

Identification of a putative protein kinase gene involved in the resistant response of sunflower to rust

By

MARIËTTE BEZUIDENHOUT

Submitted in fulfillment of the requirements for the degree

MAGISTER SCIENTIAE

In the Faculty of Natural and Agricultural Sciences

Department of Plant Sciences

University of the Free State

Bloemfontein

South Africa

2004

Study Leader:

Mr B Visser

Department of Plant Sciences

Co-Study leader:

Prof AJ van der Westhuizen

Department of Plant Sciences

I am among those who think that science has great beauty.

A scientist in his laboratory is not only a technician: he is also a child placed before natural phenomena which impress him like a fairy tale.

- Marie Curie -

List of contents

Acknowledgements	iii
List of Abbreviations	iv
List of tables and figures	vii
Chapter 1: Introduction	1
Chapter 2: Literature review	4
2.1 Introduction	4
2.2 Disease resistance genes	5
2.3 Receptor-like protein kinases	7
2.3.1 RLKs classified into several groups	8
2.3.1.1 S-domain class	8
2.3.1.2 LRR class	8
2.3.1.3 TNFR class	9
2.3.1.4 EGF class	9
2.3.1.5 PR class	10
2.3.1.6 Lectin class	10
2.3.2 Examples of RLKs involved in plant disease	10
2.3.2.1 <i>Xa-21</i>	10
2.3.2.2 <i>LRK10</i>	11
2.3.2.3 <i>At-RLK3</i>	11
2.3.2.4 <i>CHRK1</i>	11
2.3.2.5 <i>SR160</i>	12
2.3.2.6 <i>FLS2</i>	12
2.3.2.7 <i>SFR2</i>	13
2.3.3 Other protein kinases implicated in plant defense	13
2.4 MAPKs involved in signal transduction	15
2.5 Biochemical defenses	16
2.5.1 Calcium and ion channels	18
2.5.2 Reactive oxygen species	18
2.5.3 Cell wall fortification	21
2.5.4 Lipoxygenase	22
2.5.5 Jasmonic acid	23
2.5.6 Salicylic acid	23
2.5.7 Phytoalexins	24
2.5.8 G-proteins	25
2.5.9 Pathogenesis-related proteins	25

2.6 The hypersensitive reaction	28
2.7 Systemic acquired resistance	29
2.8 The interaction between sunflower and leaf rust	29
2.8.1 <i>Helianthus annuus</i>	29
2.8.2 <i>Puccinia helianthi</i>	30
2.9 Aim	32
Chapter 3: Materials and Methods	33
3.1 Materials	34
3.2 Methods	34
3.2.1 Cultivation of sunflower plants	34
3.2.2 Infection of sunflower with leaf rust (<i>Puccinia helianthi</i>)	35
3.2.3 Total RNA extraction from infected leaves	35
3.2.4 DDRT-PCR amplification of differentially expressed putative protein kinase genes	36
3.2.5 Cloning of differentially expressed cDNA fragments	39
3.2.6 Expression analysis of the D15 cDNA clone	41
3.2.7 5'-RACE of clone D15	43
3.2.8 Genomic DNA extraction	46
3.2.9 Southern blot analysis of the D15 cDNA clone	46
Chapter 4: Results	48
4.1 Infection of sunflower with leaf rust	48
4.2 Isolation of differentially expressed putative protein kinase genes	48
4.3 Cloning of the cDNA fragments	53
4.4 Expression analysis of the identified cDNA clones	56
4.5 Identification of the selected cDNA clones	60
4.6 Verification of D15 expression using RT-PCR	66
4.7 Southern Blot analysis of clone D15	67
4.8 Cloning of the full length D15 gene	70
Chapter 5: Discussion	72
Chapter 6: References	82
Summary	98
Opsomming	99

Acknowledgements

I would like to acknowledge and thank the following persons;

- Thank you! To my *study leader*, Mr Botma Visser, for your guidance, support and advice during this study.
- To my *co-study leader*, Prof AJ van der Westhuizen, for your input in making this study a success.
- To my *parents, sister and family*, thank you for love, support and understanding throughout the past two years.
- To my *friends and colleagues* in the lab, your friendship and enthusiasm about our work are precious to me.
- To *Prof ZA Pretorius*, for providing the greenhouse facilities and sunflower seeds to complete this study and to *Cornèl Bender* for assisting me with the infection procedures.
- To the *Department of Plant Sciences*, with all your friendly faces everyday.
- To the *NRF* for providing me with the finances.

List of abbreviations

A

APS	Ammoniumperoxodisulfate
ATP	Adenosine triphosphate
Avr	Avirulence

B

BLAST	Basic local alignment search tool
BTH	Benzothiadiazole
BR	Brassinosteroid
BSA	Bovine serum albumin

C

CPM	Counts per minute
CTAB	Cetyltrimethylammonium bromide

D

DDRT-PCR	Differential display RT-PCR
DMPC	Dimethyl pyrocarbonate
DMSO	Dimethylsulfoxide
dNTPs	Deoxynucleotidetriphosphates
DTE	Dithioerythritol
DTT	Dithiothreitol

E

EDTA	Ethylenedinitrilotetraacetic acid
EGF	Epidermal growth factor

H

H ₂ O ₂	Hydrogen peroxide
h.p.i.	Hours post infection
HR	Hypersensitive response

I

IPTG	Isopropyl-β-D-thiogalactopyranoside
IR	Infected resistant
IS	Infected susceptible

J

JA	Jasmonic acid
----	---------------

L

LB	Luria Bertani
LOX	Lipoxygenase
LRR	Leucine rich repeat

M

MAPK	Mitogen activated protein kinase
------	----------------------------------

N

NIL	Near isogenic lines
NO	Nitric oxide
Nonidet P40	Nonylphenolpolyethyleneglycol

P

PAL	Phenylalanine ammonia lyase
PCR	Polymerase chain reaction
PEG	Polyethyleneglycol

PG	Polygalacturonases
PGIP	Polygalacturonase inhibiting protein
PR	Pathogenesis related
PUFA	Poly unsaturated fatty acid
PVP	Polyvinylpyrrolidone

R

R	Resistance
RACE	Rapid amplification of cDNA ends
RLK	Receptor-like protein kinase
ROS	Reactive oxygen species
RT	Reverse transcriptase

S

SA	Salicylic acid
SAR	Systemic acquired resistance
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase

T

Temed	N,N,N',N',-Tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane
Tween 20	Polyoxyethylenesorbitanmonolaurat

U

UV	Ultraviolet light
----	-------------------

X

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

List of tables and figures

Table 2.1	The families of pathogenesis-related proteins.	26
Table 3.1	Nucleotide sequences of all primers used in this study.	37
Table 4.1	Genes sharing possible homologies with the selected clones.	65
Fig. 2.1	A current model for the signaling pathways initiated when bacterial avirulence gene product AvrPto binds to the Pto plant resistance gene product.	14
Fig. 2.2	Complexity of signaling events controlling activation of defense responses.	17
Fig. 2.3	A general scheme of reactive oxygen species formation and the Fenton reaction.	20
Fig. 3.1	Schematic presentation of 5'-RACE.	44
Fig. 4.1	Leaf rust infection of sunflowers.	49
Fig. 4.2	Extracted total RNA from infected resistant plants.	51
Fig. 4.3	DDRT-PCR of differentially expressed putative protein kinase genes.	52
Fig. 4.4	Selection of recombinant plasmids using α -complementation.	54
Fig. 4.5	Restriction analysis of recombinant plasmids.	55
Fig. 4.6	Expression patterns of isolated dicot cDNA fragments.	57
Fig. 4.7	Expression patterns of isolated monocot cDNA fragments.	58
Fig. 4.8	Sequence analysis of clone D15.	61
Fig. 4.9	Sequence analysis of clone M4.	62
Fig. 4.10	Sequence analysis of clone M7.	63
Fig. 4.11	Sequence analysis of clone M9.	64
Fig. 4.12	Verification of D15 gene expression using RT-PCR.	68

Fig. 4.13 Southern blot analysis of clone D15.

69

Fig. 4.14 5'-RACE of clone D15.

71

Chapter One:

Introduction...



Sunflower (*Helianthus annuus*) is a cultivated plant which is recognized worldwide for its outstanding beauty. Apart from that, it is also economically very important. Sunflower probably originated in the South West United States and Mexico area and its seed was used for food by the local inhabitants (Weiss, 2000). In the sixteenth century sunflower was introduced to European countries where it was produced for commercial uses. Sunflower seed is known for its high oil content which makes it both a healthy and a nutritious ingredient in many foods.

Sunflowers are normally cultivated in warm-tempered regions and are exposed to various pathogens. *Puccinia helianthi* causes leaf rust in sunflower. This pathogen tends to infect the leaf surface of mature plants and in severe cases, the flower head itself (Kolte, 1985). Moisture on the leaf surface provides optimum conditions for the pathogen to infect the plant in a very short time. The infection of especially young seedlings will lead to decreased yields resulting in economic losses.

Through evolutionary changes, plants have developed a resistance response which is activated when they are attacked by a pathogen (Blumwald *et al.*, 1998). A concurrence of events within the plant cell will lead to an efficient defense reaction that will protect the infected tissue as well as the rest of the plant from subsequent infections. Only those plants that are equipped with such a resistance mechanism will be able to survive (Martin, 1999). The key to the initiation of the defense response is a resistance gene that is compatible with an avirulence gene present in the pathogen (Flor, 1971). In this gene-for-gene compatibility, the plant has the ability to provide immediate resistance against the particular pathogen.

In the defense pathway, several contributing factors are responsible to relay a signal generated at plasmamembrane level to the nucleus. Such factors include receptor-like protein kinases (RLKs) that are situated on the cell membrane and are able to recognize intruding pathogens (Morris and Walker, 2003). RLKs are also able to bind an array of different molecules depending on the structure of their extracellular domain, thereby giving specificity (Shiu and Bleecker, 2001). Receptors are protein kinases which can initiate a cascade of signaling events originating from the cell membrane. If a particular protein kinase is absent, the alarm signal will not reach the nucleus and the plant defense genes would not be expressed in time to mount a full response (Shiu and Bleecker, 2001). Mitogen activated protein kinases (MAPKs) also form part of such a phosphorylation cascade and have been identified in various plant species (Jonak *et al.*, 2002). They are thought to be responsible for transferring the signal from the cell membrane-bound receptor to the nucleus.

Once the initial defense response signal has reached the nucleus, the activation of a range of biochemical defenses follow. These include the strengthening of the cell wall, reactive oxygen species production and the synthesis of substances such as salicylic acid (SA) and jasmonic acid (JA). Ultimately, pathogenesis-related proteins are produced that will provide further resistance against the pathogen attack. Plants can also induce defense mechanisms in distal areas of a plant. This is referred to as systemic acquired resistance (SAR). SAR often confers a long-lasting and non-specific resistance to a broad variety to pathogens (Ryals *et al.*, 1996).

Since protein kinases form an integral part of the signaling event, the aim of this study was to identify a putative protein kinase gene which is involved in the early resistance response when sunflower is infected by *Puccinia helianthi*. By cloning such a gene, information is gained to improve our knowledge of the roles that these protein kinases and other contributing factors play during the plant defense mechanism. It was also important to do this study, since no protein kinase gene has yet been identified in sunflower as little work is being done on this crop.

Chapter Two:

Literature review...



2.4 Introduction.

Plants are constantly challenged with fluctuations in their environment, and with pathogens and pests. Plant cells use receptors on the cell membrane to sense these environmental changes and to transduce this information via activated signaling pathways to trigger defense responses. Resistant plants are equipped with a molecular system that allows the recognition of pathogen intrusion, as well as to amplify the initial alarm signal. Here protein kinases and phosphatases play key roles in the induction of defense responses.

The early recognition of a pathogen by a plant is essential to mount an appropriate defense reaction. Plant cells are able to sense pathogen invasions by recognizing molecules derived from either a degraded plant cell wall (endogenous) or molecules directly synthesized by the pathogen (exogenous). These signal components are called elicitors and include proteins, glycoproteins, oligosaccharides and lipids. A complete set of plant defense reactions can be induced by some elicitors when they interact with specific plant receptors (Martin, 1999). Elicitors can be either race- or non-race-specific, depending on the plants in which the defense responses are activated.

Disease resistance in plants commonly requires two complementary genes. They are an avirulence (*Avr*) gene from the pathogen and a matching resistance (*R*) gene from the host. Race-specific elicitors are often products of the *Avr* genes and are specifically recognized by *R* gene products in the plant (Flor, 1971). This is called a gene-for-gene interaction which leads to resistance due to an appropriate and timely activation of the defense response.

Non-race-specific elicitors are able to activate defense responses independently of the R-genes. Recognition is probably due to high-affinity receptors located in the plasma membrane. These general elicitors include substances typically associated with basic microbial metabolism such as cell wall glucans, chitin oligomers, fatty acids, sterols and glycopeptides.

2.5 Disease resistance genes.

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 was a high priority in plants (Richter *et al.*, 1995). A common feature of these genes is that they are frequently clustered on the same chromosomal region and undergo recombination events (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 pathogen avirulence genes. This idea originated when the *Rp1* locus was found to confer race-specific resistance to *Puccinia sorghi* due to an unequal over-crossing event of the genes (Richter *et al.*, 1995). Recombinants exhibit resistance that was not present in either of the parents.

The first resistance gene cloned was *Hm1* from maize (Johal and Briggs, 1992). This gene conferred resistance to maize against race 1 strains of the fungus *Cochliobolus carbonum*. The *Hm1* gene encodes an enzyme that inactivates the host-specific HC-toxin produced by the fungus.

Several disease resistance genes have since been cloned and characterized from various plant species. The encoded proteins fall into three general classes, namely:

- Proteins consisting exclusively of a protein kinase domain such as the tomato *Pto* gene product (Zhou *et al.*, 1995);
- Proteins containing leucine rich repeats (LRR), a leucine zipper and a nucleotide binding site and/or a membrane-spanning domain (Salmeron *et al.*, 1995).
- Proteins containing a leucine zipper, an LRR and a protein kinase domain in the same protein. An example is the rice *Xa-21* gene product (Song *et al.*, 1995).

The tomato *Cf-4* and *Cf-9* R-genes giving resistance against the leaf mould fungus *Cladosporium fulvum*, belong to a large family of homologous *C. fulvum* resistance genes that include *Cf-2*, *Cf-4*, *Cf-4A*, *Cf-5* and *Cf-9* (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). The *Cf-4* and *Cf-9* genes encode polypeptides that share homology to an extracellular, membrane-anchored glycoprotein that consists mainly of LRRs (Jones *et al.*, 1994). Downstream defense signaling upon *Cf-9*-mediated recognition of *Avr9* has been studied in transgenic tobacco plants (Cai *et al.*, 2001). An oxidative burst and the activation of calcium-dependent kinases, MAPKs and ion fluxes were found to be involved in the regulation of *Cf-9/Avr9* initiated defense responses (Cai *et al.*, 2001). Each *Cf-Avr* gene combination resulted in the arrest of hyphal growth at a distinct stage of colonization, either within the substomatal cavity or in the adjacent mesophyll cell layers (Hammond-Kosack and Jones, 1996).

Examples of cloned disease resistance genes that also modulate the hypersensitive response (HR) include the *Prf* gene from tomato against *Pseudomonas syringae* (Zhou *et al.*, 1995), *Rp1-D* from maize against *Puccinia sorghi* (Boller and Keen, 1999) and the *Xa-21* gene from rice against *Xanthomonas campestris* (Song *et al.*, 1995). The latter is included in a large group of plant genes, called RLKs.

2.3 Receptor-like Protein Kinases.

RLKs are a diverse group of proteins that span the plasma membrane that allow cells to recognize and respond to their changing extracellular environment (Morris and Walker, 2003). A common feature of all plant RLKs is that each has a cytoplasmic protein kinase catalytic domain, a single membrane spanning region (transmembrane domain), an N-terminal signal sequence and an extracellular domain which varies in structure (Torii and Clark, 2000). 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 phosphorylation. The activated kinases then phosphorylates substrate proteins within the cell, resulting in the transduction of the signal (Zhang, 1998).

The first RLK in plants to be identified was ZmPK1 in maize (Walker and Zhang, 1990). The ZmPK1 amino-acid sequence is typical of the serine/threonine kinases and the putative catalytic domain is related to the Raf-family of serine/threonine kinases.

2.3.1 RLKS classified into several groups.

Based on the structural features of the predicted extracellular domain, the RLKs can be classified into several groups;

2.3.1.1 S-domain class.

The S-class of RLKs has an extracellular domain that is homologous to the self-incompatibility-locus glycoproteins (SLG) of *Brassica oleracea* (Nasrallah *et al.*, 1992). The extracellular domain consists of 12 conserved cysteine residues. It is likely that some of the cysteine residues may function as a dimerization module during the activation of the enzyme. In *Brassica*, the *SLK* gene is physically linked to the S-locus and it is proposed that SLG and SLK function together in the self-incompatibility recognition between pollen and stigma (Bower *et al.*, 1999). One role that has been proposed for SLG is to aid the putative pollen ligand in crossing the cell wall and allowing it to come into contact with the extracellular domain of the membrane-bound SRK (Goring and Rothstein, 1992). The isolation of *SLK* genes from self-incompatible plant species and their expression in vegetative tissues, indicate that RLKs play an additional role in plant development (Dwyer *et al.*, 1994). RLKs included in this class are ZmPK1 (Walker and Zhang, 1990), RLK1 (Walker and Zhang, 1993) and RLK4 (Coello *et al.*, 1999).

2.3.1.2 LRR class.

The LRR-class of RLKs is the largest class of identified plant RLKs (Torii and Clark, 2000). These leucine rich repeats occur on the extracellular domain of the protein and are tandem repeats of approximately 24 amino acids with conserved leucines. Paired cysteine molecules that

flank the LRRs most probably participate to form disulfide bonds that play a role in intermolecular assembly (Torii and Clark, 2000). LRRs are involved in protein-protein interactions and have been found in a variety of proteins (Bent, 1996). LRR-RLKs play critical roles in development and disease resistance. LRR-RLKs that regulate development are ERECTA (Torii *et al.*, 1996), CLAVATA1 (CLV1) (Clark *et al.*, 1997), brassinosteroid (BR1) (Li and Chory, 1997) and pollen receptor-like kinase 1 (PRK1) (Mu *et al.*, 1994).

2.3.1.3 TNFR class.

The maize *CRINKLY4* (*CR4*) gene product is the only member of this class. The extracellular domain contains a cysteine-rich region similar to the ligand binding domain in mammalian tumor necrosis factor receptors (TNFRs) (Becraft *et al.*, 1996). TNFRs have six conserved cysteine residues. *CR4* gene is required for proper development of the epidermis.

2.3.1.4 EGF class.

The first contact of a pathogen with a plant cell must include some form of interaction with the cell wall. The plant cell wall, or extracellular matrix, is a complex arrangement of carbohydrates and proteins (Zheng-hui *et al.*, 1996). A number of RLK proteins have been identified as integral component of the cell wall. Included is a cell wall associated receptor kinase, WAK1 that spans the plasma membrane and has a cytoplasmic kinase domain and an amino-terminal domain tightly bound to the cell wall (He *et al.*, 1996). The extracytoplasmic domain of WAK1 contains several epidermal growth factor repeats (EGFs). WAK1 could therefore mediate cell wall-cytoplasm signaling. He *et al.* (1998) found that WAK1 has an essential role in maintaining

normal plant functioning during pathogen attack. During pathogen invasion, the activation of WAK1 may either prevent damage or activate a radical scavenging system (He *et al.*, 1998).

2.3.1.5 PR class.

The only member of this class is the *Arabidopsis* PR5K (PR5-like receptor kinase). The extracellular domain of PR5K is very homologous 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 suggested a possible interaction with common or related microbial targets.

2.3.1.6 Lectin class.

The *A. thaliana* lectin-like kinase gene (*LecRK1*) has an amino-terminal membrane-targeting signal sequence and a legume lectin-like extracellular domain which is homologous to carbohydrate-binding proteins (Hervè *et al.*, 1996). It also contains a single membrane-spanning domain and a characteristic serine/threonine protein kinase domain, *LecRK1* function is not yet known, but might be involved in signal transduction (Torii and Clark, 2000).

