GST was at very low levels in the IR plants (Fig. 5.4e). The gene was down-regulated after infection followed by an up-regulation at 18 hpi. In the IS plants, a similar pattern was seen than in the IR plants, but the up-regulation followed at a later time-interval (Fig. 5.5e).

5.4 Discussion.

The infection of a plant by a pathogen could lead to big losses in crop yield. The main reason for this is that biotrophic organisms need living tissue for growth and reproduction (Mendgen and Hahn, 2002). The metabolism of the plant is thus altered since the pathogen is competing with the plant for assimilates and nutrients. This negatively impacts on the effectiveness of the carbohydrate metabolism of the plant.

In order to establish the effect that *P. helianthi* has on the photosynthetic capacity of sunflower, a non-invasive method of detection was used (Maxwell and Johnson, 2000). The principle of chlorophyll a fluorescence analysis can be explained as follows: Light energy absorbed by chlorophyll molecules has one of three fates. It can either be used to drive photosynthesis or the excess energy can be dissipated as heat or it can be re-emitted as light in the form of chlorophyll a fluorescence. The three processes are in equilibrium with each other. An increase in one would thus lead to a decrease in the other two. By measuring chlorophyll fluorescence, the photochemistry and heat dissipation can therefore be calculated (Maxwell and Johnson, 2000).

Photochemical quenching was analysed by calculating the quantum yield of PSII (ϕPSII) and the maximum quantum yield (F_v/F_m). The first parameter measures the proportion of light absorbed by the chlorophyll that is associated with PSII in photochemistry (Fig. 5.2b). It is an indication of the rate of electron transport and so indicates the efficiency of photosynthesis. A clear difference was visible between the resistant and susceptible plants with the susceptible plants clearly outperforming the resistant cultivar. This might be due to a higher level of energy needed by the resistant plants for defence related responses other than investing the energy in photosynthesis. The ϕPSII for both infected cultivars were also lower than that of the controls with IR performing the worst. This is a clear

indication that infection with the leaf rust fungus impairs the photosynthetic capacity, especially in the resistant cultivar.

When comparing the ϕPSII data to F_v/F_m it was however clear that the maximum quantum yield (F_v/F_m) for the resistant plants was at much higher levels than the ϕPSII for this cultivar (Fig. 5.2). The ϕPSII values for the resistant cultivar were also much lower than that of the susceptible plants (Fig. 5.2b). This again could be an indication that the resistant plants devoted more energy to initiate the resistance response compared to the susceptible plants. The reduction of both ϕPSII and F_v/F_m in the IR plants was in line with data showed by Bonfig *et al.* (2006) when *Arabidopsis* was infected with *P. syringae.* Both ϕPSII and F_v/F_m levels decreased at the site of infection compared to uninfected tissue. Even though the F_v/F_m and ϕPSII values for the susceptible sunflower plants showed a general increase for both IS and CS, the ϕPSII values for both were constant (Fig. 5.2). This indicated that the presence of the leaf rust pathogen also had a negative impact on photosynthesis in the IS plants. These plants were however at this stage seemingly better equipped to ensure effective photosynthesis.

The third parameter analysed was the portion of open PSII reaction centres (*q*P). This parameter is inversely proportional to NPQ. Thus, it is an indication of the quantity of light energy that is absorbed into photosynthesis via the portion of functional photosystems. While both infected cultivars showed a decrease, the IR plants however showed the biggest decrease (Fig. 5.3). This confirmed that the pathogen has a negative influence on the photosynthetic processes of both the infected resistant and susceptible plants. Similarly, *q*P values were shown to decrease when the same resistant and susceptible sunflower cultivars were treated with O_3 in an unrelated study (Grobbelaar and Mohn, 2003). While both showed a decrease in *q*P, a continued decrease in the susceptible plants indicated permanent damage to the reaction centres. The resistant plants were however able to overcome the ozone stress by repairing the photosystems. It is thus clear that the same cultivars reacted quite differently when exposed to a biotic and abiotic stress. While the resistant cultivar was able to recover after

ozone stress, pathogen infection negatively impacted on its photosynthetic capacity. The opposite is true for the susceptible cultivar.

The last parameter, non-photochemical quenching (NPQ) gives an indication of the amount of energy that is dissipated as heat. A decrease of ϕPSII usually leads to an increase in NPQ. This was evident in the study of Chou *et al.* (2000) when *Arabidopsis* was infected with *Albugo candida* where NPQ values increased but ϕPSII decreased. This indicated that the light energy that was not used for electron transport in photosynthesis, was being lost to non-photochemical processes. Similar patterns were seen in the IR and CR plants of sunflower infected with the leaf rust pathogen (Fig 5.3). NPQ reached a peak at 72 to 96 hpi which correlated with lower ϕPSII levels and then gradually decreased. An increase in NPQ might be due to a greater reduction in the activity of the Calvin-Benson cycle (Chou *et al.,* 2000). This would lead to a lower demand for ATP and NADPH which leads to an increase in the trans-thylakoid membrane pH gradient. Once the Calvin-Benson cycle is repaired, NPQ would lower as the pH gradient returns to normal (Chou *et al.,* 2000). NPQ can also be seen as a protective mechanism used by the plants. An increase in NPQ was seen in the interaction between tomato and *Botrytis cinerea* (Berger *et al.,* 2004). This was orchestrated in an attempt to protect the plant from the damaging effect of excess radiation. Despite the decrease in sunflower NPQ levels at 96 hpi, NPQ levels for the IS plants increased over time (Fig. 5.3), indicating that the electron transport was unable to recover, therefore the excess heat is dissipated.

The effect of the leaf rust fungus on sunflower was also determined on gene expression level. Several gene fragments were previously isolated since they displayed putative differential expression (Chapter 4) in the resistant sunflower cultivar upon infection with the leaf rust fungus. The gene fragments shared homology with genes whose encoded polypeptides are involved in photosynthesis-related aspects of plant metabolism. The first encoded protein, glyceraldehyde 3-phosphate dehydrogenase B (GADPH) is involved in the phosphate sugar metabolism that catalyses the reversible interconversion of

glyceraldehyde-3-phosphate and 3-phosphoglycerate (Fothergill-Gilmore and Michaels, 1993). In plants there are three types of GADPH genes namely *GapA, GapB* and *GapC* each encoding for a different type of subunit. Of the three, GapA and GapB reside in the chloroplast while GapC is cytosolic (Brinkmann *et al.,* 1987). In sunflower the *GADPH* gene fragment showed a constitutive expression in both the IR and IS plants except for a down-regulation during the earlier times of the trial (Fig. 5.4a and 5.5a). This down-regulation correlated with the time that the plants were placed in the dark dew-simulation chamber to allow rust-spore germination indicating that the *GADPH* gene was down regulated at the onset of infection due to the darkness. However it was evident that *GADPH* gene expression in the IR plants was again up-regulated earlier than in the IS plants (Fig. 5.5a). This implies that in the resistant cultivars, gene expression recovered earlier than in the susceptible plants. In an unrelated study, *GADPH* transcript accumulation in potato has also been shown to be up-regulated upon SA treatment (Laxalt *et al.,* 1996). This implies that the *GADPH* might also be elicitor responsive.

The expression of the large and small subunits of *Rubisco* was also analysed. Proteins encoded by these genes take part in the Calvin-cycle to catalyse the first step in carbon fixation (Mauseth, 1995). The photosynthetic activity of barley was tested in resistant and susceptible plants challenged with powdery mildew (Swarbrick *et al.,* 2006). The barley-powdery mildew study has shown a general down regulation of photosynthesis in the compatible interaction with reduced *RbcS* expression. Compared to the IS plants, the reduction of *RbcS* expression in the IR plants was to a lesser extent. This was speculated to be due to cell death or sugar-induced repression of gene expression (Swarbrick *et al.,* 2006). In another study, wheat exposed to O_3 also showed decreased *RbcS* transcript levels (Booker, 2004). Symptoms of O_3 injury are known to resemble senescence in plants. These include suppressed photosynthesis, chlorosis, chloroplast degeneration, necrotic lesions and leaf abscission (Pell *et al.,* 1997). When comparing these data to the expression of sunflower *RbcS*, a great resemblance was seen. A fluctuation of *RbcS* gene expression was seen in the IR plants (Fig.