2.3.2 Examples of RLKs involved in plant defense.

2.3.2.1 Xa-21.

The *Xa-21* gene in rice provides resistance to the bacterial pathogen *Xanthomonas oryzae* (leaf blight) (Song *et al.*, 1995). It encodes an RLK with a serine/threonine protein kinase domain. *Xa-21* belongs to a multi-gene family which is situated on rice chromosome 11. Leaf-blight

disease resistance is proposed to be initiated by extracellular recognition of an elicitor. Based on the animal RLK-model this recognition is mediated by the LRR-motifs (Song *et al.*, 1995). In animal receptor kinases, a ligand binds to the extracellular domain and causes receptor dimerization, the activation of the cytoplasmic kinase domain by intermolecular phosphorylation and transduction of the signal to downstream effectors.

2.3.2.2 LRK10.

LRK10 is a RLK mapped to the *Lr10* disease resistance locus in wheat (Feuillet *et al.*, 1997). *LRK10* contains a unique type of extracellular domain which is not found in known plant or animal receptor kinases. Several conserved amino acid sequences in S-domain glycoproteins and receptor-like kinases were also found in *LRK10*. This suggested that *LRK10* and S-domain proteins belong to the same superfamily of recognition molecules in plants (Feuillet *et al.*, 1997).

2.3.2.3 At-RLK3.

The *At-RLK3* gene is present as a single copy within the *Arabidopsis* genome and its transcripts are detected in root, stem, leaf and flower (Czernic *et al.*, 1999). The *At-RLK3* extracellular domain lack similarity with any other receptor-like protein kinases. The *At-RLK3* gene is activated in response to SA, oxidative stress and pathogen infection.

2.3.2.4 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 chitinase 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.

2.3.2.5 *SR160*.

LRR-RLKs also play a role in disease resistance. *SR160* is a 160-kDa systemin cell-surface receptor that was identified in *Lycopersicon peruvianum* (Scheer and Ryan, 2002). Systemin causes a cascade of intracellular signaling events leading to the release of linolenic acid from membranes and its conversion to oxylipin molecules that signal defense gene expression (Scheer and Ryan, 2002). *SR160* and *BR1* showed similarity in the percentage of conservation of amino acids in its kinase domains (Morris and Walker, 2003). This indicated that the two receptors might have a downstream intracellular signaling component in common (Scheer and Ryan, 2002).

2.3.2.6 *FLS2*.

Another LRR-RLK involved in plants defense is flagellin sensitive 2 (*FLS2*) (Gomez-Gomez *et al.*, 1999). Plants carry systems to sense bacterial flagellin. An early transcriptional activation was found in *Arabidopsis* after treatment with *flg22*, a peptide corresponding to the most conserved domain of flagellin (Navarro *et al.*, 2004). The *flg22-FLS2* interaction leads to the production of reactive oxygen species (ROS) and the activation of MAPKs (Gomez-Gomez *et al.*, 1999).

2.3.2.7 *SFR2*.

The S-gene family receptor 2 (*SFR2*) is a novel member of the *Brassica* S-gene family (Pastuglia *et al.*, 1997). *SFR2* is induced in response to wounding and pathogen infection. *SFR2* mRNA also accumulates

rapidly after treatment with SA (Pastuglia *et al.*, 1997). This SFR2 induction pattern indicates that this gene plays a role in the defense signal transduction pathway.

2.3.3 Other protein kinases implicated in plant defense.

The *Pto* gene from tomato encodes a functional cytoplasmic serine/threonine protein kinase that interacts directly with the avirulence *AvrPto* protein to confer resistance to bacterial speck disease (Loh and Martin, 1995). *Pto* belongs to a small gene family which is situated on tomato chromosome five of tomato (Martin *et al.*, 1993).

Pto kinase activity is required for its role in disease resistance and *Pto* undergoes intra-molecular autophosphorylation on several sites (Zhou *et al.*, 1995). *Pto-AvrPto* recognition is postulated to activate the *Pto*-kinase and induce phosphorylation, including that of downstream components, which leads to the activation of defense responses (Zhou *et al.*, 1995) (Fig. 2.1). The *Fen* gene is located close to the *Pto* gene on chromosome 5 (Salmeron *et al.*, 1996). Similarity exists between fenthion-induced necrosis and the pathogen-induced HR (Salmeron *et al.*, 1996). This raised the possibility that fenthion is structurally similar to the elicitor molecule produced by the avirulent bacterium. Another gene, *Prf*, has been identified that is required for both *Pto*-mediated resistance and fenthion sensitivity. This gene encodes a protein with LRR, nucleotide binding site and leucine zipper motifs (Salmeron *et al.*, 1996).

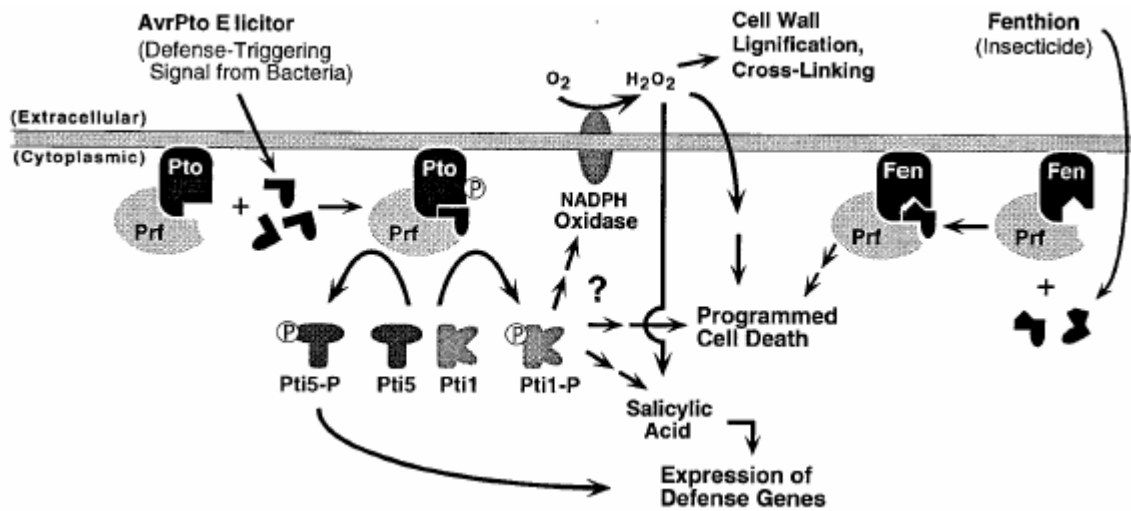


Figure 2.1. A model for the signaling pathways initiated when bacterial avirulence gene product AvrPto binds to the Pto plant resistance gene product Pto. Pto interacts with Prf, Pti1 and transcription factors Pti4, Pti5 and Pti6. Upon binding AvrPto, Pto autophosphorylates and then phosphorylates Pti1 and at least one transcription factor. Pti is involved in the signal transduction cascade, leading to the hypersensitive response. Pti 4, 5, 6 interact with promoters of genes encoding PR-proteins to initiate PR-gene expression (Bent, 1996).

Pto and *Fen* were isolated using map-based cloning and were found to belong to a clustered gene family of five to seven members (Zhou *et al.*, 1995). *Pto*-related sequences have also been detected in a wide spectrum of plant species. Thus, the signaling pathway involving a *Pto*-like gene may be widely conserved in the plant kingdom (Zhou *et al.*, 1995). In addition to Avr*Pto*, *Pto* also interacts with several plant proteins in the yeast two-hybrid system. To date two of these, *Pti1* (serine-threonine kinase) and *Pti4* (DNA binding protein), have been found to be specific phosphorylation targets for *Pto*. Such components are the protein kinase *Pti1* and transcription factors *Pti4*, *Pti5* and *Pti6*. (Zhou *et al.*, 1997).

2.4 MAPKs involved in signal transduction.

MAPK signaling cascades are utilized in yeast and mammals to convert extracellular stimuli into intracellular responses (Jonak *et al.*, 2002). MAP kinases are activated by MAP kinase kinases (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 MAP kinase kinase kinase (MAPKKK). Recent studies provided evidence that MAP kinases are also involved in plant signaling pathways, particularly during the activation of stress-associated responses (Sessa and Martin, 2000).

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 encodes an amino-acid sequence with high similarity to 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 activated rapidly upon treatment with

benzothiadiazole (BTH), as well as with dichloroisonicotinic acid, probenzole, 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 defense responses.

Protein phosphorylation is thus responsible for the modification of protein 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 defenses

A plant under pathogen attack (Fig. 2.2) triggers a multicomponent defense response pathway (Scheel, 1998). The activation of this response requires 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 defense genes that are responsible for the physiological processes that cause disease resistance.

In some cases resistance is manifested by the appearance of necrotic lesions localized at the site of infection. This localized cell death is termed the hypersensitive response (Scheel, 1998). The HR limits the spread of the pathogen throughout the infected plant by killing the infected cells.

The molecular response associated with HR include the production of ROS, the transient opening of ion channels, cell wall fortifications, the production of antimicrobial phytoalexins and the synthesis of

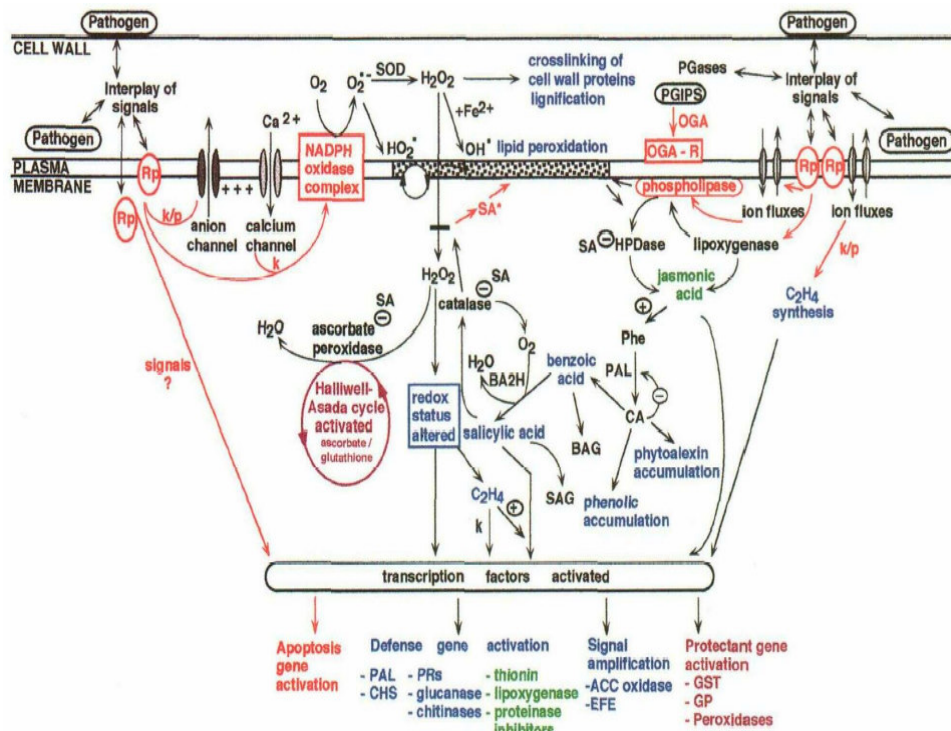


Figure 2.2 Complexity of signalling events controlling activation of defence responses

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; HO₂, hydroperoxyl radical; HPDase, hydroxyperoxide dehydrase; GP, glutathione peroxidase; GST, glutathione S-transferase; k, kinase; O₂⁻, superoxide anion; OH[•], hydroxyl radical; OGA and OGA-R, oligalacturonide fragments and receptor; P, phosphatase; PAL, phenylalanine ammonia-lyase; PGases, polygalacturonases; PGIPS, plant polygalacturonic acid inhibitor proteins; Phe, phenylalanine; PR, pathogenesis related; Rp, plant receptor protein; SA and SAG, salicylic acid and salicylic acid glucoside; SA*, SA radical; and SOD, superoxide dismutase (Hammond-Kosack and Jones, 1996).

pathogenesis-related (PR) proteins such as glucanases and chitinases (Scheel, 1998). Once the earliest defense responses have been activated, complex biochemical pathways within the responding cells are activated as new signaling molecules are generated.

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⁺ and Ca²⁺ were registered (Nürnbergger *et al.*, 1994). Ion channels that are activated as a result of an activated receptor together with an increased Ca²⁺ uptake, were shown to precede the oxidative burst (Zimmerman *et al.*, 1997). Transgenic tobacco cell cultures expressing the Ca²⁺ sensitive bioluminescent protein aequorin, showed an elevation of cytosolic Ca²⁺ levels after treatment with various non-specific elicitors (Chandra and Low, 1997). The kinetics of Ca²⁺ accumulation and application of Ca²⁺ ion channel blockers placed Ca²⁺ upstream of an oxidative burst and preceding HR.

2.5.2 Reactive oxygen species.

The production of ROS is another early response detected shortly after an attack by either a virulent or avirulent pathogen (De Gara *et al.*, 2003). A second prolonged ROS production, the oxidative burst, occurs in cells attacked by avirulent pathogens (De Gara *et al.*, 2003). The two-phase kinetics of ROS production is typical of incompatible plant-pathogen interactions that are characterized by HR. 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 superoxide (O_2^-). Apoplastic superoxide dismutase (SOD) iso-enzymes are then responsible for hydrogen peroxide (H_2O_2) production by means of superoxide dismutation (Fig. 2.2).

Mitochondria are also believed to be a major site for ROS production. Superoxide ions are generated during mitochondrial oxidative phosphorylation at complex III, the major site for mitochondrial ROS production (Fleury *et al.*, 2002). This site catalyses the conversion of molecular oxygen to the superoxide anion radical (O_2^-) by the transfer of a single electron to molecular oxygen. The inhibition of the respiratory chain, due to a lack of oxygen or the presence of an inhibitor such as cyanide or antimycin A, increases the ubi-semiquinone free radical level in the normal catalytic mechanism of complex III.

Both O_2^- and H_2O_2 are only moderately reactive and can cause cellular damage (Hammond-Kosack and Jones, 1996). 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 to the hydroperoxyl radical (HO_2^-). It can cross membranes easily and has the ability to attack fatty acids directly, resulting in membrane damage (Hammond-Kosack and Jones, 1996).

H_2O_2 can undergo the Fenton reaction in the presence of Fe^{3+} (Hammond-Kosack and Jones, 1996). This gives rise to an extremely destructive hydroxyl free radical (OH^-). It can initiate self-perpetuating lipid peroxidation (Fig. 2.3). If H_2O_2 enters the cell cytoplasm in sufficient concentrations, it could react with intracellular metal ions to give OH^- which is known to fragment DNA by site-specific attack. For this reason,

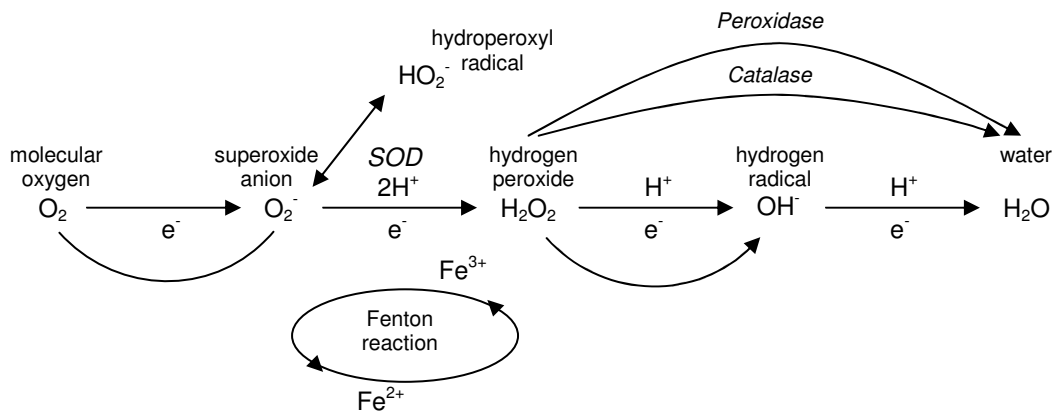


Figure 2.3. A general scheme of reactive oxygen species formation and the Fenton reaction (Hammond-Kosack and Jones, 1996).

ROS production can lead to damage to both the host and pathogen cells (Hammond-Kosack and Jones, 1996).

Nitric oxide (NO) is well known as a signal 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, two defense related genes PR-1 and phenylalanine ammonia lyase (PAL) were 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 O₂⁻ 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 defense gene activation (Durner *et al.*, 1998).

2.5.3 Cell wall fortification.

The fortification of the plant cell wall can increase resistance against microbes. 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*, 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 protoplasting 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 defenses such as the

production of phytoalexins, lytic enzymes and other antimicrobial proteins (Tenhaken *et al.*, 1995). More-over, 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.

Microbes produce a number of cell wall hydrolyzing enzymes, including pectinases, cellulases, xylanases and polygalacturonases (PGs). PGs are believed to contribute to cell wall softening by some necrotrophic fungi. Polygalacturonase-inhibiting proteins (PGIPs) inhibit PGs (De Lorenzo *et al.*, 2001) and have similar kinetics to PR-proteins. PGIPs may slow down the rate of hyphal extension so that other components of the defense response can be more effectively deployed (De Lorenzo *et al.*, 2001). PGIPs also possess a LRR domain similar to that predicted for several of the cloned R-gene products.

Rapid callose deposition in cell walls is frequently associated with sites of pathogen incompatibility (Tenhaken *et al.*, 1995). Callose deposition also occur when plant cell cultures are challenged with pathogen-derived elicitors or when plant tissue is mechanically wounded. The constitutive plasma membrane-localized callose synthase enzyme catalyses the formation of this β -1,3-glucan polymer and requires both increased levels of the primer β -furfuryl- β -glucoside and Ca^{2+} fluctuations. Blockage of plasmodesmata with callose is an essential component in the defense response and is required to impede cell-to-cell movement of viruses (Hammond-Kosack and Jones, 1996).

2.5.4 Lipoxygenase.

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 increase the induction of hypersensitive cell death (Montillet *et al.*, 2002).

2.5.5 Jasmonic acid.

An insect or microbial pathogen attack will lead to an interaction of elicitors with receptors which will initiate the octadecanoid-based pathway (Blechert *et al.*, 1995). In this pathway, JA is formed from the C₁₈ fatty acid linolenic acid. JA levels in leaves of *Vicia faba* increase immediately after infestation with *Spodoptera littoralis* (Framer and Ryan, 1992). The jasmonate induction was transient and decreased to background levels 4 h after the initial challenge. One can therefore assume that the lipid-derived signal compound, JA, is intracellularly induced and subsequently metabolized within this system.

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 defense related responses, as it is involved in signal transduction pathways in response to wounding and ultra violet (UV) radiation of intact plants.

2.5.6 Salicylic acid.

SA is a phenolic acid which induces defense responses upon primary infection and is instrumental in the activation of *PR*-genes (Chen *et al.*, 1995). Endogenous SA levels following TMV infection of tobacco

increased specifically during the resistance response (Malamy *et al.*, 1990). Moreover, 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. These results suggested that SA might play a role in the development of systemic as well as local resistance (Chen *et al.*, 1995).

An absolute requirement for SA has been demonstrated in *R* gene mediated resistance against various viruses, bacteria and fungi (Ryals *et al.*, 1996). Transgenic tobacco and *Arabidopsis* lines have been made that constitutively express a bacterial *nahG* gene encoding the enzyme salicylate hydroxylase (Gaffney *et al.*, 1993). Salicylate hydroxylase converts SA to catechol and these transgenic plants have markedly reduced levels of SA. The lack of SA accumulation in these *nahG*-expressing plants correlated with weakened local *R* gene mediated resistance responses, as well as a block in the induction of various defense genes (Delaney *et al.*, 1994). However, in tomato-*C. fulvum* interactions, the presence of the *nahG* gene does not compromise *Cf* gene-mediated resistance. Clearly, the role of SA in defense is complex and may also differ from species to species (Hammond-Kosack and Jones, 1996).