5.4c), whereas gene expression in the IS plants were at much lower levels (Fig. 5.5c). This indicated that *RbcS* might be more prone to be influenced by environmental changes such as pathogen attack. The low levels of *RbcS* gene expression in the IS plants might also explain the fluorescence data where photosynthetic capacity were shown to deteriorate as NPQ levels increased and *q*P decreased (Fig. 5.3). The expression of *RbcL* was constitutive in both the IR and IS plants, except for a down-regulation at 30 hpi in the IS plants (Fig. 5.4b and 5.5b).

The third analysed gene was *psbA*. Within PSII, the *psbA* gene encodes the D1 protein which in combination with the D2 subunit, provides a backbone to which several co-factors such as quinones are attached (Mauseth, 1995). In both the IR and IS plants, the gene was constitutively expressed. Judging by these results, the pathogen does not seem to influence *psbA* expression for the maintenance of the PSII reaction centres.

The expression of the *GST* gene has been known to increase following pathogen infection (Wagner *et al.,* 2002). Stress-inducible GST also have glutathione peroxidase activity, thereby protecting the cells from oxidative injury by detoxifying organic peroxides of fatty and nucleic acids (Dixon *et al.,* 2002). These organic peroxidases are synthesized in plants during processes such as photosynthesis and pathogen attack (Mauch and Dudler, 1993). The failure to remove such oxidative molecules would lead to damage in the plant cells. The expression of *GST* was low in both the IR and IS plants (Fig. 5.4e and 5.5e) with a down-regulation after infection of both the resistant and susceptible plants. However, elevated and constitutive gene expression was seen from 18 hpi in the IR plants. A similar pattern was seen in the IS plants where gene expression was up-regulated at 24 hpi where after a constant level of gene expression was seen through the trial. As shown with the fluorescence data, a sharp increase in electron transport was seen in the IS plants (Fig. 5.2b). Cases have been shown where higher electron transport levels led to higher ROS production (Tschiersch *et al.,* unpubl.). As the *GST* gene is involved in the detoxification of ROS in the

plant cell, higher expression levels of *GST* in the IS plants correlated with the higher rate of electron transport. The higher ROS production in the IS plants is related to a study where the consistent accumulation of ROS production was shown in both compatible and incompatible interactions, when barley was infected with powdery mildew (Huckelhoven and Kogel, 2003).

As a pathogen invades a plant, the carbon-metabolism is influenced (Scholes, 1996). This is caused by a higher demand for carbohydrates by the pathogen that needs nutrients for its own growth as well as for the plant when providing resistance. Photosynthesis at its optimum functional level is required to provide this energy. In this study, chlorophyll fluorescence data indicated that the electron transport rate in the resistant plants is functioning at a lower level compared to the susceptible plants. In addition to higher levels of heat dissipation in the IS plants and lower levels of *RbcS* gene expression, the higher electron transport rate in the IS plants indicated that photosynthesis was not utilized for carbon fixation only but also for ROS production, as evident by the higher *GST* gene expression in the IS plants.

Combined, the obtained results not only indicated the need for optimum photosynthetic capacity in the IR plants to both initiate and maintain an effective defence response, but also suggested that the susceptible infected plants could increase their photosynthetic as a means to increase the ROS concentrations. Future research will further investigate this possibility.

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Chapter Six:

General Discussion

Plants are comprised of different cell and tissue types. These are required for the specialised functions of plant growth, development and reproduction. Regarding metabolism, plant organs can be divided into two types namely source or sink tissues. Source tissues are mature leaves that are able to produce an excess of assimilates which are transported to sink tissues that are unable to produce enough assimilates for themselves (Berger *et al.,* 2004). The different sink tissues are constantly competing for assimilates. The distribution of assimilates are determined by the relative strength of the sink tissue and can also be influenced by biotic and abiotic factors.