2.5.7 Phytoalexins.

Phytoalexins are low molecular weight, lipophilic, antimicrobial compounds that accumulate rapidly around sites of incompatible pathogen infections (Blechert *et al.*, 1995). It reacts in response to an extensive array of biotic and abiotic elicitors. Phytoalexin biosynthesis occurs after a diversion of primary metabolic precursors into novel secondary metabolic pathways. This diversion often arises from the

induction 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 ($G\alpha$) that has two domains. One contains a predominantly alpha helical secondary structure, while the other contain a GDP/GTP binding site, GTP hydrolytic activity and a covalently attached lipid that anchors this subunit to the bi-layer (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 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 defense responses could be G-protein mediated through plasmamembrane delimited pathways (Scheel, 1998).

2.5.9 Pathogenesis-related proteins.

PR-proteins (Table 2.1) are proteins encoded by the host plant whose expression is induced specifically in pathological or related situations

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	<i>Arabidopsis</i> THI2.1	thionin
PR-14	Barley LYP4	lipid-transfer protein

(Van Loon and Van strien, 1999). These enzymes do not only accumulate locally in the infected leaf, but are also induced systemically when associated with the development of SAR (Van Loon and Van Strien, 1999). PR-proteins belong to the family of 'stress-inducible' proteins. It was first discovered in tobacco. More than fourteen major classes are known (Van Loon and Van Strien, 1999).

Several PR-proteins possess either antifungal or antibacterial activity *in vitro* (Van Loon and Van Strien, 1999). The degradation or alteration of the fungal cell wall 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).

Sunflower PR-proteins share similar characteristics to most PR-proteins (Jung *et al.*, 1993). These 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.

In response to treatment with aspirin, sunflower plants produce proteins that possess all of the general properties of the PR-proteins (Jung *et al.*, 1993). Acidic PR-1, PR-3 and PR-5 and basic PR-2 and PR-5 proteins were detected in sunflower (Jung *et al.*, 1993). All of these proteins are excreted into the intercellular spaces. The possibility of PR-proteins accumulating inside the cells is unlikely, since no such proteins were detected in protoplasts when isolated from leave discs that produced PR-proteins. β -1,3-glucanase and chitinase activity was found in the same partially purified sunflower extracts. Their kinetics of induction was very similar to that in tobacco.

The mechanisms involved in defense responses also include the synthesis of chitinases (Robert *et al.*, 2002). These are a group of defense molecules for which a direct activity against pathogens has been demonstrated (Robert *et al.*, 2002). These enzymes are divided into six classes and each class is characterized by the different properties of the chitinases it includes (Melchers *et al.*, 1994).

2.6 The hypersensitive reaction.

The HR is defined as the death of host cells within a few hours after pathogen contact (Hammond-Kosack and Jones, 1996). The presence of the HR can range from a single cell to spreading necrotic areas. HR is a common mechanism deployed by plants against the attack of various pathogens (Tenhaken *et al.*, 1995). The incompatible plant responses are frequently associated with the appearance of necrotic flecks containing dead plant cells at the sites of attempted pathogen attack. ROS, JA, SA and proteins all are involved in the activation of the HR. HR plays a crucial role in disease resistance. If the pathogen form haustorial associations with the host, plant cell death would deprive the pathogen of access to further nutrients (Tenhaken *et al.*, 1995).

The production of H₂O₂ resembles inflammation responses in the immune system and the mechanism of H₂O₂ action in hypersensitive cell death may be related to apoptotic cell death in animals. The hypersensitive cell death in plants might then be a primitive eukaryotic cell defense mechanism from which both inflammation responses in the immune system and programmed cell death have evolved (Jabs and Slusarenko, 2000).

2.7 Systemic acquired resistance.

SAR is a secondary defense 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 defenses to prevent the spreading of infection. Mechanisms involved in SAR are 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).

Plant defense mechanisms are of great importance to the plants' immune system. These mechanisms include both the HR and SAR. After a pathogen attack, such as *P. helianthi*, these responses are necessary for the sunflower plant to minimize any further infection.

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 West 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 the warm-tempered regions, 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). Two insects are specific pests on sunflower. Grasshoppers, particularly *Zonocerus* spp in Africa causes damage, as well as the stem-borer, *Heteronychus* spp in eastern Africa (Weiss, 2000). The most damage is caused by pathogens that attack the bud, flower head or developing seeds of young plants. Feeding punctures can also lead to secondary infection by pathogenic fungi, in particular *Rhizopus* head rot (Weiss, 2000). Another important pathogen of sunflower is *Puccinia helianthi*, which causes leaf rust.

2.8.2 *Puccinia helianthi*.

A common and serious disease of sunflowers is leaf rust. This fungus can lead to a 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 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. Leaves with many pustules may wilt due to water loss through the ruptured leaf surface. Uredial pustules occur on both the upper and the 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. The aecia generally occur in groups of three to eight and are surrounded by a broad chlorotic border (Kolte, 1985).

Urediospores usually germinate within 4 h after inoculation. A germ

tube is issued from one (rarely from two) 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 penetrates through the stomata into the substomatal cavity. An H-shaped vesicle is formed into which the appressorium then empties its contents. Vesicle formation follows about 12 hours after inoculation (Sood and Saxton, 1970).

Haustorium development follows 24 h after inoculation. 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 than in susceptible plants (Sood and Saxton, 1970).

Mycelial growth is rapid in susceptible plants and reaches the lower epidermis within 96 h after inoculation. Mature hyphae form cushions of sporogenous tissue under the upper and lower epidermis. Urediospores are formed at about 144 h after inoculation. 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 Aim.

The commercially important sunflower plant is exposed to various pathogens including *P. helianthi* which causes leaf rust and results in a lower crop yield. Resistant plants are equipped with an early detection system that launches appropriate defenses. Upon activation of this

pathway, biochemical elements such as ROS, SA, JA and PR-proteins are synthesized that lead to HR and SAR (Scheel, 1998). In this pathway some key components, such as protein kinases, play an essential role. This role can determine whether the plant can defend itself against the pathogen or not. The aim of this study was therefore to identify any protein kinase gene that plays a key role in the early defense response of resistant sunflower plants against leaf rust.

Chapter Three:

Materials and Methods...

3.1 Materials:

Sunflower (*Helianthus annuus*) seeds, resistant [GH99PHRR3(VII)] and susceptible [GH99S37-388(VII)] to leaf rust (*Puccinia helianthi*), were used in this study. The S37-388RR susceptible sunflower cultivar was used to propagate the leaf rust. The UVPhe2 strain of *P. helianthi* leaf rust was used for all infections, as it is known to cause severe infection in sunflower cultivars.

Escherichia coli JM 109 [*endA1*, *recA1*, *gyrA96*, *thi*, *hsdR17* (*r_k⁻*, *m_k⁺*), *relA1*, *supE44*, Δ (*lac-proAB*), (*F'*, *traD36*, *proAB*, *laql^qZ Δ M15*)] competent cells (Promega) were used for the transformation experiments.

3.2 Methods:

3.2.1 Cultivation of sunflower plants.

Fifteen sunflower seeds of each cultivar were planted in a 2:1 soil mixture of peat moss and potting soil in seedling trays and germinated at $\pm 24^{\circ}\text{C}$ in a greenhouse. When the seedlings were 2-3 cm in height, they were transplanted into pots containing potting soil and fertilized three times per week with a hydroponic nutrient solution (6.5% N, 2.7% P, 13.0% K, 7.0% Ca, 2.2% Mg, 7.5% S, 15% Fe, 0.024% B, 0.024% Mn, 0.005% Zn, 0.002% Cu, 0.001% Mo).

The S37-388RR susceptible sunflower cultivar was treated with $30 \mu\text{g}\cdot\text{ml}^{-1}$ meloic hydrozide when the seedlings were 2 weeks old to retard the growth of the plants, to simplify the propagation of the leaf rust.

3.2.2 Infection of sunflower with leaf rust (*Puccinia helianthi*).

The S37-388RR susceptible plants were inoculated with leaf rust spores when the plants were two weeks old. A concentrated solution of rust spores that were resuspended in kerosene oil was sprayed under high pressure onto the dorsal and ventral sides of the leaves. The plants were left to dry for 30 min and were then placed in a dark Dew-simulation-chamber for 16 h to allow the rust to germinate at 22°C to 24°C. The plants were then moved to the greenhouse with normal day and night cycles. Mature rust spores were collected two weeks later. A concentrated suspension of these rust spores in water containing 0.05% (v/v) polyoxyethylensorbitanmonolaurat (Tween 20) was used to similarly infect the resistant and susceptible sunflower cultivars. The control plants were sprayed with water containing 0.05% (v/v) Tween 20. 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 another 24 h. The infected leaves were randomly harvested, quickly frozen in liquid nitrogen and stored at -80°C.

3.2.3 Total RNA extraction from infected leaves.

Distilled water was treated with 0.1% (v/v) dimethylpyrocarbonate (DMPC), left overnight and then autoclaved at 121°C for 20 min. All pestles, mortars and spatulas were first washed with soap and then in 10% (w/v) sodium dodecyl sulphate (SDS) in DMPC treated water. It was wrapped in aluminum foil and oven-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.

Plant material was ground into a fine powder in liquid nitrogen, transferred to 1.5 ml micro-centrifuge tubes and stored at -80°C.

Total RNA was extracted from 0.5 g ground sunflower tissue using the Tripure isolation reagent (Roche) according to the manufacturers' specifications. The RNA was finally dissolved in 100 µl DMPC treated water. The concentration was determined as described (Sambrook *et al.*, 1989) and expressed as µg.ml⁻¹.

A 1% (w/v) agarose gel containing 0.5 µg.ml⁻¹ ethidium bromide in 0.5 × TAE [20 mM hydroxymethylaminomethane-HCl (Tris-HCl) pH 8, 0.5 mM ethylenedinitrilotetraacetic acid (EDTA), 0.28% (v/v) acetic acid] was used to separate the RNA. Five hundred ng total RNA was dissolved in 0.015% (w/v) bromophenol blue, 2.5% (w/v) ficoll and resolved at 12 V.cm⁻¹ using 0.5 × TAE as running buffer. The RNA was visualized under UV illumination and photographed.

3.2.4 DDRT-PCR amplification of differentially expressed putative protein kinase genes.

The Titan one tube reverse transcription polymerase chain reaction (RT-PCR) system (Roche) was used to amplify differentially expressed putative protein kinase genes. In order to amplify these genes, two degenerate primers were designed (Table 3.1). Protein kinases contain a catalytic domain with residues that are highly conserved (Hanks *et al.*, 1988). Bovis 22 coded for the consensus amino acid sequence of subdomain VIb of the kinase domains of RLKs from monocotyledonous plants, while Bovis 23 coded for the similar subdomain from dicotyledonous plants. During the RT-PCR reaction, an anchored oligo-dT primer (Bovis 32) containing an additional 5' tail sequence was used for first strand cDNA synthesis. During the PCR step, Bovis 39,

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

Primer	Sequence	T _m -value
Bovis 22	5' GAY ATH AAR CCN CAY AAY 3'	46.4
Bovis 23	5' GAY GTN AAR CCN GAR AAY 3'	49.7
Bovis 26	5' CAA CTT TCG ATG GTA GGA TAG 3'	51.3
Bovis 27	5' CTC GTT AAG GGA TTT AGA TTG 3'	49.4
Bovis 32	5' GAA GAA TTC TCG AGC GGC CGC TTT TTT TTT TTT TTT TVN 3'	65.5
Bovis 39	5' GAA GAA TTC TCG AGC GGC 3'	53.9
Bovis 48	5' TCG CCG GAG AAT GTG ATT T 3'	55.0
Bovis 70	5'-/Phos/ AAC AAA CAA TTG CCC 3'	43.9
Bovis 71	5' TTA GTG AAT AGT GAC TCG CC 3'	51.6
Bovis 72	5' AAA TGA AAC AAG GGT GGT C 3'	52.8
Bovis 73	5' GTC GTT GTT CAA CCA ACT CC 3'	54.1
Bovis 74	5' ATC ATT CCA CCA CTC ATC GT 3'	53.7
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		

whose sequence was identical to this 5'-tail, was used in combination with Bovis 22 and 23 respectively to amplify putative protein kinase genes.

The RT-PCR reactions were done using total RNA isolated from resistant sunflower plants harvested at different time intervals after infection (3.2.3). Each reaction contained 5 ng total RNA, 2.5 pmol of Bovis 22, Bovis 32 and Bovis 39 respectively, 2.5 mM MgCl₂, 6 mM dithiothreitol (DTT), 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dTTP, 0.5 mM dCTP and 10 µCi [α -³²P]dCTP, 4 mM Tris-HCl pH 7.5, 100 mM KCl, 0.1 mM EDTA, 0.5% (v/v) Tween 20, 0.5% (v/v) 4-nonylphenolpolyethylenglycol (Nonidet P40), 10% (v/v) glycerol and 1 µl Expand high fidelity enzyme mix (Roche) per 25 µl reaction. In the second set of reactions, Bovis 22 was replaced with Bovis 23. The amplification regime for the reactions was one cycle at 37°C for 30 min and 94°C for 2 min. Twenty-five cycles then followed at 94°C for 10 sec, 37°C for 1 min, 68°C for 4 min. Ten more cycles followed where an additional five seconds was added to the extension steps with a final cycle of 68°C for 7 min.

A 4% (v/v) denaturing poly-acrylamide gel was used to separate the RT-PCR products. The gel consisted of 8 M urea and 4% (v/v) Long ranger (FMC Bioproducts) gel solution in 0.6 × TBE (6.48 mM Tris-HCl pH 8, 6.48 mM boric acid, 0.144 mM EDTA). The gel was polymerized by adding 4.5 µl.ml⁻¹ ammoniumperoxodisulfate (APS) and 0.55 µl.ml⁻¹ N,N,N',N',tetramethylethylenediamin (Temed). A 0.6 × TBE running buffer was used for the separation of the fragments. The amplified cDNA was dissolved in 0.0125% (w/v) bromophenol blue, 50% (v/v) formamide and 0.0125% (w/v) orange G, boiled for 5 min and then loaded onto the gel.

The cDNA was resolved for 2 1/4 h at 60 W, the gel dried and exposed to a X-ray film for six days.

3.2.5 Cloning of differentially expressed cDNA fragments.

After development, the X-ray film was studied and all the differentially expressed cDNA bands were marked and numbered. The film was aligned with the dried acrylamide gel and the marked bands were cut from the gel and placed in microcentrifuge tubes. To each gel piece, 100 µl TE (10 mM Tris-HCl pH 8, 1 mM EDTA) was added. The gel was ground to a fine paste using a glass rod that was first rinsed in 50% (v/v) HCl and then washed with water. The tubes were incubated at room temperature for 10 min and then boiled for 10 min. After centrifuging the tubes for 10 min at 12 000xg, the supernatant was transferred to a new tube and stored at -20°C.

The cDNA bands were re-amplified using the same primer combinations as previously described, but omitting Bovis 32. The PCR reactions consisted of 1-2 µl of the recovered cDNA, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01% (w/v) gelatin, 25 pmol of the respective primers, 0.25 mM deoxyribonucleotidetriphosphates (dNTPs), 2.5 U Taq-polymerase and 10% (v/v) dimethyl sulfoxide (DMSO). The amplification conditions were one cycle at 94°C for 5 min and then 94°C for 1 min, 44°C for 30 sec and 72°C for 1 min, which was repeated 30 times. The last step was for 10 min at 72°C.

The PCR-products were resolved on a 1.2% (w/v) agarose gel (3.2.3). The amplified fragments were cut from the gel and purified with the GFX PCR DNA and gel band purification kit (Amersham) as specified by

the manufacturer. The DNA was finally eluted in 50 µl water and lyophilized.

The re-amplified fragments were cloned into the pGEM-T Easy vector (Promega) according to the manufacturers' specifications. To the cDNA fragments, 12.5 ng pGEM-T easy vector DNA was added in the presence of 30 mM Tris-HCl pH 7.8, 10 mM MgCl₂, 10 mM DTT, 1 mM adenosine triphosphate (ATP), 10% (w/v) polyethylene glycol 4000 (PEG 4000) and 3.0 Weiss units T4 DNA ligase. The tubes were incubated overnight at 4°C.

E. coli JM109 was transformed with the ligation mixture using ultracompetent cells from Promega. To each 10 µl ligation reaction, 25 µl competent cells were added and mixed. The tubes were incubated on ice for 20 min and then heat shocked for 50 sec at 42°C. After adding 950 µl LB-medium [1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl], the tubes were incubated at 37°C for 1½ h while shaking vigorously.

The cells were finally 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). The plates were incubated overnight at 37°C (Sambrook *et al.*, 1989).

White colonies were inoculated in 5 ml LB-medium containing 50 µg.ml⁻¹ ampicillin and shaken at 37°C overnight. Plasmid DNA was extracted from the cells using the GFX micro plasmid prep kit (Amersham) according to the manufacturers' specifications.

To confirm the presence of an insert in the plasmids, 5 µl of each extract was digested with 10 U *EcoRI* in the presence of 50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 100 mM NaCl, 1 mM dithioerythritol (DTE) for 1 h at 37°C. The digested plasmid was then resolved on a 1% (w/v) agarose gel (3.2.3).

3.2.6 Expression analysis of the D15 cDNA clone.

To confirm the induced expression of the isolated cDNA fragments, reverse Northern blots were performed. Of each recombinant plasmid, 200 ng DNA was digested with 10 U *SacI* in the presence of 33 mM Tris-acetate pH 7.9, 10 mM Mg-acetate, 66 mM potassium-acetate, 0.5 mM DTT at 37°C for 1 h. A recombinant plasmid containing the actin gene was included as an internal control and was treated similarly. After boiling the samples for 5 min, an equal volume 6 × SSC (0.9 M NaCl, 0.09 M sodium citrate, pH 7.0) was added to each sample. The samples were then transferred to a nylon membrane using a slot blot apparatus. The membrane was washed 3 times with 6 × SSC and then dried at 70°C for 15 min.

The nylon membranes were quenched in 50% (v/v) formamide, 5 × SSPE [0.75 M NaCl, 0.05 M NaH₂PO₄ pH 7.4], 0.5% (w/v) SDS, 5 × denhardtts [1% (w/v) polyvinylpyrrolidone (PVP), 1% (w/v) Ficoll, 1% (w/v) bovine serum albumin (BSA)] and 1 µg.ml⁻¹ salmon-sperm DNA for 2 h at 42°C.

First strand cDNA probes were prepared from total RNA isolated from tissue harvested for the first four time-intervals of both infected resistant (IR) and infected susceptible (IS) plants. For each time-interval, 15 µg total RNA was used to purify mRNA using the mRNA capture kit (Roche) according to manufacturers' specifications. First strand cDNA was

synthesized when 1 × ImProm-II™ Reaction buffer (Promega), 3 mM MgCl₂, 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dTTP, 25 μCi [α -³²P]dCTP and 200 U reverse transcriptase (Promega) were added to the captured mRNA. The reactions were incubated at 42°C for 1 h to synthesize first strand cDNA. The reactions were denatured for 5 min at 94°C, the denatured probe was added to fresh hybridization solution which was then added to the membranes. Hybridization was done overnight at 42°C.

The membrane was first washed twice with 20 ml wash buffer 1 [2 × SSC (0.3 M NaCl, 0.03 M sodium citrate), 1% (w/v) SDS] for 5 min at 25°C and then twice with 20 ml wash buffer 2 [0.5 × SSC (0.075 M NaCl, 0.0075 M sodium citrate), 1% (w/v) SDS] for 15 min at 50°C. The hybridization signals were quantified using a scintillation counter when each hybridized cDNA clone was cut from the membrane and counted using the Cerenkov-channel of a liquid scintillation counter. Actin was used as the standard value and the cpm value of each clone was normalized to that of actin. These values for each time-interval were then expressed relative to that of time 0 h.

Four clones were selected on the basis of their induction profiles and the cloned inserts were sequenced at the University of Stellenbosch. The plasmid DNA was first deleted from the obtained sequences, where after the sequences were translated to amino acid sequences using (www.au.expasy.org/tools/dna.html). The largest open reading frame for each clone was used to search for homologous sequences on NCBI using the basic local alignment search tool (BLAST) tool (www.ncbi.nlm.nih.gov/). The four sequences with the highest homology to each clone were re-aligned with each respective clone using CLUSTALW (www.ebi.ac.uk/clustalw/) to confirm

the homology. A primer complementary to the 5' end of clone D15 called Bovis 48 (Table 3.1), was then designed (<http://seq.yeastgenome.org/cgi-bin/web-primer>) to confirm the expression of the gene using RT-PCR.

As a control, the 18S rRNA was amplified using Bovis 26 and 27 as primer (Table 3.1). The RT-PCR reactions were done as described (3.2.4) using 10 ng total RNA and 25 pmol of each primer. The amplification regime for the reactions was 42°C for 30 min, followed by 25 cycles at 94°C for 10 sec, 48°C for 30 sec and 68°C for 2 min. Ten more cycles followed where an additional 5 seconds were added to the extension steps, followed by a final step at 68°C for 10 min.

The expression of clone D15 was similarly characterized. The amplification regime was the same as for 18S rRNA, but with an annealing temperature of 56°C. Bovis 32 was used for first strand cDNA synthesis, while Bovis 39 and 48 were only added after the RT step was completed (Table 3.1). The clone's expression was tested in both infected resistant and susceptible plants.

3.2.7 5'-RACE of D15 clone.

In order to identify the full length gene, the unknown 5' end region of clone D15 was amplified using the 5' Full RACE core set kit (Takara Bio Inc) according to the manufacturers' specifications, but with a few modifications.

Primers designed to perform RACE included a 5' phosphorylated primer for the reverse transcription of infected resistant (IR) total RNA (Fig. 3.1a)

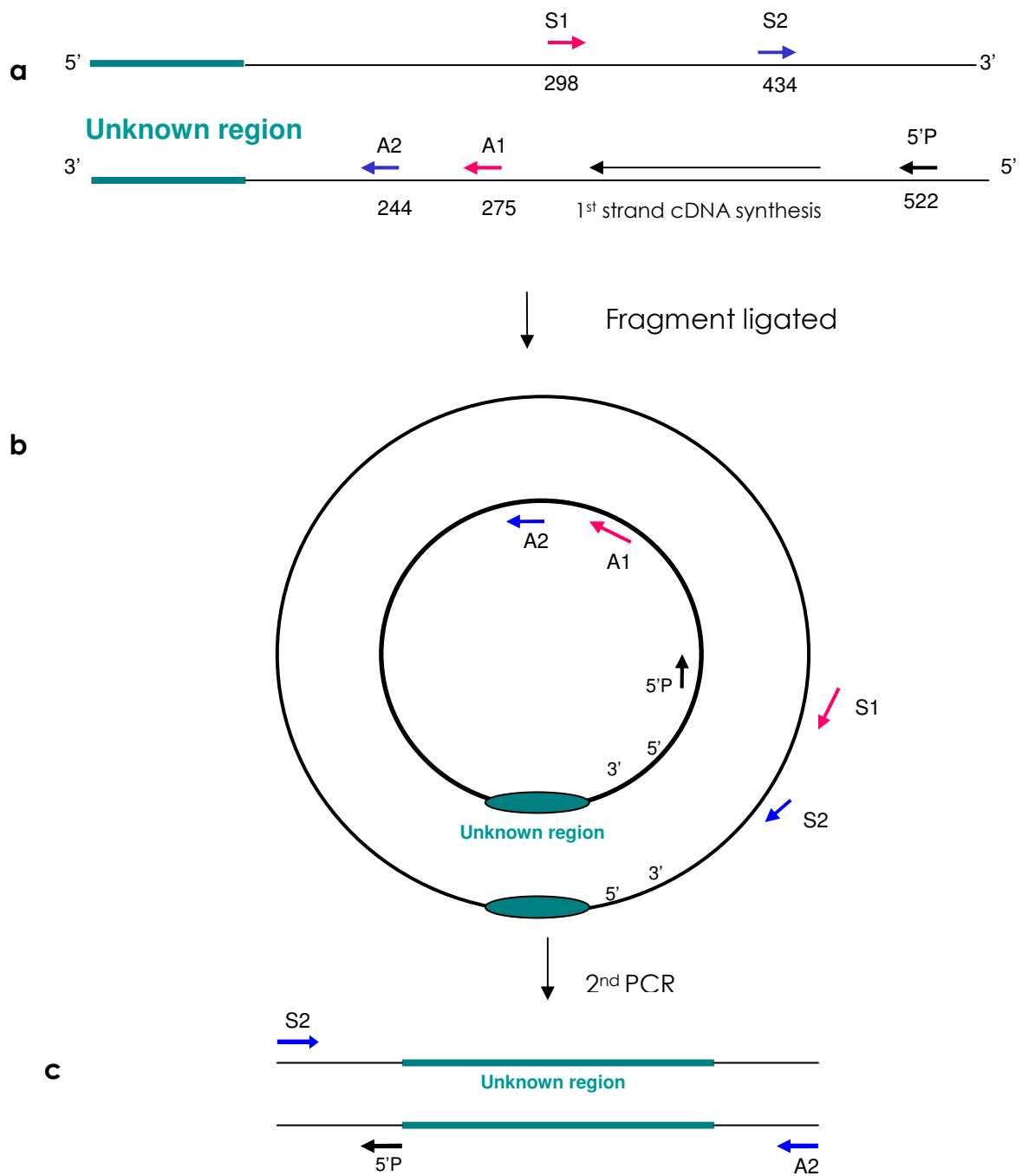


Fig. 3.1 Schematic presentation of 5'-RACE. The primers and its direction of amplification are indicated by the arrows. The orientation of the designed primers is indicated in (a), the results of the ligation reaction are indicated in (b), while the amplification strategy is indicated in (c).

As this primer has a phosphate-group linked to the 5' end, it would enable the single-stranded cDNA fragment to be self ligated at its ends or to form concatemers (Fig. 3.1b). Two sense (Bovis 71 and Bovis 72) and two anti-sense (Bovis 73 and Bovis 74) (Table 3.1) primers were also designed to perform the first and second strand amplification steps (Fig. 3.1c). First strand cDNA synthesis and the ligation of the amplified single-strand cDNA fragment, were performed according to the manufacturers specifications. After the ligation of the cDNA fragment, the first amplification step (PCR-1) was done using 1 µl of the ligation mixture in the presense of 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01% (w/v) gelatin, 0.4 mM dNTPs, 2.5 U Taq-polymerase, 10% (v/v) DMSO and 20 pmol of Bovis 71 and Bovis 73 each. The amplification conditions were one cycle of 94°C for 3 min and 25 cycles of 94°C for 30 sec, 51°C for 30 sec and 72°C for 2 min. The second amplification step (PCR-2) was done similarly by using 1 µl of the PCR-1 reaction and Bovis 72 and Bovis 74 as primers (Table 3.1). The annealing temperature was 52°C. An additional third amplification step was done using 1 µl of PCR-2 and Bovis 72 and Bovis 74.

The products of the PCR reactions were resolved on an agarose gel (3.2.3) and the single 2 kb DNA fragment from PCR-3 was purified from the gel using the GFX PCR DNA and gel band purification kit (Amersham) as specified by the manufacturer. The fragment was cloned into the pGEM-T easy vector (3.2.5) and then again digested from it to confirm the successful cloning thereof. The fragment was then sequenced at the University of Stellenbosch. Sequence analysis was performed as described (3.2.6).

3.2.8 Genomic DNA extraction.

Genomic DNA was extracted from resistant and susceptible sunflower cultivars. Frozen plant material was resuspended in 0.05 M cetyltrimethylammoniumbromide (CTAB), 1.4 M NaCl, 0.1 M Tris-HCl pH 8, 20 mM EDTA and 10 $\mu\text{g}\cdot\text{ml}^{-1}$ RNase. The mixture was incubated at 60°C for 30 min, centrifuged at 12 100 \times g for 10 min and the supernatant extracted once with chloroform. After centrifugation, the DNA was precipitated with two volumes ice cold 100% (v/v) EtOH for 2 h at -20°C. After centrifugation, the DNA pellet was washed once with 70% (v/v) EtOH, air dried and finally dissolved in distilled water. The concentration was determined and expressed as $\mu\text{g}\cdot\text{ml}^{-1}$ (Sambrook *et al.*, 1989).

3.2.9 Southern blot analysis of the D15 cDNA clone.

Isolated genomic DNA (25 μg) from resistant and susceptible plants respectively was digested with 80 U *SacI* as described (3.2.6) and 80 U *EcoRI* (3.2.5) respectively for 36 h. The samples were lyophilized, resuspended in 60 μl distilled water containing 0.015% (w/v) bromophenol blue, 2.5% (w/v) Ficoll and resolved on a 0.8% (w/v) agarose gel (3.2.3). As a control, 10 ng *EcoRI* digested plasmid containing clone D15 was also resolved on the gel. The gel was photographed and the DNA denatured in 3 M NaCl, 0.4 M NaOH for 45 min. The DNA was transferred to a nylon membrane using 1.5 M NaCl, 0.4 M NaOH for 4 h (Chomczynski, 1992). The membrane was neutralized in a 0.2 M sodium phosphate buffer pH 6.8 for 10 min and dried at 70°C for 15 min. The membrane was then pre-hybridized in 10 ml Ultra sensitive hybridization buffer (Ambion) for 1 h at 42°C.

The probe was prepared by digesting 2 µg of the recombinant D15 plasmid DNA with 20 U *EcoRI* as described (3.2.5). The digested plasmid DNA was resolved on an agarose gel (3.2.3) and the insert purified from the gel using the GFX DNA and gel band purification kit (Amersham) according to the manufacturers' specifications. The DNA was eluted in a final volume of 50 µl. The DNA fragment was denatured at 95°C for 5 min and cooled on ice for 5 min. The denatured DNA was then labeled with the Rediprime II random prime labeling system (Amersham) using 40 µCi [α -³²P]-dCTP. The labeled probe was purified using a Sephadex G75 column according to Sambrook *et al.* (1989).

After denaturing the 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 membrane was washed twice with 25 ml wash buffer 1 [2 × SSC, 0.1% (w/v) SDS] at 42°C for 5 min and then twice with 25 ml wash buffer 2 [0.1 × SSC, 0.1% (w/v) SDS] at 42°C for 15 min. The membrane was then exposed to an x-ray film for 7 days. After developing the x-ray film, it was photographed and the approximate sizes of the hybridized signals were calculated.

Chapter Four:

Results...



4.1 Infection of sunflower with leaf rust.

In order to clone a protein kinase gene involved in the perception of leaf rust in sunflower, resistant and susceptible sunflowers were infected with the leaf rust fungus (Fig. 4.1). The uninfected control plants remained healthy with no visible disease symptoms for the duration of the study. No observable differences were found in the rust germination efficiencies on the leaves of susceptible and resistant plants shortly after infection. 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 6 days post infection. These cinnamon-brown pustules were surrounded by a chlorotic border which indicated that the chloroplasts had degenerated in the infected and neighboring cells. In contrast with the highly infected susceptible plants, the infected resistant plants showed signs of infection but the effects of the disease were limited. Chlorotic flecks were visible on the resistant leaves, but far less rust pustules were formed. Judging by the conditions of the plants 2 weeks after infection, the resistant plants were in a much healthier condition than the susceptible plants. Since the infection of the plants was optimal, the infected resistant plants were deemed suitable to be used for the subsequent cloning of differentially expressed genes.

4.2 Isolation of differentially expressed putative protein kinase genes.

In order to isolate differentially expressed genes using differential display RT-PCR (DDRT-PCR), total RNA was extracted from the infected resistant plants harvested at different time-intervals (Fig. 4.1). Since the success of DDRT-PCR depended on the quality of the template mRNAs, 500 ng total RNA extracted from leaf tissue harvested at different time intervals was resolved on an agarose gel to assess the

a



b



Fig. 4.1 Leaf rust infection of sunflowers. In (a), leaves from uninfected (left) and infected (right) resistant plants are shown. (b) represents leaves from the susceptible cultivar that was similarly treated.

quality and quantity thereof. As can be seen in Fig. 4.2, the uniform intensities of the resolved RNA meant that the concentrations of the different extractions were accurate and consistent. Furthermore, several discrete bands were visible in each lane. These bands represented the different rRNA molecules present in each cell. The fact that the bands were intact with a slight smear present in the background, indicated that the extracted RNA was of good quality with very little degradation that occurred. The visible background smear represented the mRNA molecules. The extracted RNA was therefore used for further studies.

DDRT-PCR was used to amplify and identify putative protein kinase genes from sunflower that are involved in the perception of the leaf rust fungus shortly after infection. RNA from infected resistant plants was used to perform the DDRT-PCR. Differentially expressed cDNA fragments were visible for both the monocot and dicot primer combinations used during the DDRT-PCR (Fig. 4.3). A total of 33 differentially expressed cDNAs were selected, 12 for the monocot primer combination and 21 for the dicot primer combination. Hence the specificity of the primers in monocot and dicot plants, the fragments amplified by these two primers will be referred to as monocot and dicot fragments. These fragments were up-regulated at different time-intervals after infection with a subsequent down regulation at a later stage. The monocot and dicot primer combinations yielded differentially expressed cDNA fragments whose expression was up-regulated as early as 3 h.p.i. These up-regulated levels continued for up to 21 h.p.i. Most of the identified monocot cDNAs were smaller than 831 bp, while most of the dicot cDNAs were larger than 1904 bp.

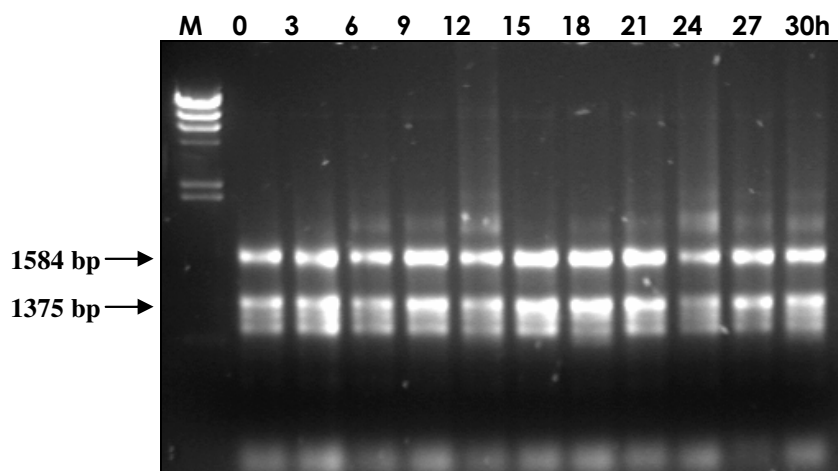


Fig. 4.2 Extracted total RNA from infected resistant plants.
The time-intervals used and DNA marker size (M) are as indicated.

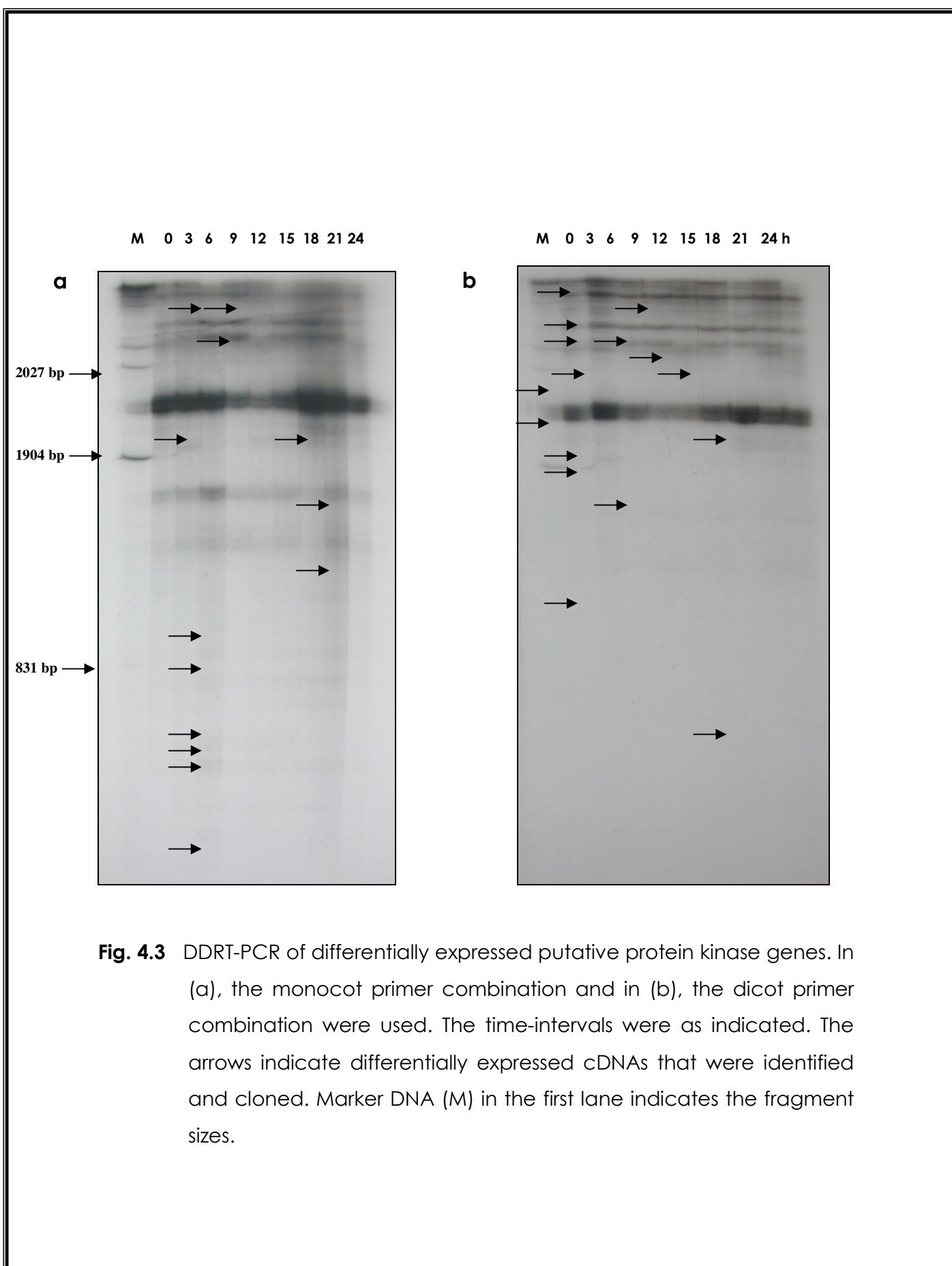


Fig. 4.3 DDRT-PCR of differentially expressed putative protein kinase genes. In (a), the monocot primer combination and in (b), the dicot primer combination were used. The time-intervals were as indicated. The arrows indicate differentially expressed cDNAs that were identified and cloned. Marker DNA (M) in the first lane indicates the fragment sizes.

4.3 Cloning of the cDNA fragments.

After recovering the cDNAs from the dried gel, they were re-amplified using the respective primer combinations as for the DDRT-PCR. Four of the selected cDNAs however failed to be re-amplified and were subsequently discarded. The amplified PCR products were ligated into the pGemT-Easy plasmid vector. After *E. coli* was transformed with the ligation mixture, white and blue colonies were obtained for all the cloned fragments (Fig. 4.4). While white colonies represented bacterial cells containing recombinant plasmids, the blue ones contained non-recombinant plasmids. For each cloned fragment, three colonies were selected for further analysis.