When a plant is invaded by a pathogen, the source-sink relationship plays a crucial role. A biotrophic pathogen depends entirely on living tissue for its own reproduction (Mendgen and Hahn, 2002). Upon infection, the pathogen would attempt to alter the plants carbohydrate metabolism for its own benefit. The haustoria used by biotrophic pathogens as infection structures, have been shown to provide the pathogen of nutrients by absorbing it from the plant tissue (Panstruga, 2003). The uptake of sucrose from powdery mildew infected *Arabidopsis* source leaves indicates that the pathogen acts as an additional sink (Fotopoulus *et al.,* 2003). As hexoses serves as substrates for the pathogen, it might also function as signals in the plant leading to signal transduction of defence responses and the reduction of photosynthesis (Herbers *et al.,* 1996). Therefore, the pathogen should inhibit hexose-signalling in plants by either increasing the uptake thereof or by blocking the signal transduction pathway. This was found when the pea pathogen *Mycospharella pinodes* was shown to rapidly deactivate the gene encoding phenylalanine ammonia-lyase (PAL) (Wada *et al.,* 1995). PAL plays a role in plant defence responses as it synthesizes phenolic compounds including SA and phytoalexins (Hahlbrock and Scheel, 1989).

The disturbance of the source-sink transition is not without consequence for the process of photosynthesis as the process declines upon pathogen attack (Tang *et al.,* 1996). The effect of the leaf rust pathogen on the photosynthetic capacity of resistant and susceptible sunflower cultivars was determined by measuring

chlorophyll fluorescence. A decline of general photosynthesis was seen in pathogen infected plants that was occurring at a faster rate in the IR plants compared to the IS plants. Decreased efficiency (ϕPSII) of sunflower photosystem II was more evident in the resistant plants compared to the susceptible plants. The available photosystems were also declining at a faster rate in the IR plants than in the IS plants. This was similar to other plant-pathogen interactions where the declining rate of photosynthesis was faster during the incompatible interaction compared to the compatible interactions (Swarbrick *et al.,* 2006).

Furthermore, the level of energy dissipated as heat measured by NPQ in the IR plants, were at an overall steady level compared to the IS plants that showed a drastic overall increase in NPQ over time. Combining this with the higher levels of ϕPSII in the susceptible plants, an alternation in the metabolic status of these cells is indicated. Meng *et al.* (2002) demonstrated that general sink tissues of tobacco leaves had a higher carbon demand thus an increase in electron transport. Meng *et al.* (2002) also demonstrated that the source-tobacco leaves had a slower induction of ϕPSII thus implying that the resistant cultivar showing a lower ϕPSII, has the ability to control the invading pathogen with other resistant related factors such as *R*-genes. On the other hand, the increased NPQ level combined with the steady levels of ϕPSII of the IS plants indicate that less light energy is incorporated into the photosystems. This indicates higher levels of stress as no specific resistance components are present.

Combining the fluorescence data with gene expression analysis, the effect of the pathogen regarding photosynthesis was further confirmed. Gene expression of *RbcS* differed greatly between the IR and IS plants (Fig. 5.4c and 5.5c). Levels of gene expression were at much lower levels in the IS than IR plants. However, the genes were again up-regulated at later time-intervals in both IR and IS, but at lower levels in IS. An accumulation and/or a change in the flux of sugars within a leaf has been shown to act as signals that down-regulate gene expression (Koch,

1996). It has been suggested that this mechanism may contribute to the down-regulation of *RbcS* in infected leaves (Chou *et al.,* 2000; Berger *et al.,* 2004).

The *GST* gene expression was also shown to be induced at time intervals after infection in both IR and IS plants. The gene is known to increase following pathogen infection (Wagner *et al.,* 2002) acting as a substance to protect the cells from oxidative injury. It has been suggested that an increase in electron transport was the consequence of sink-tissue demand in *Blumeria graminis* infected barley (Swarbrick *et al.,* 2006). Higher electron transport rates were however not paralleled by higher CO_2-fixation, instead higher ROS production was observed (Tschiersch *et al.,* unpubl.). This might explain the up-regulation of *GST* gene expression in the IS plants that showed higher electron transport rates during chlorophyll fluorescence. Higher ROS production has also been shown in other compatible interactions (Huckelhoven and Kogel, 2003). Thus the down-regulation of *RbcS* and the up-regulation of *GST* implicate that ROS production is a means for resistance against the leaf rust pathogen in the susceptible plants.

A plant's surveillance system regarding a pathogen attack is based on the early recognition of the invading organism (Dangl and Jones, 2001). The plant is equipped with several mechanisms which are used to provide a means of resistance against the invading pathogen. Recognition of the pathogen is accomplished by the detection of elicitors such as peptide-, oligosaccharide- and lipid-based molecules either from the pathogen itself or from the degrading plant cell wall (Romeis, 2001). The gene-for-gene interaction where an *R*-gene from the plant and an *Avr*-gene from the pathogen is present provides a sufficient resistance response against the pathogen (Flor, 1971). The *Xa21* gene, is a functional LRR receptor-like protein kinase gene encoding for an extracellular and transmembrane domain that provides resistance in rice against *Xanthomonas oryzae* pv. *oryzae* (Song *et al.,* 1995).

Also involved in the recognition of pathogen derived molecules are RLKs that are bound to the plasmamembrane and able to provide resistance against an

invading pathogen. RLKs share a family of highly conserved catalytic domains that have been predicted to phosphorylate serine and threonine residues (Hanks *et al.,* 1988) and in some cases tyrosine residues (Mu *et al.,* 1994). Based on the diverse functions of the different extracellular domains, plant RLKs have been grouped into different subfamilies (Shiu and Bleecker, 2001) where the RLKs of each class have a functional role.

The abundance of RLKs and the diversity thereof imply common signalling cascades in response to different types of extracellular signals (Walker, 1994). SA is known to play a key role in the plant defence reaction and is instrumental in the activation of *PR*-gene expression (Klessig *et al.,* 2000). SA is known to induce a specific group of protein kinases such as *SFR2* (Pastuglia *et al.,* 1997), *WAK1* from *Arabidopsis* (He *et al.,* 1998) and *RLK3* from *Arabidopsis* (Czernic *et al.,* 1999). Both *WAK1* and *RLK3* are also up-regulated by pathogen infection. The encoded protein from *RLK3* contains two copies of the C-X8-C-X2-C motif as identified by Takahashi *et al.* (1998). This motif is known as the DUF26 domain (Chen, 2001). Phylogenetic analysis based on the catalytic domain of RLKs indicated that the C-X8-C-X2-C containing RLKs showed similarity to the S-domain class of RLKs (Ohtake *et al.,* 2000).

In this study, a gene fragment was obtained that showed good homology to a hypothetical protein containing a DUF26 domain (Fig. 4.4). In the interaction between sunflower and *P. helianthi,* the NBS6 gene expression was induced in both the resistant and susceptible cultivars (Fig. 4.12), as well as SA (Fig. 4.13). The up-regulation regarding SA also correlated with findings by Ohtake *et al.* (2000) where the *RKC1* protein kinase containing the DUF26 domain was inducibly expressed in *Arabidopsis* upon treatment with SA.

The NBS6 gene fragment was further implicated to be involved in the sunflower-*P. helianthi* interaction when it was inducibly expressed in resistant plants treated with menadione (Fig. 4.13). Various plant RLKs are induced in pathogen-related defence responses that are often associated with a rapid oxidative burst (Lamb

and Dixon, 1997). Therefore, the possibility exists that the DUF26 domain might play a role in assisting the regulation of the oxidative burst. Together, these results clearly implicate NBS6 to play a general defence role in sunflower after infection. However, this role can only be confirmed once the complete gene has been cloned.

Following pathogen perception, the stress signal is further transduced throughout the cytosol. Many signalling cascades have been implicated to be responsible for signal transduction. One such protein kinase cascade involves several MAPKs (Jonak *et al.,* 2002). The signal is transduced through a cascade that involves three different MAPKs. Each subsequent MAPK is phosphorylated and de-phosphorylated thus carrying the signal throughout the cytosol. This triggers subsequent cellular responses such as ion fluxes, synthesis of ROS and changes in gene transcription (Romeis, 2001). A sufficient resistance response would ultimately lead to the HR (Hammond-Kosack and Jones, 1996).

A putative protein kinase gene has been identified in sunflower upon the infection with the leaf rust pathogen. The *HaLRD15* gene showed high homology to a calcineurin B-like interacting protein kinase 16 (CIPK) identified in *Populus trichocarpa* (Fig. 3.11). In plants, calcium plays an important role in the regulation of gene transcription (Gilroy and Trewavas, 2001). Many external stimuli can lead to changes in the levels of Ca^{2+} (Sanders *et al.,* 2002). It serves as a second messenger and its specificity is decoded by Ca^{2+} sensing proteins which interact with their target proteins to relay the signal. Such sensor proteins are the CBL proteins (Luan *et al.,* 2002). CBLs are known to have an EF-motif that serves as a structural basis for calcium binding which interacts specifically with a group of serine/threonine protein kinases namely CIPKs (Sanchez-Barrena *et al.,* 2005).