To confirm the presence of the cloned inserts in the recombinant plasmids, the inserts were digested from the recombinant plasmid DNA with *EcoRI* and resolved on an agarose gel. On the first gel (Fig. 4.5a), the first two lanes represent non-recombinant plasmid DNA that was undigested (ud) (lane 1) and digested (d) with *EcoRI* (lane 2). Each recombinant plasmid produced two fragments. The larger of the two was the same size as the digested non-recombinant plasmid, indicating that the top fragment represented the plasmid DNA. Therefore, all the smaller fragments were insert DNA that was digested from the different plasmids. The monocot fragments (M2 - M13) that were digested from the recombinant plasmids showed a decline in size which correlated with the DDRT-PCR gel profile from which the fragments were re-amplified (Fig. 4.5a). Clones M2 and M3 each produced two insert fragments after digestion. This could be due to an internal *EcoRI* restriction site within the insert which will result in two smaller bands after digestion. All the other clones produced a single insert fragment. The 21 dicot fragments are shown in Fig. 4.5(b). The inserts showed a similar digestion profile as the monocot fragments,

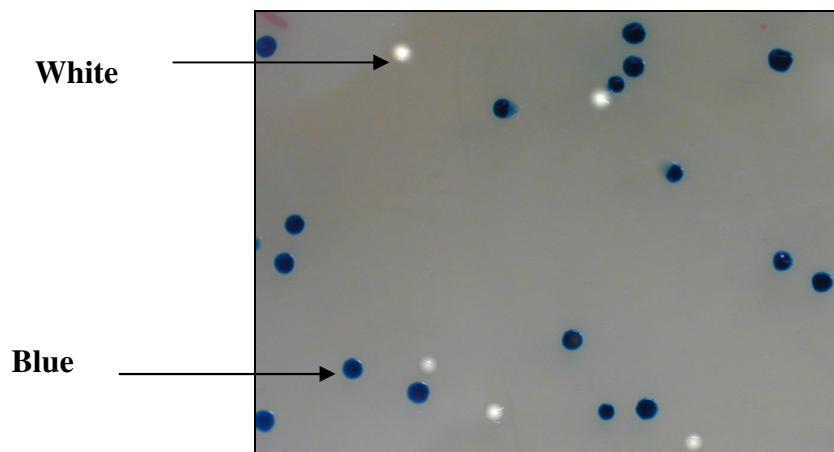


Fig. 4.4 Selection of recombinant plasmids using α -complementation. White colonies contain recombinant plasmids and blue colonies contain non-recombinant plasmids.

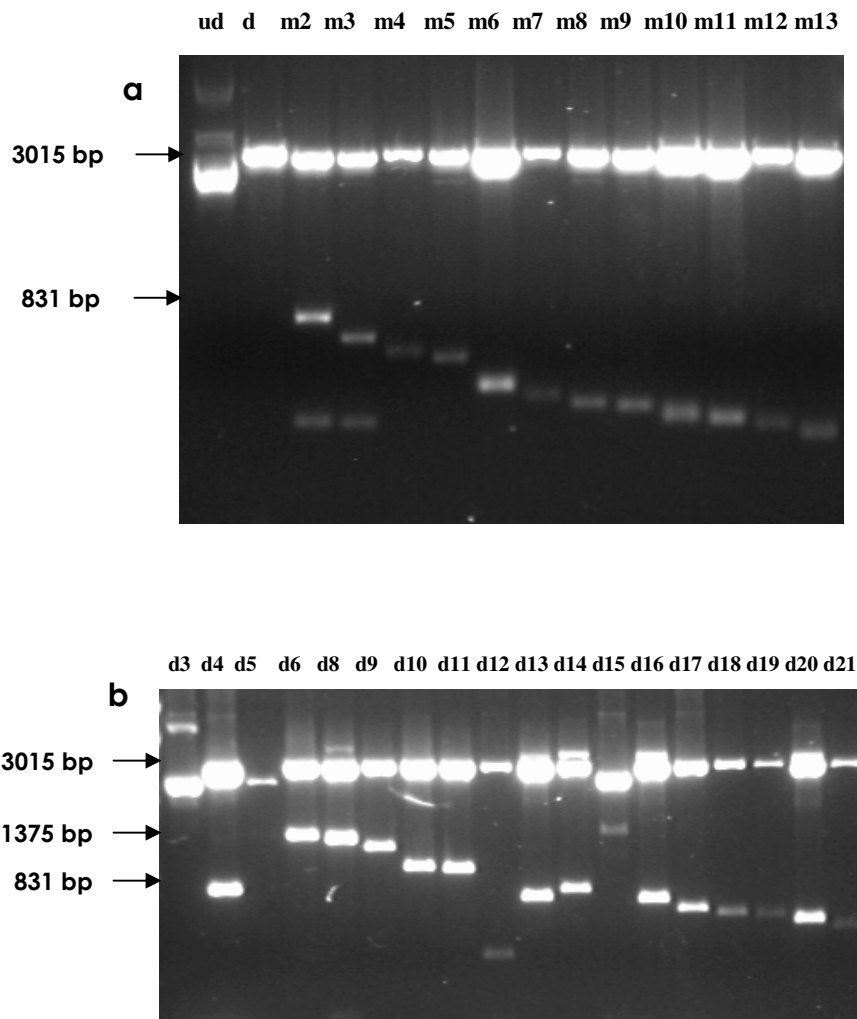


Fig. 4.5 Restriction analysis of recombinant plasmids. In (a), recombinant plasmids containing the monocot cDNA fragments digested with *Eco*RI. In (b), recombinant plasmids containing the dicot cDNA fragments digested with *Eco*RI. The size of the digested plasmid DNA is as indicated.

which also correlated with the DDRT-PCR gel. Clone D13 was again slightly smaller than D14, also probably due to an internal site *EcoRI* restriction site. The second fragment was however probably very small and was therefore not visible on the gel. Clones D4 and D12 had smaller inserts than expected. Since the digestion yielded only a single insert fragment, one can assume that an internal *EcoRI* restriction site cleaved the insert internally, yielding a single visible fragment consisting of the two equally sized fragments. Clones D3, D5 and D15 were most probably undigested, as the plasmid DNA were similar in size than the undigested control plasmid DNA in lane 1 of Fig. 4.5b.

4.4 Expression analysis of the identified cDNA clones.

Since DDRT-PCR is known to produce false positives, the expression of each identified cDNA clone was reconfirmed using reverse Northern blot analysis. This was done using different cDNA probes that were prepared using total RNA isolated from infected resistant (IR) and infected susceptible (IS) plants respectively. The aim was to determine whether the cDNA fragments were expressed differently in the IR and IS plants. The expression of all the cloned cDNA fragments was expressed relative to that of the actin gene which was used as an internal control. By doing this, it was possible to accurately quantify the expression levels of the cloned cDNA fragments.

Of the dicot fragments, clone D15 showed a rapid and significant 2.5 fold induction 6 h.p.i. with an immediate down regulation 9 h.p.i. (Fig. 4.6). This induction was limited to the resistant plants with the susceptible plants showing constant expression of the gene. The other dicot clones showed very little changes of gene expression in IR and IS plants. Several of the dicot clones did show induced expression in

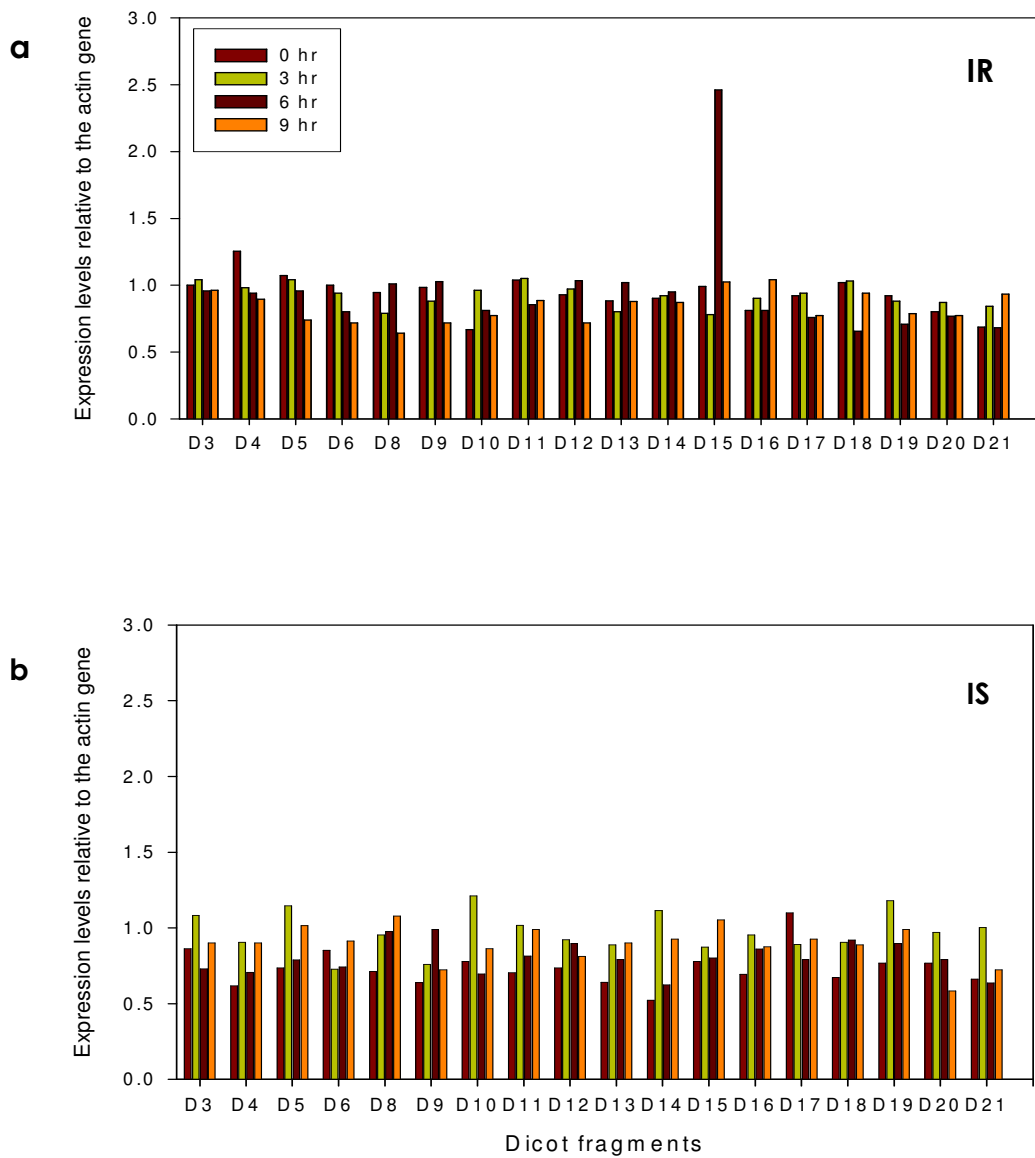


Fig. 4.6 Expression patterns of the isolated dicot cDNA fragments. In (a), RNA from infected resistant plants and in (b), RNA from infected susceptible plants were used to generate the cDNA probes. The clones used were as indicated.

the IR, but not in the IS plants. The most obvious was D14 whose expression level almost doubled at 3 h.p.i. in IS plants. Clones D5, D10, D14, D19 and D21 also showed potential, as they were up- and down-regulated within very short time spans.

In contrast to the dicot fragments, most of the monocot fragments showed a greater level of induction of gene expression in the IS plants compared to that in the IR plants (Fig. 4.7). Based on their unique induction profiles, three clones, M4, M7 and M9 were selected for further study. Clone M4 showed an induction of expression as early as 3 h.p.i., with an immediate down regulation at 6 h.p.i. in the susceptible plants. No such induction was seen in the resistant plants. Clones M7 and M9 were selected since both showed induced expression in both resistant and susceptible plants at 3 h.p.i. In the case of M9, the induced expression was three hours earlier in the susceptible plants compared to the resistant plants.

It is important to mention the low levels of induced expression that was found. Even though the induced levels were not that big, it must be kept in mind that the number of cells involved in the induced expression of these genes was only a fraction of the total number of cells harvested. This induced expression levels will therefore be much higher should one work for instance on cell suspension cultures. Any doubling in the induced expression of a gene can therefore be significant. Because of their unique expression patterns, it was thus decided to concentrate the remainder of the study on clones D15, M4, M7 and M9. Later studies would however also include clones like D5, D10, D14 and M13, since their specific induction could help to elucidate the defense system of sunflower.

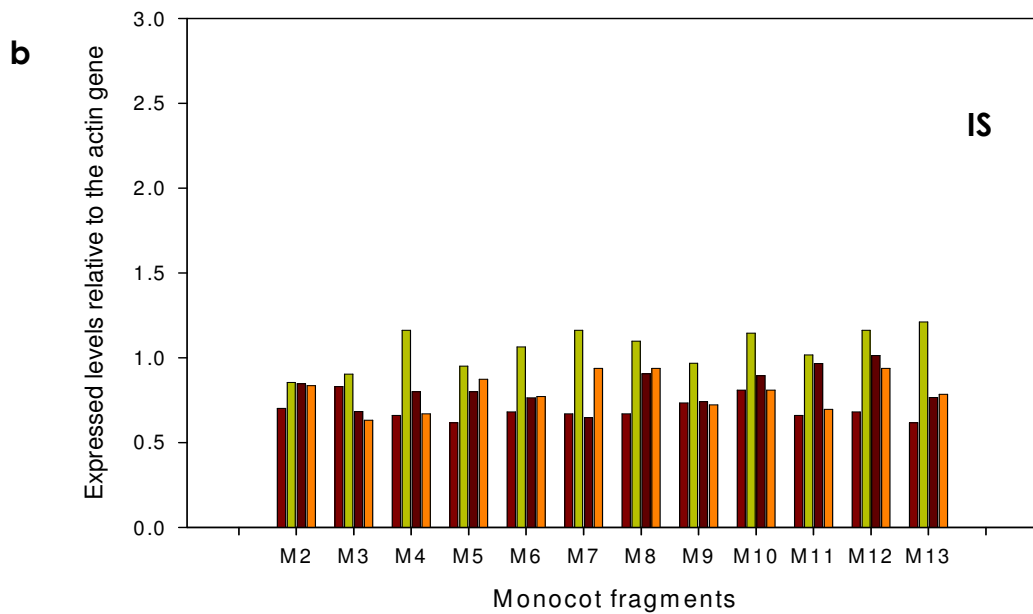
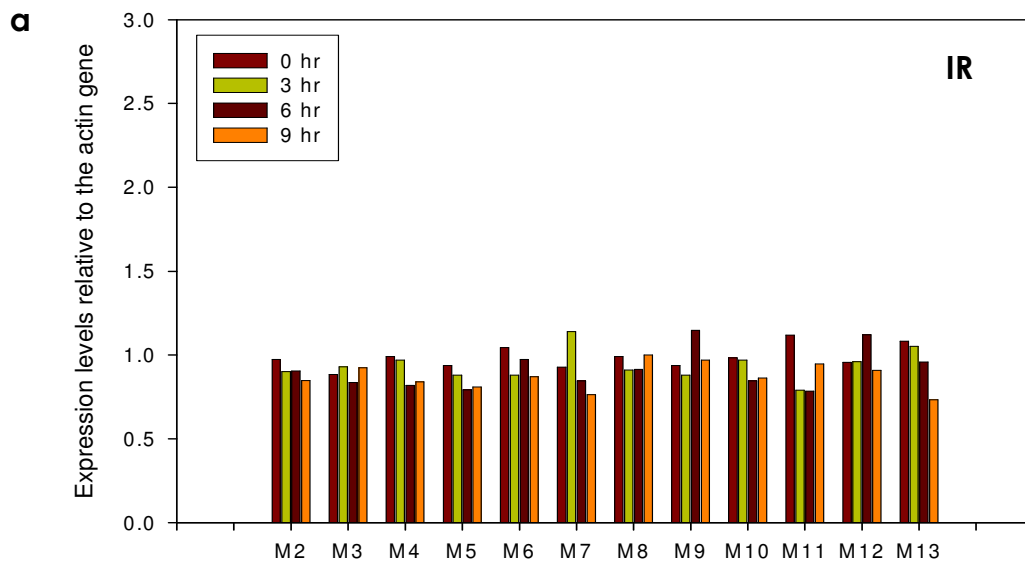


Fig. 4.7 Expression patterns of the isolated monocot cDNA fragments. In (a), RNA from infected resistant plants and in (b) RNA from infected susceptible plants were used to generate the cDNA probes. The clones used were as indicated.

4.5 Identification of the selected cDNA clones.

Once the four clones were selected, the inserts were sequenced to identify them. Every clone's nucleotide sequence included a poly-A tail on the 3'end (Fig. 4.8 – 4.11). This indicated that the 3'end of each gene was successfully cloned. However, it has come to light that an exact sequence, that match that of primer Bovis 39, were present on the 5'end of each clone's sequence. This indicated that the primer combinations during the re-amplification of each gene did not successfully work, but rather that Bovis 39 annealed on both the 3'end and 5'end during amplification of the gene.

After translating the nucleotide sequences, the amino acid sequences were used to search for homologous genes in the Genbank database. For each clone, the genes showing the best homology are shown in Table 4.1. The homology found between the amino acid sequence of each clone and the Genbank sequences were confirmed when each clone was re-aligned with the four possible homologous proteins given in Table 4.1.

The first clone, D15, had a nucleotide sequence of 612 bp and the longest open reading frame of the translated sequence coded for a polypeptide of 99 amino acids (Fig. 4.8). On nucleotide level it shared highest similarity with a *Cf-9/Avr9* rapidly elicited protein from *Nicotiana tabacum* (Genbank Accession nr. AAP03879, 41% identity). The second highest similarity to this clone was a locus (At2g30360) encoding a protein kinase gene from *Arabidopsis thaliana* (Genbank Accession nr. AAP31926). The clone also showed similarity to putative protein kinase genes in *Arabidopsis thaliana* and *Oryza sativa* (Table 4.1).



Fig. 4.8. Sequence analysis of clone D15. In (a), the nucleotide sequence of D15 and in (b) the translated amino acid sequence of the clone is given. In (c), the clones translated sequence was aligned with the four amino acid sequences to which it showed homology. The asterisks (*) indicate nucleotides which are identical in all sequences, the (:) means that conserved substitutions have been observed while the (.) is indicative of semi-conserved substitutions. The stop codon is printed in bold.

a)

```
GAAGAATTCTCGAGCGGCTCAGGTTGGTTTTATCGATGAAAGTCAACGT
GTTGTCGAGGCTCGTCCACTAGATTCCAGGTGGCGGCTCTGGGCGC
TTGCTAACGACACTTGAAGACGCTAAATCTAAATATCCTAAAGTTGACC
CCAATGACCTGCCCTATTATGCATGGATCTTGTGTATCAATACTACTAC
TTGTAGATGGCTTTGGCTTCAAACCTTGCAAACCTATTACCCCTTGTGAAAC
AAATAAAGTATCAAGATGCGCTGGTTGAAGCTGCATGGCCATTAGGCA
GTGCAATTGAAGCAGTGCATCAAACAATGCACTTGAAGCAGTGTAAAG
AAGCGGTCATTAACACTACATATGACTCTACCATTACCAGTTGTATCATGAA
TTAATAAACCGTAGGTTATCGTTAGATCATCATGCGGTTAATGCTCTAAGG
TATCGGAAGACGCGGAAGCCAAATGAAGTATGTTCTTTTGTATATTGATG
CATTCTAGTTTGTCTGTATTTAAGCAAAAAGTCATCTATGTAATAAAAAATA
AAGAAGTTTGGTTTCGTCGAAAAAAAAAAAAAAAAAAGCGCGCCGCT
CGAGAATTCTCA
```

b)

```
KNSRAAQVGFIDESQRVVEARPLDFQVAALGACLTTLE
DAKSKYPKVDPNDLPYLCMDLVYQYTLTLLVDGFGFKPWQ
TITLVKQIKYQDALVEAAWPLGSAIEAVSSNNALEAV Stop
```

c)

```
AA023007   DGQKNL FVASFFFDRAAEAGFVDPNSPVAIVRPADFEDA AKQACQTKLENAKSTYPRVEE 415
AAG22044   DGQKNL FARSFFFYRAAEAGFADPKSPVAKVRPVD FEKA AKLACQPKLEDAKSTYPNVEE 394
AAF26805   GGQKKM FVASFFFDRAAEAGFVDPNQPVAEVRPLDFEKAANKACNMRMEEGKSKFPRVEE 412
AAN15648   GGQKNM FVASFFFDRAAEAGFVDPKQPVATVRPMDFEKA AKKACSMKLEEGKSTFPLVEE 420
M4         ---KN-----SRAAQVGFIDESQRVVEARPLDFQVAALGACLTTLEDAKSKYPKVDP 49
          *:          ***:.* ** * .. * . ** ** *: ** ** *:.* ** .:* *
          *:          ***:.* ** * .. * . ** ** *: ** ** *:.* ** .:* *

AA023007   GNLPYLCMDLVYQYTLTLDVDFGFLYPWQETITLVKVKYDDALVEAAWPLGSAIEAVSSTB- 474
AAG22044   GNLPYLCMDLVYQYTLTLDVDFGFLDQMQQITLVKQVKYHDSLVEAAWPLGSAIEAVSSIQB 454
AAF26805   DNLPYLCMDLVYQYTLTLDVDFGLKPSQITITLVKVKYGDYAVEAAWPLGSAIEAVSS-- 470
AAN15648   ENLPYLCMDLVYQYTLTLDVDFGFLGPSQITITLVKVKYGDQAVEAAWPLGSAIEAVSSPB- 479
M4         NDLPYLCMDLVYQYTLTLDVDFGFLKPWQITITLVKQIKYQDALVEAAWPLGSAIEAVSSNNA 109
          :*****:*****: ***: * *****:.* ** * *****:*****
```

Fig. 4.9. Sequence analysis of clone M4. In (a), the nucleotide sequence of M4 and in (b) the translated amino acid sequence of the clone is given. In (c), the clones translated sequence was aligned with the four amino acid sequences to which it showed homology. The asterisks (*) indicate nucleotides which are identical in all sequences, the (:) means that conserved substitutions have been observed while the (.) is indicative of semi-conserved substitutions. The stop codon is printed in bold.

a)

```

GAAGAATTCTCGAGCGGCAATTAGGGTGGTTATCAAGCCACAACAACC
ACCGAAAGAGAAGAAAAAGCAACGTCAAGGATGTTTTGTGAAC TTGTTT
GCACAAGGAACTCACTTGTCTGGGATAAAAATAAACAGCGAGTTTGT
CGTATTGAGTTCAACTGTAAC TTAACGTTTAAAGATTGATAGAATCGGCTG
CCGTCGTTAGTAAATAGGCAAGCAGAGAAAGTATATTTGTTGAAATATATA
TATATTTGTTTCGATTATGCATGTTTAACTATAACCATTTTGTATGATATCAACG
CTGAAATAGCTACTTGTAAATCAGTCCGAAAAAAAAAAAAAAAAAAGCG
GCCGCTCGAGAATTCTCA

```

b)

```

KNSRAAIRVVIKPPKEKKKQRQGCFVNLFCRKLTC
LG Stop

```

c)

```

AJ496228  KLDLREDKHFLADHPGLVPVTTAQGEELRKQIGAAYYIECSSKTQNVKAVFDAAIKVVI 180
M7        -----KNSR-----AAIRVVI 11
                :* :      ***:***

AJ496228  KPPQKQKEKKKQRRGCLMNVMCGRKLVCLK 210
M7        KPPQPPKEKKKQRQCFVNLFCRKLTC LG 41
                ** *  *****:*:.*:.* ***.**

```

Fig. 4.10. Sequence analysis of clone M7. In (a), the nucleotide sequence of M7 and in (b) the translated amino acid sequence of the clone is given. In (c), the clones translated sequence was aligned with the four amino acid sequences to which it showed homology. The asterisks (*) indicate nucleotides which are identical in all sequences, the (:) means that conserved substitutions have been observed while the (.) is indicative of semi-conserved substitutions. The stop codon is printed in bold.

a)

```

GAAGAATTCTCGAGCGGCAATCAGGCGTGGTTGTCGGAGGAGTTGGAT
ATTACAAAGCAAGAGAGGCTAAGATGGGTGCAAAGAATTATATGATCT
ACAGCTACTGCTCAGATTCTAAGAGGTTCCACAAGGTTTCCTCCTGAGT
GTCAAATGGCATAAAAAGTAGTCCATTGAGTGTTGGGCCCAAATATATT
CTTCTATGTATCTAGTTCCCTTAAGGTTAAAGTTGATTATTTTTCATATTATTGT
AGCTATCATCATCAATAAAAATTTAAAGTTCTGTCAAAAAAAAAAAAAAAA
GCGGCCGCTCGAGAATTCTCA

```

b)

```

EEFSSGNQAWLSEELDITKQERLRWVQKNYMIYSYCSDS
KRFPQGFPEEQ MA Stop

```

c)

```

BAB86890      N-NPSSSNVWVLSSELDITKQERLRWVQKNYMIYNYCSDTKRFPQGFPESECNTA----- 291
AAS46241      N-SAASTSN-SWLNFEELDNSTQERLRWVQKNYMYDYCTDSKRFPQGFPEADCVQNIPTM 297
AAF80591      KKSPSASPSNAWLNFEELDNSTQERMRWVQKNYMIYNYCADLKRFPQGFPEPCSVA----- 284
CAA10231      NSQSSTSNNAWLNKEDLDFTRQERLRWVQKNYMIYNYCTDTKRFPQGFPEECAATKA--- 292
M9            --EEFSSGNQAWLSEELDITKQERLRWVQKNYMIYNYCSDSKRFPQGFPEEQMA----- 53
               .  : : .  * * . : * * * * * : * * * * * : * * * * * : * * * * * : * * *

```

Fig. 4.11. Sequence analysis of clone M9. In (a), the nucleotide sequence of M9 and in (b) the translated amino acid sequence of the clone is given. In (c), the clones translated sequence was aligned with the four amino acid sequences to which it showed homology. The asterisks (*) indicate nucleotides which are identical in all sequences, the (:) means that conserved substitutions have been observed while the (.) is indicative of semi-conserved substitutions. The stop codon is printed in bold.

Table 4.1. Genes sharing possible homologies with the selected clones.
No identity values were given by the program used in the case of "-".

Clone	Accession nr.	Homologies	Expect	Identity (%)
D15	AAP03879	Avr9/Cf9 rapidly elicited protein (<i>Nicotiana tabacum</i>)	3e-15	41%
	AAP31926	Protein kinase At2g30360 (<i>Arabidopsis thaliana</i>)	6e-11	-
	AAC27394	Putative Protein kinase (<i>Arabidopsis thaliana</i>)	7e-05	-
	AAU03103	Protein kinase gene (OsPK4) (<i>Oryza sativa</i>)	8e-04	-
M4	AAO23007	Apyrase-like protein (<i>Medicago truncatula</i>)	1e-36	70%
	AAF26805	Apyrase (Atapy 1) (<i>Arabidopsis thaliana</i>)	4e-36	-
	AAG22044	PsAPY2 (<i>Pisum sativum</i>)	4e-33	-
	AAN13648	Apyrase (<i>Arabidopsis thaliana</i>)	3e-34	-
M7	CAD42725	Putative Rac Protein (<i>Nicotiana tabacum</i>)	1.4	44%
M9	CAA10231	Xyloglucan endotransglycosylase (<i>Fagus sylvatica</i>)	4e-16	73%
	AAF80591	Xyloglucan endotransglycosylase 2 (<i>Asparagus officinalis</i>)	4e-15	69%
	BAB86890	Syringolide induced protein (<i>Glycine max</i>)	8e-15	71%
	AAS46241	Xyloglucan endotransglycosylase-hydrolyse (XTH3) (<i>Lycopersicon esculentum</i>)	1e-13	67%

The second clone, M4, had a nucleotide sequence of 594 bp (Fig. 4.9). The longest open reading frame of the translated sequence coded for a polypeptide of 113 amino acids. This clone showed sequence similarity with an Apyrase-like protein identified in *Medicago truncatula* (Genbank Accession nr. AAO23007, 69% identity). The clone also showed similarity to Apyrase genes from *Pisum sativum* and *Arabidopsis thaliana* (Table 4.1).

Clone M7 showed 44% homology with only one protein, namely a putative Rac protein from *Nicotiana tabacum* (Genbank Accession nr. CAD42725). This clone had a 318 bp nucleotide sequence with the largest open reading frame being 41 amino acids in length (Fig. 4.10).

The last clone, M9, had a 330 bp nucleotide sequence with a 53 amino acid open reading frame (Fig. 4.11). This clone showed highest homology to a Xyloglucan endotransglycosylase (XET) found in *Fagus sylvatica* (Genbank Accession nr. CAA10231, identity 71%). Homology with a XET gene from *Asparagus officianalis* and *Lycopersicon esculentum* was also found, respectively. This clone also showed homology with a syringolide induced protein in *Glycine max*.

4.6 Verification of D15 gene expression using RT-PCR.

Clone D15 was selected from the four sequenced clones for further analyses, since it shared homology with several protein kinase genes (Table 4.1). It was induced as early as 6 h.p.i in the IR plants, but not at all in the IS plants. The expression of the clone was verified using RT-PCR. As a control, the 18S rRNA fragments were also amplified from IR and IS plants. The intensities of the amplified 18S rRNA fragments were used to quantify the expression of clone D15. This was done by dividing each intensity-value of clone D15 by the corresponding values of 18S rRNA.

The expression of the gene at each time-interval was further quantified relative to time 0 h. By normalizing the expression values of D15, an attempt was made to eliminate all possible experimental errors.

When the amplification profile of D15 was analyzed, a noticeable difference could be seen between its expression in IS and IR plants respectively (Fig. 4.12). An 8.5 fold induction of expression occurred at 3 h.p.i. in the IR plants with a second even stronger 31 fold induction at 15 h.p.i. This stronger expression was found for up to 21 h.p.i. In contrast, the expression of clone D15 was down regulated in the IS plants immediately after infection. This down regulation continued for the duration of the study.

It is important to note that the RT-PCR results indicated both an earlier and more pronounced induction of D15 expression than what was found in the reverse Northern blot. It must also be kept in mind that the reverse Northern blot was performed with only the first four time-intervals after infection. It is thus possible that the maximum values for the reverse Northern blot would only be obtained if more time-intervals were analysed. This clearly indicates a role for D15 in the defense response of sunflower against leaf rust.

4.7 Southern Blot analysis of clone D15.

The next step in the analysis of clone D15 was to do a Southern blot. The aim was to determine the copy number of the gene, as well as to see whether any differences are found between the gene in the susceptible and resistant plants. Digested genomic DNA from susceptible and resistant plants was resolved on an agarose gel (Fig. 4.13a).

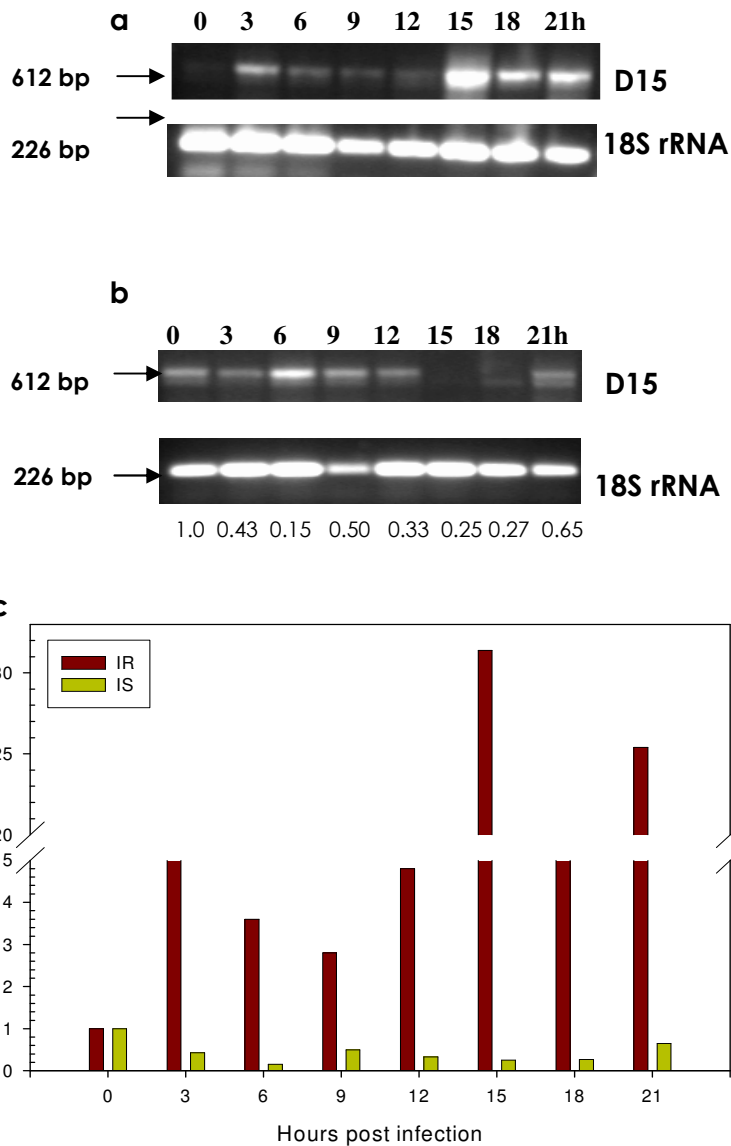


Fig. 4.12. Verification of D15 gene expression using RT-PCR. In (a), the induction profile of the gene in IR RNA (top frame) according to the induction of the 18S rRNA fragments (bottom frame). The induction values are indicated below. In (b), the induction profile of the gene in IS RNA (top frame) according to the induction of the 18S rRNA fragments (bottom frame). The induction values are indicated below. In (c), the induction values are plotted on a graph to visualize the difference in gene expression.

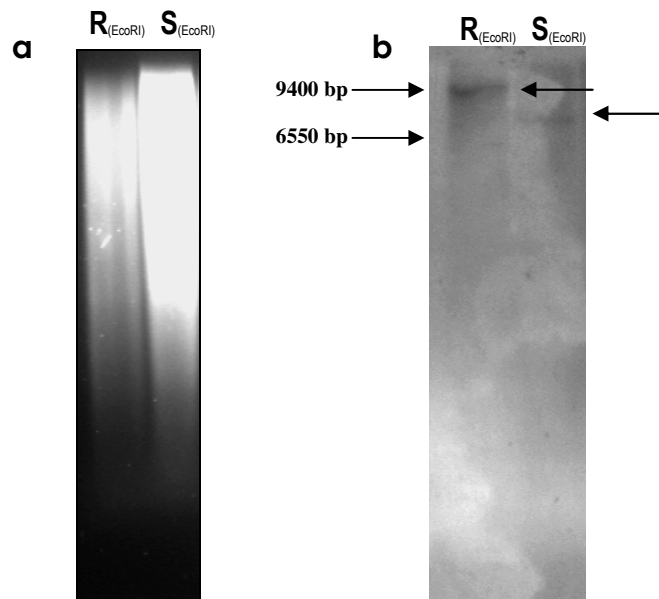


Fig. 4.13. Southern blot analysis of clone D15. In (a), DNA from resistant (R) and susceptible (S) plants digested with *EcoRI* was resolved on an agarose gel. In (b), the x-ray showing the hybridization signals with clone D15 is presented. The hybridizing fragments are indicated with arrows together with their respective approximate sizes.

After hybridization at high stringency with clone D15, a single hybridizing band can be seen in the resistant and susceptible plants when digested with *EcoRI* (Fig. 4.13b). The hybridized fragment in the resistant plants was larger than the fragment in the susceptible plants. This indicates that some chromosomal rearrangement occurred within the susceptible or resistant plants. In addition, since the hybridization was done at high stringency, the single hybridizing fragment in both susceptible and resistant plants indicates that D15 is a single copy gene in sunflower. The fact that a fragment hybridized in both resistant and susceptible plants, confirmed the RT-PCR results, since the D15 gene was expressed in IR and IS plants, even though it was not induced in the IS plants.

4.8 Cloning of the full length D15 gene.

As clone D15 had showed strong induction levels in the IR plants only, it was attempted to clone the full length gene thereof using a 5'-RACE Kit. By using two sets of specially designed primers in three different PCR reactions, their fragments of approximately 950 bp, 1580 bp and 1900 bp respectively, were amplified (Fig. 4.14a). The single 1900 bp fragment that was amplified during the final PCR reaction was cloned into a plasmid vector. It was proven that the plasmid does indeed contain an insert, as it was successfully digested from the plasmid DNA using restriction enzymes (Fig. 4.14b). However, after several attempts the fragment could not be sequenced in time for completion of the study. This would however be done in the near future to identify the type of protein kinase that was cloned.

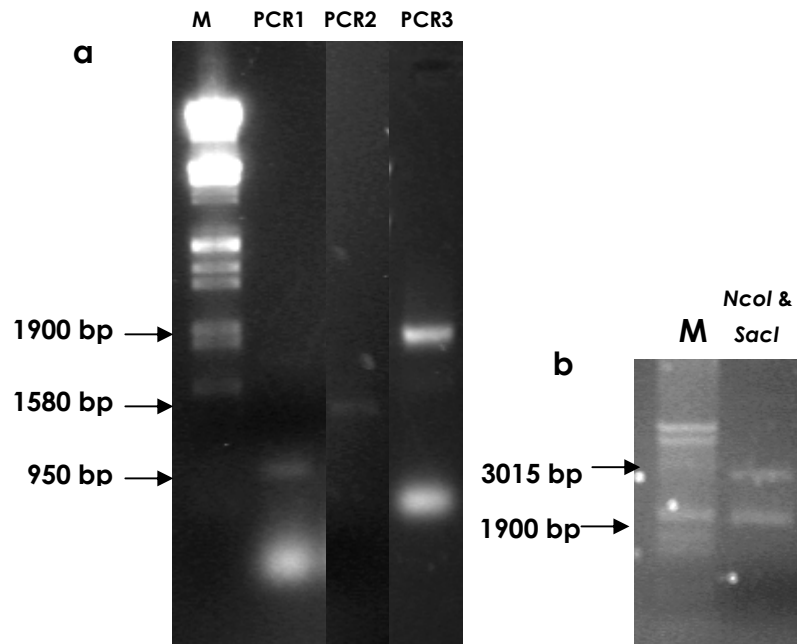


Fig. 4.14 5'-RACE of clone D15. In (a), the amplification products of all three PCR reactions are shown. In (b), a recombinant plasmid containing the 1.9 kb insert that were digested with *NcoI* and *SacI*. The 1.9 kb insert and the empty plasmid is indicated with an arrow. Marker DNA (M) in the first lane is an indication of the fragment sizes.

Chapter Five:

Discussion...

Environmental changes provide a constant challenge to plants. The pathogenic invasion of plants is a frequent stress factor that plants need to adjust to. The early recognition of pathogens and pests by a plant cell is essential to mount an appropriate defense reaction. Resistant plants have developed effective defense signaling pathways which are activated upon the intrusion of a potential pathogen. These pathways, which are initiated at the cell wall and cell membrane, incorporate several classes of proteins and important phosphorylating cascades which amplify the initial alarm signal leading to an appropriate defense reaction.

Protein kinases form part of these signaling pathways. When these protein kinases are absent, signal transduction will be terminated and disease will follow. Several protein kinases, such as RLKs and MAP kinases, are involved in these signaling events. The MAPK signaling cascade include up to three different kinases that are actively phosphorylated during signal transduction in stress-associated responses (Sessa and Martin, 2000). By transferring these stress-related signals through the cell, multi-step defense responses are induced which include the rapid production of ROS, the strengthening of the cell wall as well as the synthesis of pathogenesis-related proteins (Jonak *et al.*, 2002). These processes ultimately lead to the hypersensitive response and cell death, thus preventing the pathogen from further infecting the plant tissue.