CIPKs have a 24-amino acid motif (NAF) within the C-terminal non-kinase region of CIPKs that is required to mediate a CBL-CIPK interaction (Albrecht *et al.,* 2001). The CBL-proteins regulate CIPK via the Ca^{2+} which is transferred by the EF-motif (Luan *et al.,* 2002). This makes the CBL-CIPK interaction a novel plant-

specific signal transduction system. Several CIPK-CBL interactions were identified to be involved in a large array of signalling processes where Ca^{2+} is involved (Luan *et al.,* 2002). *CIPKs* expression has been implicated to be induced upon several stress responses such as salinity and drought (Romeis *et al.,* 2001). A *CIPK* gene identified in *Pisum sativum, PsCIPK*, showed an up-regulation after Ca^{2+} treatment (Mahajan *et al.,* 2006). Induced gene expression of *PsCIPK* were also shown upon wounding, NaCl and SA treatment (Mahajan *et al.,* 2006).

Furthermore, the structural properties of *HaLRD15* such as the presence of a NAF domain and various phosphorylation sites such as CK1 and GSK3 as well as a MAPK docking site added to the similarity between *HaLRD15* and a *CIPK* (Table 3.3). The presence of these phosphorylation sites indicates that the protein has the potential to play some role in the signal transduction processes. To analyse the gene's involvement in signal transduction, the expression thereof was tested in different sunflower cultivars. Even though the gene was shown to be present in all the resistant and susceptible cultivars, it was shown to be inducibly expressed in only one resistant cultivar (Fig. 3.15). The gene was further implicated to be involved in the defence response when it was inducibly expressed in the same resistant sunflower cultivar upon MeJA treatment (Fig. 3.20). It is therefore clear that the *HaLRD15* gene has the means to participate in the defence response of sunflower to the leaf rust pathogen. It was shown to be cultivar specific as well as having the potential for being part of a phosphorylation process.

Combined, results obtained in this study have implicated the involvement of three factors in the defence response of sunflower upon infection with the leaf rust pathogen. These were, the putative protein kinase gene (*HaLRD15*), the gene fragment containing the DUF26 domain and the role that photosynthesis plays in the plant-pathogen interaction. Future research will concentrate to prove the involvement of the encoded polypeptides of the cloned cDNAs in the defence response.

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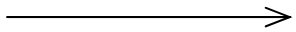

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Summary:

Plants are equipped with a surveillance system which enables the plant to recognize an invading pathogen. After recognition, the stress signal is conveyed throughout the plant cell and subsequent defence responses are activated.

Protein kinase genes encode proteins which play important roles in the relay of the initial signal. The aim of this study was to characterise some aspects of the defence response of sunflower upon infection with *P. helianthi*. One such aspect was the identification of a putative protein kinase gene from the resistant sunflower cultivar. The *HaLRD15* gene showed good homology (e-value $7e^{-151}$) to a Calcineurin B-like protein kinase (CDPK). The virtual protein encoded by the *HaLRD15* gene contained various phosphorylation sites and a MAPK docking site. These sites could lead to the protein being phosphorylated thereby conveying the stress signal throughout the plant cell. Further analyses of *HaLRD15* in different sunflower cultivars indicated that the gene was inducible expressed in only one resistant cultivar, even though gene copies were present in all the tested cultivars. This led to the conclusion that *HaLRD15* could be essential factor needed in the defence response.

Furthermore, an attempt was made to obtain putative disease resistance genes which are differentially expressed in sunflower after infection by using primers specific for the nucleotide binding site of resistance genes. One such gene fragment obtained was *NBS6*. The polypeptide sequence of *NBS6* showed homology to a hypothetical protein containing a DUF26 domain. Further analysis of *NBS6* showed that the gene is up-regulated by defence response related chemicals such as MeJA and menadione. Literature have implicated that receptor-like protein kinases containing DUF26, were involved in the defence responses in the plant-pathogen interaction, more specifically in the response of plants upon oxidative stress.

As photosynthesis related genes were also differentially expressed, the effect of the leaf rust pathogen on the photosynthetic capacity of both resistant and susceptible cultivars was investigated by using chlorophyll fluorescence. The

electron transport rate of the infected resistant plants was shown to be much lower than that of the infected susceptible plants. However the energy dissipated as heat (NPQ) was shown to increase over time in the infected susceptible plants. The expression of various genes such as *glutathione S-transferase* and the large and small subunit of *Rubisco* were finally analysed. The infected susceptible plants were shown to have a higher down-regulation of the *RbcS* gene than the infected resistant plants. The higher electron transport rate of the infected susceptible plants in combination with the low induction levels of *RbcS* as well as a higher induction of *GST* during the early intervals, led to the conclusion that the susceptible plants increase the electron transport system to produce higher amounts of reactive oxygen species, thereby leading to the higher induction of *GST* expression.

Keywords: *Puccinia helianthi*, protein kinases, plant defence, CIPK, DUF26, chlorophyll lfluorescence.

Opsomming:

Plante is in staat om potensiële patogene te herken deur middel van ‘n baie effektiewe deteksiesisteem. Dit kan lei tot die aktivering van die plant se verdedigingssisteem. Dit is egter afhanklik van die oordraging van seine vanaf die plasmamembraan van die sel tot in die kern. Proteïen kinases is nou betrokke by die oordraging van sodanige seine.

Die doel van die studie was om sekere aspekte van die verdedigingsrespons van sonneblomme na infeksie met *P. helianthi* te ondersoek. Die eerste was om die rol van `n gekloneerde proteïen kinasegeen, *HaLRD15*, tydens die verdedigingsrespons te bevestig. Die geen het hoë homologie (e-waarde 0) met `n calcineurin B-tipe protein kinase (CDPK) getoon. Die gekodeerde polipeptied besit verskeie fosforileringsgebiede, asook `n MAPK vashegtingsgebied. Die teenwoordigeheid van die gebiede impliseer dat HaLRD15 betrokke is by seinoordraging aangesien dit self gefosforileer kan word. Die uiting van die geen was in slegs een van die getoetsde kultivars geïnduseer, ten spyte daarvan dat dit in al die kultivars teenwoordig was. Die gevolgtrekking wat dus gemaak was, is dat HaLRD15 `n essensiële komponent van die algemene verdedigingsrespons van sonneblomme kan wees.

Tweedens was daar gepoog om weerstandsgene wat differensieël in geïnfekteerde sonneblomme tot uiting kom, te kloneer. Priemstukke spesifiek vir die nukleotiedbindingsgebied is daarvoor gebruik. *NBS6* was een van die klone wat vir verdere analiese gekies was. Die gekodeerde polipeptied was homoloog aan `n hipotetiese protein wat `n DUF26 gebied besit het. Die geen se uiting was deur beide MeJA en menadioon wat verdedigingsverwante chemikalieë is, aangeskakel. Uit die literatuur was dit duidelik dat proteïene wat die gebiede besit, betrokke is by die verdedigingsrespons tydens verskeie plant-patogeen interaksies, maar veral tydens oksidatiewe spanning.

Laastens is daar ook ondersoek ingestel na die invloed wat die patogeen op die fotosintetiese vermoë van beide vatbare en weerstandbiedende sonneblomme gehad het. Chlorofil-fluoresensie was hiervoor gebruik. Die elektronoordragtempo van die geïnfekteerde weerstandbiedende plante was

laer as die van die vatbare kultivar. Laasgenoemde het egter met die verloop van die studie verhoogde hitteverlies (NPQ) getoon. Verder is die uiting van verskillende gene soos *GST*, *RbcL* en *RbcS* ook bepaal. In vergelyking met die geïnfekteerde weerstandbiedende plante, was die uitingsvlakke van *RbcS* laer in die vatbare plante. Dit, tesame met die verhoogde elektronoordrag, het gelei tot die afleiding dat vatbare plante tydens infeksie die elektronoordragsisteem moontlik eerder vir die produksie van reaktiewe suurstofspesies gebruik. Dit lei dan ook tot die verhoogde uiting van *GST* soos tydens die studie waargeneem.

Sleutelwoorde: *Puccina helianthi*, proteïen kinase, plant verdediging, CIPK, DUF26, chlorofil fluoresensie.