Protein kinases involved in plant defense, include the *Pto* gene that was identified in tomato (Zhou *et al.*, 1997). This gene provides the plant with resistance against bacterial speck disease. The gene encodes a cytosolic serine/threonine kinase that interacts with other proteins, probably by transphosphorylating them. The ability of the *Pto* gene

product to trigger defense mechanisms was confirmed when the overexpression of *Pto* in transgenic tomato plants conferred broad resistance against different pathogens that resulted in the activation of various defense responses (Tang *et al.*, 1998). It was found that *Pto* physically interact with the *AvrPto* protein coming from the pathogen (Scofield *et al.*, 1996; Tang *et al.*, 1996). The activation of *Pto* by *AvrPto* is due to conformational changes in *Pto* when the two proteins bind to each other. This binding might expose certain domains which were previously unavailable for autophosphorylation or transphosphorylation by an additional protein kinase (Tang *et al.*, 1998). By the phosphorylation of these protein kinases, a signal transduction pathway is then initiated.

In the general signaling pathway are also included; resistance genes and RLKs such as *LRK10* in wheat (Feuillet *et al.*, 1997), that is contributing to the signaling pathway. An example of a resistance gene is *Xa-21* that provides resistance to the bacterial pathogen *Xanthomonas oryzae* (Song *et al.*, 1995).

During this study, the main aim was to identify a protein kinase gene from the resistant sunflower cultivar after it was infected with leaf rust. This was important, as no protein kinase gene has previously been identified in sunflower. In addition, any protein kinase gene whose expression is induced shortly after infection, will most probably be involved in the signaling events leading to the activation of the plant defenses. Should this protein kinase gene be inducibly expressed only in the IR plants, but not the IS plants, it would most definitely be linked to the disease resistance mechanism of sunflower against leaf rust.

In the search for a putative protein kinase gene, two degenerate primers were designed that were specific for the conserved subdomain VIb region of all protein kinase genes (Hanks *et al.*, 1988). During the RT-PCR step, these primers were used in combination with an anchored oligo-dT primer containing an additional 5'-tail sequence. After the clones were sequenced, it came to light that the original methodology of using the kinase domain specific primers, was not entirely successful. It was found that the cloned cDNA fragments were amplified, with Bovis 39 annealing on both the 5'- and 3'-end of each clone (Fig. 4.8 – Fig. 4.11). This meant that the kinase domain specific primers were not effective to target protein kinase genes expressed in sunflower. Despite this flaw, several cDNAs that were inducibly expressed upon infection, were still efficiently amplified and subsequently cloned.

Of the four cDNAs chosen for further study, clone D15 was of special interest. This clone was induced at a very early stage after infection most probably indicating that it is involved in the very early signaling events (Fig. 4.6). As this clone showed great potential, its sequence was determined (Fig. 4.8). It was found that clone D15 shared homology with several protein kinase genes identified in both *Arabidopsis thaliana* and *Oryza sativa* (Table 4.1). The clone also showed homology with a rapidly elicited protein induced in the Cf9/Avr9 interaction in tobacco (Navarro *et al.*, 2004). The Cf9/Avr9 interaction in tomato that was shown in Jones *et al.* (1994) is part of the classical gene-for-gene interaction (Flor, 1971). Since D15 showed homology with another gene directly involved in a plant/pathogen interaction governed by the resistance-avirulence interaction, it holds great promise to be directly involved in the interaction between sunflower and leaf rust.

When sequences are aligned using BLAST, an expectation value (E) is given (Table 4.1). The E-value needs to be negative to give a good indication that the aligned sequences are related. The lower the E-value, the more significant the alignment is. Judging by the low E-values given for clone D15's alignment, it is indicative that the similarities are significant.

Further studies revealed that clone D15 is indeed stronger expressed within the IR plants than in the IS plants (Fig. 4.12). Even more interesting was the fact that the expression of the cDNA clone was induced twice within 15 h after infection of the IR plants. The second stronger induction of the cDNA clone is an indication that the gene is possibly involved in the activation of secondary defense mechanisms as part of SAR in the uninfected parts of the plant. The second induction would ensure that whatever the role the gene is playing during the resistance response, it would have a longer lasting effect in combating the invading pathogen. This led to the assumption that clone D15 play an important role in the activation of both local and systemic plant defenses in IR sunflower. This was again emphasized by the fact that clone D15 gene expression in the IS plants is not only uninduced, but it is indeed actively repressed.

Since clone D15 was expressed in both the IR and IS plants (Fig. 4.6) it was assumed that the gene was present in both the resistant and susceptible plants. A Southern blot confirmed this (Fig. 4.13). What was however significant was the fact that the hybridization profile differed between the two plants. A polymorphism of the gene seems to exist between these two cultivars since the hybridized fragments differed in size. Such naturally occurring variations in nucleotide sequences can occur throughout the genome. As these two cultivars are near isogenic

lines (NILs), a discontinuous phenotype might exist between the two. The variation between the two cultivars might be due to chromosomal rearrangements where a part of the gene's sequence in the susceptible plants got distorted. Through evolution or chromosomal mutations, the 5'-end of the gene might have been lost or even the promoter region could have been inactivated. This could explain why it was possible to amplify the 3'-end of the gene from both plants using RT-PCR, but why the gene was differentially expressed in the IR and IS plants.

An attempt was further made to clone the full length D15 gene. This was successfully done when a sequence of approximately 1900 bp was amplified after RACE (Fig. 4.14). Judging by the length of this sequence, it was assumed that this was indeed the full length gene, since when combined with the first obtained sequences (Fig. 4.8), the full length gene would be 2788 bp in length. It's however disappointing that after several attempts, the fragment could not be sequenced. Further analyses to identify this gene will continue in the future as it holds great promise.

Finally one has to consider the importance of the gene in sunflower when infected with leaf rust. The fact that the gene is not unique to the resistance plants, but is also expressed in the susceptible plants, led to the conclusion that the difference in expression pattern could be due to one of the following. The first is that chromosomal rearrangements have occurred. This could also have affected the promoter structure, thereby affecting expression in the susceptible plants. In addition, secondary factors could influence its expression. For example, a factor uniquely linked to the disease resistance locus of the resistant plants can directly induce clone D15 gene expression but since the locus is

absent in the susceptible plants, its expression was not activated. Alternatively, a repressor protein produced by the pathogen, could actively shut down clone D15 gene expression in the susceptible plants, but does not affect the expression in the resistant plants. Future research will however clarify which scenario is the correct one.

When one considers the other cloned cDNA fragments, the monocot fragments had the opposite expression profile compared to the dicot fragments. Most of the monocot fragments were stronger expressed in the IS plants compared to the IR plants. These included clones such as D3, D17 and D14 (Fig. 4.6) as well as M3 and M8 (Fig. 4.7). One reason for this might be that the activated defense response in the IR plants suppresses the expression of the genes in the IS plants. They could however be part of a general defense response inhibited in the IS plants. It might be that these clones are only induced at a later time-interval and not within the first four time-intervals as indicated with the reverse Northern blots. Therefore, the stronger induction of each clone might be due to the plant responding in every way that it can to combat the pathogenic infection.

Several of the identified clones did not show any changes of expression at all (Fig. 4.6 – Fig. 4.7). This was for both the monocot and dicot fragments. It is however possible that since the reverse Northern blots were done for only the first four time-intervals, the induced expression of these genes will be found only later on in the study.

Of the monocot clones that were selected, clone M4 showed very high homology to an Apyrase-like protein (Fig. 4.9). Homology were found with Apyrase genes from *Medicago truncatula* (Navarro-Gochicoa *et al.*, 2003), *Arabidopsis thaliana* (Steinebrunner *et al.*, 2000) and *Pisum*

sativum (Chen *et al.*, 1987). These homologies were significant as all the E-values are very small.

Apyrases are enzymes that are able to hydrolyze both γ - and β -phosphates from ATP and ADP respectively (Plesner, 1995). The pea apyrase was initially purified from nuclei and was reported to play a role in mediating the uptake of phosphates from the extracellular matrix surrounding the cell (Thomas *et al.*, 1999). These enzymes are membrane-associated and it has also been reported that apyrases hydrolyze extracellular ATP (Thomas *et al.*, 1999). The physiological relevance of the degradation of extracellular ATP had been demonstrated in at least two other systems (Todorov *et al.*, 1997).

It is important to note that the mobilized phosphate groups are then available to take part in phosphorylation reactions which occur during the defense responses (Thomas *et al.*, 1999). The relevance of these results are however unclear at this stage regarding this study. The reason for this is that clone M4 showed preferential induced expression in the IS plants, while the expression in the IR plants remained constant. It is therefore difficult to understand why mobilized phosphate groups are only found in the IS plants, where as it is absent in the IR plants where the active defense is initiated. This needs to be further investigated.

The third clone (M7) had homology with only one sequence (Fig. 4.10), namely a putative Rac protein which was previously identified from *Nicotiana tabacum* (Morel *et al.* 2004). As with the previous clone, homology was only found within a short region of the amino acid sequence. Although the E-value is above zero, it was still thought to be

significant since only a very short amino acid sequence was used for alignment.

The Rac protein is a low M_r G-protein that is similar to the neutrophils in humans and plays an essential role as a regulator of NADPH-oxidase (Bokoch, 1994; Irani and Goldschmidt-Clermont, 1998). Rac proteins belong to the conserved Rho-family of small GTPases (Watson and Yang, 1993). These proteins are believed to play an important role in the production of ROS during the defense reaction (Hammond-Kosack and Jones, 1996). A good example of such a GTP-binding protein is *OsRac1*, which is a rice homolog of the human Rac-proteins (Kawasaki *et al.*, 1999). It has been proven that *OsRac1* induces ROS production in transformed rice cells. By following this route for ROS production, it can initiate cell death in the plant-pathogen interactions as part of the HR.

It has also been reported that Rho-related proteins binds to the activated CLAVATA1 (CLV1) receptor protein which is a RLK (Trotochaud *et al.*, 1999). CLV1 are involved in cell proliferation and organ formation. The plant GTPase, Rop, associates with the CLV1 kinase domain to form a complex when CLV1 is activated via phosphorylation. The Rop protein is then activated and could in turn activate a MAPK pathway (Hirt, 1997). The fact that CLV1 is an RLK leads to the possibility that the Rac-protein can interact with other RLKs as well to activate the defense response via a signal transduction cascade. Although the Rac-protein did not show a relative high level of induction of expression during this study, it still holds great potential for further studies, since it was induced in both IS and IR plants. Maybe similar to D15, RT-PCR expression analysis of clone M7 will show that the induced expression of the gene is bigger than what was found during the reverse Northern blot. This will however need to be tested.

With the fourth clone (M9), homology was found with a xyloglucan endotransglycosylase (XET) (Fig. 4.11) that has previously been identified in *Fagus sylvatica* (Lorenzo *et al.*, 1999), *Asparagus officinalis* (Donoghue *et al.*, 2001) and *Lycopersicon esculentum*. After alignment, a relative high homology with low E-values was found (Table 4.1).

Xyloglucan is a soluble hemicellulose with a backbone composed of glucose which binds noncovalently to cellulose, thereby coating and cross-linking the adjacent cellulose microfibrils (Hayashi, 1989; McCann *et al.*, 1990). During plant processes such as fruit ripening, hydrolytic enzymes are produced that partially degrades the cell wall to allow expansion (Fry *et al.*, 1992). However, after expansion, the cell walls need to be repaired. A rapid reinforcement of the cell wall must follow this expansion. Therefore, all xyloglucan-metabolizing enzymes play a central role in the processes to remodel the cell wall (Fry, 1989). For this purpose, transglycosylating enzymes namely XETs, are involved in the cross-linking of the cell wall to strengthen it (Johansson *et al.*, 2004).

Although these enzymes are involved in processes such as fruit ripening etc., it is also possible that they strengthen and repair damaged cell walls during plant-pathogen interactions and therefore play a role in the defense response. This might also explain as to why clone M9 are induced similarly in both the resistant and susceptible plants, even though the expression levels were not very high.

To conclude, four different cDNA fragments were identified based on their induced expression profiles in both IR and IS plants. Of the four, three of the clones showed homology with genes that were previously

implicated to be involved in plant defense. The specific involvement of each clone need however be further investigated and confirmed.

Of the four, the putative protein kinase gene perhaps holds the most promise. This is due to its unique expression pattern in IR plants that was completely absent in the IS plants. In addition, several protein kinases have in the past been shown to be involved in plant defense, either as receptors or as MAPKs.

It can therefore be concluded that the aim of the project was met, in that a putative protein kinase gene was cloned whose induced expression was unique to the IR plants. The timing of the induced expression of the gene also implicated that the encoded protein takes part in the earliest events following infection, most probably as part of the signal transduction event. Future results would need to prove its identity as well as the function thereof in sunflower which is infected with leaf rust.

Chapter Six:

References...



- Becraft P.W., Stinard P.S. and McCarty D.R.**, 1996. CRINKLY4: A TNFR-like receptor kinase involved in maize epidermal differentiation. *Science*. 273: 1406-1409.
- Bent F.A.**, 1996. Plant disease resistance genes: Function meets structure. *The Plant Cell*. 8: 1757-1771
- Blechert S., Brodschelm W., Holder S., Kammere L., Kutchan T.M., Mueller M.J., Xia Z. and Zenk M.H.**, 1995. The octadecanoid pathway: signal molecules for the regulation of secondary pathways. *Proceedings of the National Academy of Sciences of the USA*. 92: 4099-4105.
- Blumwald E., Aharon G.S. and Lam B.C.H.**, 1998. Early signal transduction pathways in plant-path interactions. *Trends in Plant Science*. 3: 342-346.
- Bokoch G.M.**, 1994. Regulation of the human neutrophil NADPH oxidase by the Rac GTP-binding proteins. *Current Opinion in Cell Biology*. 6: 212-218.
- Boller T. and Keen N.T.**, 1999. Resistance genes and the perception and transduction of elicitor signals in host-pathogen interactions. Slusarenko, A.T., Fraser, R.S.S. and van Loon, L.C. (eds), *Mechanisms of Resistance to Plant Diseases*. Kluwer Academic Publishers. Dordrecht/Boston/London.
- Bower M.S., Matias D.D., Fernandes-Carvalho E., Mazzurco M., Gu T., Rothstein S.J. and Goring D.R.**, 1996. Two members of the Thioredoxin-h family interact with the kinase domain of a Brassica S-locus receptor kinase. *The Plant Cell*. 8: 1641-1650.

- Cai X., Takken F.L.W., Joosten M.H.A., P.J.G.M., De Wit,** 2001. Specific recognition of AVR4 and AVR9 results in distinct patterns of hypersensitive cell death in tomato, but similar patterns of defense-related gene expression. *Molecular Plant Pathology*. 2: 77-86.
- Chandra S. and Low P.S,** 1997. Measurement of Ca²⁺ fluxes during elicitation of the oxidative burst in aequorin-transformed tobacco cells. *Journal of Biological Chemistry*. 272: 28274-28280.
- Chen Y.R., Datta N. and Roux S,** 1987. Purification and partial characterization of a calmodulin-stimulated nucleoside triphosphate from pea nuclei. *Journal of Biological Chemistry*. 262: 10689-10694.
- Chen Z. Malamy J., Henning J., Conrath U., Sanchez-Casas P., Silva H., Ricigiano J. and Klessig D.F,** 1995. Induction, modification and transduction of salicylic acid signal in plant defense responses. *Proceedings of the National Academy of Sciences of the USA*. 92: 4134-4137.
- Chomczynski P,** 1992. One-hour downward alkaline capillary transfer for blotting of DNA and RNA. *Annals of Biochemistry*. 201: 134-139.
- Clark S.E., Williams R.W and Meyerowitz E.M,** 1997. The CLAVATA1 gene encodes a putative receptor kinase that controls shoot and floral meristem size in *Arabidopsis*. *Cell*. 59: 575-585.
- Coello P., Sassen A., Haywood V., Davis K.R. and Walker J.C,** 1999. Biochemical characterization and expression of *RLK4*, a receptor-like kinase from *Arabidopsis thaliana*. *Plant Science*. 142: 83-91.

- Czernic P., Visser B., Sun W., Savoure A., Deslandes L., Marco Y., Van Montagu M. and Verbruggen N,** 1999. Characterization of an *Arabidopsis thaliana* receptor-like protein kinase gene activated by oxidative stress and pathogen attack. *The Plant Journal*. 18: 321-327.
- De Gara L., de Pinto M.C. and Tommasi F,** 2003. The antioxidant systems vis-à-vis reactive oxygen species during plant-pathogen interaction. *Plant Physiology and Biochemistry*. 41: 863-870.
- Delaney T.P, Uknes S., Vernooij B., Friedrich L., Weymann K., Negrotto D., Gaffney T., Gut-Rella M., Kessman H., Ward E. and Ryals J,** 1994. A central role of salicylic acid in plant disease resistance. *Science*. 266: 1247-1250.
- Delledonne M., Yiji X., Dixon R.A. and Lamb C,** 1998. Nitric oxide functions as a signal in plant disease resistance. *Nature*. 394: 585-588.
- De Lorenzo G., D'Ovidio R. and Cervone F,** 2001. The role of polygalacturonase-inhibiting proteins (PGIPs) in defense against pathogenic fungi. *Annual Review of Phytopathology*. 39: 313-335.
- Desikan R., Neill S.J. and Hancock J.T,** 2000. Hydrogen peroxide-induced gene expression in *Arabidopsis thaliana*. *Free Radical Biology and Medicine*. 28: 773-778.
- Dixon M.S., Jones D.A., Keddie J.S., Thomas C.M., Harrison K. and Jones J.D.G,** 1996. The tomato *Cf-2* disease resistance gene and six homologs show pronounced allelic variation in leucine-rich repeat copy number. *The Plant Cell*. 10: 1915-1925.

- Durner J., Wendehenne D. and Klessig D,** 1998. Defense gene induction in tobacco by nitric oxide, Cyclic GMP and cyclic ADP-ribose. *Proceedings of the National Academy of Sciences of the USA.* 95: 10328-10333.
- Dwyer K.G., Kandasamy M.K., Mahosky D.I., Acciai J., Kudish B.I., Miller J.E., Nasrallah M.E. and Nasrallah J.B,** 1994. A superfamily of S locus-related sequences in Arabidopsis: Diverse structures and expression patterns. *The Plant Cell.* 6: 1829-1843.
- Farmer E.E. and Ryan C.A,** 1992. Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. *The Plant Cell.* 4: 129-134.
- Feuillet C., Schachermayr G. and Keller B,** 1997. Molecular cloning of a new receptor-like kinase gene encoded at the Lr10 disease resistance locus of wheat. *The Plant Journal.* 11: 45-52.
- Fleury C., Mignotte B. and Vayssiere J,** 2002. Mitochondrial reactive oxygen species in cell death signaling. *Biochimie.* 84: 131-141.
- Flor H.H,** 1971. Current status of the gene-for-gene concept. *Annual Review of Phytopathology.* 9: 275-296.
- Fry S.C,** 1989. The structure and functions of xyloglucan. *Journal of Experimental Botany.* 40: 1-11.
- Fry C., Smith R.C., Renwick K.F., Martin D.J., Hodge S.K. and Matthews J,** 1992. Xyloglucan endotransglycosylase, a new wall-loosening enzyme activity from plants. *Biochemistry Journal.* 282: 821-828.

- Gaffney T., Friedrich L., Vernooij B., Negrotto D., Nye G., Uknes S., Ward E., Kessman H. and Ryals J**, 1993. Requirement of salicylic acid for the induction of systemic acquired resistance. *Science*. 261: 754-756.
- Gomez-Gomez L., Felix G. and Boller T**, 1999. A single locus determines sensitivity to bacterial flagellin in *Arabidopsis thaliana*. *The Plant Journal*. 18: 277-284.
- Goring D.R. and Rothstein S.J**, 1992. The S-locus receptor kinase gene in a self-incompatible *Brassica napus* line encodes a functional serine/threonine kinase. *The Plant Cell*. 4: 1273-1281.
- Hammond-Kosack K.E. and Jones J.D.G**, 1996. Resistance gene-dependent plant defense responses. *The Plant Cell*. 8: 1773-1791.
- Hanks S.K., Quinn A.M. and Hunter T**, 1988. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science*. 241: 42-52.
- Hayashi T**, 1989. Xyloglucans in the primary cell wall. *Annual Review of Plant Physiology and Plant Molecular Biology*. 40: 139-168.
- He Z., Fujiki M. and Kohorn B.D**, 1996. A cell wall-associated receptor-like protein kinase. *The Journal of Biological Chemistry*. 271: 19789-19793.
- He Z., He D. and Kohorn B.D**, 1998. Requirement for the induced expression of a cell wall associated receptor kinase for survival during the pathogen response. *The Plant Journal*. 14: 55-63.

- Hervè C., Dabos P., Galaud J.P., Rouge D. and Lescure B,** 1996. Characterization of an *Arabidopsis thaliana* gene that defines a new class of putative plant receptor kinases with an extracellular lectin-like domain. *Journal of Molecular Biology*. 258: 778-788.
- Hirt H,** 1997. Multiple roles of MAP kinases in signal transduction in plants. *Trends in Plant Science*. 2: 11-15.
- Hu X., Bidney L., Yalpani N., Duvick J.P., Crasta O., Folkerts O. and Lu G,** 2003. Overexpression of a gene encoding hydrogen peroxide-generating oxalate oxidase evokes defense responses in sunflower. *Plant Physiology*. 133: 170-181.
- Hwang H., Kim H., Yu H., Oh M., Lee I. and Kim S,** 2003. Gene encoding pathogenesis-related 10 protein of *Lithispermum erythrorhizon* is responsive to exogenous stimuli related to the plant defense system. *Plant Science*. 165: 1297-1302.
- Irani K. and Goldschmidt-Clermont P.J,** 1998. Ras, superoxide and signal transduction. *Biochemical Pharmacology*. 55: 1339-1346.
- Jabs T. and Slusarenko A.J,** 2000. The hypersensitive response. Slusarenko, A.J., Fraser, R.S.S., Van Loon, L.C. (eds). *Mechanisms of Resistance to Plant Diseases*. Kluwer Academic Publishers. Netherlands.
- Johal G.S. and Briggs S.P,** 1992. Reductase activity encoded by the *Hm1* disease resistance gene in maize. *Science*. 258: 985-987.

- Johansson P., Brumer H., Baumann M.J., Kallas A.M., Hendriksson H., Denman S.E., Teeri T.T. and Jones T.A.**, 2004. Crystal structures of a poplar xyloglucan endotransglycosylase reveal details of transglycosylation acceptor binding. *The Plant Cell*. 16: 874-886.
- Jonak C., Okrèsz L., Bogre L. and Hirt H.**, 2002. Complexity, cross talk and integration of plant MAP kinase signaling. *Current Opinion in Plant Biology*. 5: 415-424.
- Jones A.M.**, 2002. G-protein-coupled signaling in *Arabidopsis*. *Current Opinion in Plant Biology*. 5: 402-407.
- Jones D.A., Thomas C.M., Hammond-Kosack K.E., Balint-Kurti P.J. and Jones J.D.G.**, 1994. Isolation of the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* by transposon tagging. *Science*. 266: 789-793.
- Jung J.L., Fritig B. and Hahne G.**, 1993. Sunflower (*Helianthus annuus*) pathogenesis-related proteins. *Plant Physiology*. 101: 873-880.
- Kawasaki T, Henmi K, Ono E, Hatakeyama S, Iwano M, Satoh H and Shimamoto K.**, 1999. The small GTP-binding protein Rac is a regulator of cell death in plants. *Proceedings of the National Academy of Sciences of the USA*. 96: 10922-10926.
- Kim Y., Lee J.H., Yoon G.M., Cho H.S., Park S., Suh M.C., Choi D., Ha H.J., Liu J.R. and Pai H.**, 2000. CHRK1, a chitinase-related receptor-like kinase in tobacco. *Plant Physiology*. 123: 905-915.
- Kolte S.J.**, 1985. *Diseases of Annual Edible Oilseed Crops*. CRC Press, Inc. Florida, pp 9-20.

- Li J. and Chory J**, 1997. A putative leucine-rich repeat receptor kinase involved in Brassino-steroid signal transduction. *Cell*. 90: 929-938.
- Loh Y.T. and Martin G.B**, 1995. The *Pto* bacterial resistance gene and the *Fen* insecticide sensitivity gene encode functional protein kinases with serine/threonine specificity. *Plant Physiology*. 108: 1735-1739.
- Lorenzo O., Calvo A., Nicolas C., Nicolas G. and Rodriguez D**, 1999. Isolation, sequence analysis and expression characteristics of a cDNA (Accession No. AJ130885) encoding a Xyloglucan endotransglycosylase of *Fagus sylvatica* L. seeds. *Plant Physiology*. 119: 1148-1148.
- Malamy J., Carr J.P., Klessig D.F. and Raskin I**, 1990. Salicylic acid: A likely endogenous signal in the resistance response of tobacco to viral infection. *Science*. 250: 1002-1004.
- Martin G.B**, 1999. Functional analysis of plant disease resistance genes and their downstream effectors. *Current Opinion in Plant Biology*. 2: 273-279.
- Martin G.B., Brommonschenkel S.H., Chunwongse J., Frary A., Ganai M.W., Spivey R., Wu T., Earle E.D. and Tanksley S.D**, 1993. Map-based cloning of a protein kinase gene conferring disease resistance in tomato. *Science*. 262: 1432-1436.
- McCann M.C., Wells B. and Roberts K**, 1990. Direct visualization of crosslinks in the primary plant cell wall. *Journal of Cell Science*. 96: 323-334.

Melchers L.S., Apotheker-de Groot M., van der Knaap J.A., Ponstein A.S., Sela-Buurlage M.B., Bol J.F., Cornelissen B.J.C., van den Elzen P.J.M. and Linthorst H.J.M., 1994. A new class of tobacco chitinases homologous to bacterial exo-chitinases displays antifungal activity. *The Plant Journal*. 5: 469-480.

Montillet J., Agnel J., Ponchet M., Vailliau F., Roby D. and Triantaphylides C., 2002. Lipoyxygenase-mediated production of fatty acid hydroperoxides is a specific signature of the hypersensitive reaction in plants. *Plant Physiology and Biochemistry*. 40: 633-639.

Morel J., Fromentin J., Blein J.P., Simon-Plas F. and Elmayan T., 2004. Rac regulation of NtrbohD, the oxidase responsible for the oxidative burst in elicited tobacco cells. *Plant Journal*. 37: 282-293.

Morris E.R. and Walker J.C., 2003. Receptor-like protein kinases: The keys to response. *Current Opinion in Plant Biology*. 6: 339-342.

Mu J., Lee H. and Kao T., 1994. Characterization of a pollen-expressed receptor-like kinase gene of *Petunia inflata* and the activity of its encoded kinase. *The Plant Cell*. 6: 709-721.

Nasrallah M.E., Kandasamy M.K. and Nasrallah J.B., 1992. A genetically defined trans-acting locus regulates S-locus function in *Brassica*. *The Plant Journal*. 2: 497-506.

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

- Navarro-Gochicoa M.T., Camut S., Niebel A. and Cullimore J.V,** 2003. Expression of the Apyrase-like APY1 genes in roots of *Medicago truncatula* is induced rapidly and transiently by stress and not by *Sinorhizobium meliloti* or nod factors. *Plant Physiology*. 131: 1124-1136.
- Nurnberger T., Nennstiel D., Jabs T., Sacks W.R., Halbrock K. and Scheel D,** 1994. High affinity binding of a fungal oligopeptide elicitor to parsley plasma membranes triggers multi defense responses. *Cell*. 78: 449-460.
- O'Donoghue E.M., Somerfield S.D., Sinclair B.K. and Coupe S.A,** 2001. Xyloglucan endotransglycosylase: a role after growth cessation in harvested asparagus. *Australian Journal of Plant Physiology*. 28: 349-361.
- Parniske M. and Jones J.D.G,** 1999. Recombination between diverged clusters of the tomato Cf-9 plant disease resistance gene family. *Proceedings of the National Academy of Sciences of the USA*. 96: 5850-5855.
- Pastuglia M., Roby D., Dumas C. and Cock J.M,** 1997. Rapid induction by wounding and bacterial infection of an S gene family receptor-like kinase gene in *Brassica oleracea*. *The Plant Cell*. 9: 49-60.
- Plesner L,** 1995. Ecto-ATPases: identities and functions. *International Review of Cytology*. 158: 141-214.
- Richter T.E., Pryor T.J., Bennetzen J.L. and Hulbert S.H,** 1995. New rust resistant specificities associated with recombination in the *Rp1* complex in maize. *Genetics*. 141: 373-381.

Robert N., Roche K., Lebeau Y., Breda C., Boulay M., Esnault R. and Buffard D, 2002. Expression of grapevine chitinase genes in berries and leaves infected by fungal or bacterial pathogens. *Plant Science*. 162: 389-400.

Ryals J.A., Neuenschwander U.H., Willets M.G., Molina A., Steiner H. and Hunt M.D, 1996. Systemic acquired resistance. *The Plant Cell*. 8: 1809-1819.

Salmeron J.M., Oldroyd E.D., Rommens M.T., Scofield S.R., Kim H., Lavelle D.T., Dahlbeck D. and Staskawicz B.J, 1996. Tomato *Prf* is a member of the leucine-rich repeat class of plant disease resistance and lies embedded within the *Pto* kinase gene cluster. *Cell*. 86: 123-133.

Sambrook J., Fritsch E.F. and Maniatis T, 1989. Molecular cloning: a laboratory manual, 2nd ed. Ford, N., Nolan, C., Ferguson, M. (eds). Cold Spring Harbor Laboratory Press. Cold Spring Harbor, New York.

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

Scheer J.M. and Ryan C.A, 2002. The systemin receptor SR160 from *Lycopersicon peruvianum* is a member of the LRR receptor kinase family. *Proceedings of the National Academy of Sciences of the USA*. 99: 9585-9590.

Scofield S.R., Tobias C.M., Rathjen J., Chang j.H., Lavelle D.T., Michelmore R.W. and Staskawicz B.J, 1996. Molecular basis of gene-for-gene specificity in bacterial speck disease of tomato. *Science*. 274: 2063-2065.

Sessa G and Martin G.B, 2000. Protein kinases in the plant defense response. Kreis, M. and Walker, J.C. (eds). *Advances in botanical Research*. Academic Press. San Diego/San Francisco/New York/Boston/London/Sydney/Tokyo.

Shiu S.H. and Bleecker A, 2001. Plant receptor-like kinase gene family: diversity, function and signaling. *Science's Signal Transduction Knowledge Environment*. 113: RE22.

Song F. and Goodman R.M, 2002. *OsBIMK1*, a rice MAP kinase gene involved in disease resistance responses. *Planta*. 215: 997-1005.

Song W., Wang G., Chen L., Kim H., Pi L., Holsten T., Gardner J., Wang B., Zhai W., Zhu L., Fauquet C. and Ronald P. 1995. A receptor kinase-like protein encoded by the rice disease resistance gene, *Xa21*. *Science*. 270: 1804-1806.

Sood P.N. and Sackston W.E, 1970. Studies on sunflower rust. VI. Penetration and infection of sunflowers susceptible and resistant to *Puccinia helianthi* race 1. *Canadian Journal of Botany*. 48: 2179-2181.

Steinebrunner I.A., Jeter C.R., Song C. and Roux S.J, 2000. Molecular and biochemical comparison of two different apyrases from *Arabidopsis thaliana*. *Plant Physiology and Biochemistry*. 38: 913-922.

Sticher L., Mauch-Mani B. and Metraux J.P, 1997. Systemic Acquired Resistance. *Annual Review of Phytopathology*. 35: 235-270.

- Takken F.L.W., Schipper D., Nijkamp H.J.J. and Hille J,** 1998. Identification and Ds-tagged isolation of a new gene at the *Cf-4* locus of tomato involved in disease resistance to *Cladosporium fulvum* race 5. *The Plant Journal*. 14: 401-411.
- Tang X., Frederick R.D., Zhou J., Halterman D.A., Jia Y. and Martin G.B,** 1996. Initiation of plant disease resistance by physical interaction of AvrPto and Pto kinase. *Science*. 274: 2060-2063.
- Tang X., Xie M., Kim Y.J., Zhou J., Klessig D.F. and Martin G.B,** 1998. Overexpression of Pto activates defense responses and confers broad resistance. *Plant Cell*. 11: 15-29.
- Tenhaken R., Levine A., Brisson L.F., Dixon R.A. and Lamb C,** 1995. Function of the oxidative burst in hypersensitive disease resistance. *Proceedings of the National Academy of Sciences of the USA*. 92: 4158-4163.
- Thomas C., Sun Y., Naus K., Lloyd A. and Roux S.,** 1999. Apyrase functions in plant phosphate nutrition and mobilizes phosphates from extracellular ATP. *Plant Physiology*. 119: 543-551.
- Thomas C.M., Jones D.A., Parniske M., Harrison K., Balint-Kurti P.J., Hatzixanthis K. and Jones J.D.G,** 1997. Characterization of the tomato *Cf-4* gene for resistance to *Cladosporium fulvum* identifies sequences that determine recognitional specificity in *Cf-4* and *Cf-9*. *The Plant Cell*. 9: 2209-2224.
- Todorov L., Mihaylova-Todorova S., Westfall T., Sneddon P., Kennedy C., Bjur R., Westfall D.,** 1997. Neuronal release of soluble nucleotidases and their role in neurotransmitter inactivation. *Nature*. 387: 76-79.

Torii K.U. and Clark S.E., 2000. Receptor-like kinases in plant development. Kreis, M. and Walker, J.C. (eds). *Advances in botanical Research*. Academic Press. San Diego/San Francisco/New York/Boston/London/Sydney/Tokyo. *Advances in Botanical Research*. Academic press, San Diego.

Torii K.U., Mitsukama N., Oosumi T., Matsuura Y., Yokohama R., Whittier R.F. and Komeda Y., 1996. The *Arabidopsis* ERECTA gene encodes a putative receptor protein kinase with an extracellular leucine-rich repeat. *The Plant Cell*. 8: 735-746.

Trochoud A.E., Hao T., Wu G., Yang Z. and Clark S.E., 1999. The CLAVATA1 receptor-like kinase requires CLAVATA3 for its assembly into a signaling complex that induces KAPP and Rho-related protein. *The Plant Cell*. 11: 393-405.

Tsuji J., Jackson E.P., Gage D.A., Hammerschmidt R. and Somerville S.C., 1992. Phytoalexin accumulation in *Arabidopsis thaliana* during the hypersensitive response to *Pseudomonas syringae* pv *syringae*. *Plant Physiology*. 98: 1304-1309.

Van Loon L.C. and Van Strien E.A., 1999. The families of pathogenesis-related proteins, their activities and comparative analysis of PR-1 type proteins. *Physiological and Molecular Plant Pathology*. 55: 85-97.

Walker J.C. and Zhang R., 1990. Relationship of a putative receptor protein kinase from maize to the S-locus glycoproteins of *Brassica*. *Nature*. 345: 743-746.

Wang X., Zafian P., Choudhary M. and Lawton M, 1996. The PR5K receptor protein kinase from *Arabidopsis thaliana* is structurally related to a family of plant defense proteins. *Proceedings of the National Academy of Sciences of the USA*. 93: 2598-2602.

Watson J.C. and Yang Z, 1993. Molecular cloning and characterization of rho, a ras-related small GTP-binding protein from the garden pea. *Proceedings of the National Academy of Sciences of the USA*. 90: 8732-8736.

Weiss E.A, 2000. *Oilseed Crops 2nd Edition*. Blackwell Science, United Kingdom, pp 205-243.

Zhang X, 1998. Leucine-rich repeat receptor-like kinases in plants. *Plant Molecular Biology Reporter*. 16: 301-311.

Zheng-hui H., Fujiki M. and Kohorn B.D, 1996. Cell wall-associated receptor-like protein kinase. *The Journal of Biological Chemistry*. 271: 19789-19793.

Zhou J. Loh Y. Bressan R.A. and Martin G.B, 1995. The tomato gene *Pti1* encodes a serine/threonine kinase that is phosphorylated by Pto and is involved in the hypersensitive response. *Cell*. 83: 925-935.

Zhou J., Tang X. and Martin G.B, 1997. The Pto kinase conferring resistance to tomato bacterial speck disease interacts with proteins that bind a cis-element of pathogenesis-related genes. *European Molecular Biology Organization*. 16: 3207-3218.

Zimmerman S., Nurnberger T., Frachisse J.M., Wirtz W., Guern J., Hedrich R. and Scheel D, 1997. Receptor-mediated activation of a plant Ca^{2+} -permeable ion channel involved in pathogen defense. *Proceedings of the National Academy of Sciences of the USA*. 94: 2751-2755.

Summary...

Sunflower (*Helianthus annuus*) is a commercially important crop plant. Susceptible plants infected with leaf rust (*Puccinia helianthi*) leads to great yield losses. The aim of this study was to identify putative protein kinase genes from a resistant sunflower cultivar that are involved in the early resistance response when the plant is infected with leaf rust.

Several differentially expressed cDNA fragments were isolated from infected resistant sunflower. One of these identified clones (D15) showed an 8.5 fold induction of gene expression 3 h.p.i. in infected resistant sunflower, while the expression in the susceptible infected cultivar was repressed. A second 31 fold activation was found 15 h.p.i. This second induction most probably forms part of induced SAR in the uninfected plant tissue. This cDNA clone showed homology to several protein kinase genes. In addition, the gene is present as a single copy within the genomes of susceptible and resistant sunflowers. A polymorphism in size was however evident between the genes present in the two cultivars.

Three other cDNA clones were also selected on the basis of their expression profiles. These cDNAs showed homology to Apyrases, a Rac-protein and Xyloglucan endotransglycosylases respectively. The involvement of all four cDNAs in the activation of plant defense however still needs to be confirmed.

Keywords: *Helianthus annuus*, *Puccinia helianthi*, DDRT-PCR, protein kinase gene, plant defense.

Opsomming...

Sonneblom (*Helianthus annuus*) is 'n kommersieël belangrike gewas. Vatbare kultivars wat met blaarroes (*Puccinia helianthi*) geïnfekteer is, lei tot grootskaalse verliese. Die doel van hierdie studie was om 'n proteïenkinasegeen te identifiseer wat in die weerstandbiedende kultivar 'n belangrike rol speel tydens die verdedigingsreaksie wanneer die plant met blaarroes geïnfekteer word.

Verskeie differensieël uitgedrukte cDNA fragmente is uit geïnfekteerde weerstandbiedende sonneblomme geïsoleer. Een van die klone (D15) het 'n 8.5 voudige induksie van uiting slegs 3 ure na infeksie in die geïnfekteerde weerstandbiedende plante getoon. Die uitdrukking van hierdie geen was egter aktief in die geïnfekteerde vatbare kultivar onderdruk. 'n Tweede induksie van die geen was ook 15 ure na infeksie in die weerstandbiedende plante gevind. Hierdie tweede induksie vorm moontlik deel van die indusering van sistemiese verworwe weerstand in die ongeïnfekteerde weefsel. Hierdie kloon toon homologie met verskeie proteïenkinasegene uit verskillende plante. Die D15-geen kom as 'n enkelkopie voor in beide die weerstandbiedende en vatbare kultivars. 'n Moontlike polimorfisme is gevind deurdat die gehybridiseerde fragmente in grootte verskil in die twee kultivars.

Drie ander cDNA klone is ook geselekteer na aanleiding van hul induksieprofiële. Hierdie klone toon homologie met Apirases, 'n Rac-proteïen en 'n Xyloglukanendotransglikosilase onderskeidelik. Die betrokkenheid van al vier hierdie cDNAs tydens die aktivering van plant verdediging moet egter verder nog bevestig word.

Sleutelwoorde: *Helianthus annuus*, *Puccinia helianthi*, DDRT-PCR, proteïenkinasegeen, plant verdediging.