Characterization of At-RLK3, a putative receptor-like protein kinase from *Arabidopsis thaliana*.

by

Botma Visser

submitted in fulfillment of the requirements for the degree

Philosophiae Doctor

in the Faculty of Natural and Agricultural Sciences
Department of Plant Science
University of the Free State
Bloemfontein
South Africa

2004

Promoter:

Prof GHJ Pretorius
Department of Hematology
University of the Free State
Bloemfontein
South Africa

Co-promoters:

Prof AJ van der Westhuizen
Dept of Plant Sciences
University of the Free State
Bloemfontein
South Africa

Prof N Verbruggen
Laboratory of Plant Physiology
and Molecular Genetics
Free University of Brussels
Brussels
Belgium

Prof BA Prior
Department of Microbiology
University of Stellenbosch
Stellenbosch
South Africa

Genesis 1: 11 – 12

God said, "I command the earth to produce all kinds of plants, including fruit trees and grain." And that's what happened. The earth produced all kinds of vegetation. God looked at what He had done, and it was good."

Revelations 21: 1

"I saw a new heaven and a new earth. The first heaven and the first earth had disappeared, and so had the sea."

Table of Contents

List of Figures

Chapter 3

Chapter 4

Chapter 5

Chapter 6

List of Tables

Chapter 2

Chapter 3

Chapter 4

Abbreviations

A	absorbency
ABA	abscisic acid
ABRE	ABA-responsive element
ACC	1-aminocyclopropyl-1-carboxylic acid
APS	ammonium peroxydisulfate
ATP	adenosine triphosphate
Avr	avirulence
BAP	benzylacylpurine
BPB	bromophenol blue
BL	brassinolide
bp	base pair
BSA	bovine serum albumin
CIM	callus inducing medium
CRR	cysteine-rich repeat
DAG	diacylglycerol
dATP	deoxyadenosine triphosphate
DEPC	diethylpyrocarbonate
DMSO	dimethyl sulfoxide
DEX	dexamethazone
dNTPs	deoxyribonucleotide triphosphates
2,4-D	2,4-dichlorophenoxyacetic acid
DRE	drought-responsive element
DTE	dithioerythritol
DTT	dithiotreitol
DUF	domain of unknown function
ECM	extracellular matrix
EDTA	(ethylenedinitrilo) tetraacetic acid

EGF	epidermal growth factor
EGTA	ethyleneglycolbis (aminoethylether) tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
EtBr	ethidium bromide
GA	gibberellic acid
GM	germination medium
H_2O_2	hydrogen peroxide
HEPES	4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid
HR	hypersensitive reaction
HSE	heat shock element
2-IP	2-isopentinyladenine
IAA	indole acetic acid
IgG	immunoglobulin
IP_3	inositol 1, 4, 5-triphosphate
IPTG	isopropylthio-ß-D-galactoside
JA	jasmonic acid
KAPP	kinase associated protein phosphatase
kb	kilobase pair
kDa	kilodalton
KID	kinase interacting domain
KLH	keyhole limpet hemocyanin
LRR	leucine rich repeat
LZ	leucine zipper
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
MeJA	methyl jasmonate
MES	2-[N-morpholino] ethanesulfonic acid
MOPS	3-[*N*-morpholino] propanesulfonic acid
MS	Murashige and Skoog
NAA	α-naphthaleneacetic acid
NBS	nucleotide binding site
NLS	nuclear localization signal

NO	nitric oxide
Nonidet P40	octylphenolpoly (ethyleneglycolether)
ORF	open reading frame
PA	phosphatidic acid
PCD	programmed cell death
PCR	polymerase chain reaction
PEG	polyethylene glycol
PGIP	polygalacturonase-inhibiting protein
Pipes	1,4-piperazine diethanesulfonic acid
PLC	phospholipase C
PLD	phospholipase D
PMSF	phenylmethylsulfonyl fluoride
ppb	parts per billion
PR	pathogenesis related
PSK	phytosulfokine
PVP	polyvinylpyrrolidone
R-gene	disease resistance gene
RLK	receptor-like protein kinase
RLP	receptor-like protein
ROS	reactive oxygen species
RPK	receptor protein kinase
RT	reverse transcription
RT-PCR	reverse transcribed polymerase chain reaction
SA	salicylic acid
SAR	systemic acquired resistance
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS poly-acrylamide gel electrophoresis
SEM	shoot elongation medium
SI	self-incompatibility
SIM	shoot inducing medium
SLG	self-incompatibility-locus glycoproteins
SOM	shoot overlay medium
TCA	trichloroacetic acid
TEMED	N, N, N', N'-tetramethylethylendiamine

TF	transcription factor
TM	transmembrane
TNFR	tumor necrosis factor receptor
Tris	tris(hydroxymethyl)aminomethane
Triton X-100	octylphenol decaethylene glycol ether
Tween 20	polyoxyethylenesorbitan monolaurate
U	units
$V.cm^{-1}$	volts per centimeter
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Acknowledgements

At the end of a study like this, one is overcome with emotion and gratitude towards so many that has contributed to make this a success. I would therefore like to thank the following for their contributions:

- First and most important, my Father in Heaven, Jesus Christ His Son and the Holy Spirit for Their guidance, love and for the ability and wisdom that was bestowed upon me to complete the study.
- My promoter, Prof GHJ Pretorius. Oubaas, over the years you have taught me the novelty and elegance of Molecular Genetics. For me, you are the ultimate Molbol. Thank you for your friendship over all these years.
- My co-promoters:
  - Prof A van der Westhuizen. Thank you Prof for the help and wisdom for the many times when I did not know what to do next. I look forward to many years of close collaboration in the future.
  - Prof N Verbruggen. Nathalie, as I have mentioned before, the chance to work in your lab was both a life-changing and eye-opening experience for me. It was only then that I realized that Molecular Genetics and Plant Physiology walk hand in hand. I hope to plough back what I learned into the training of future students.
  - Prof B Prior. Thank you Prof for opening the door that allowed me to work overseas. It was valuable time spent.
- Marisa, Colette, Carita and Henlo. You have really sacrificed a lot especially during the last year. Thank you for your unconditional love and support, it means the world to me.
- My late father and my mother. You voluntarily missed out on so many opportunities just to allow us, your children, to get the best education and future possible. Thank you.

- My colleagues, friends and students at the department of Plant Sciences. We have always been a great group of people together that shared laughter and sadness, pleasure and pain. Where ever we go in life, the years spent together will always hold good memories.

I also wish to thank the following institutions:

- The department of Plant Sciences, as well as the University of the Free State for the use of their facilities.
- The Laboratorium voor Genetika, Vlaams Interuniversitair Instituut voor Biotechnologie, Universiteit Gent in Belgium for the time spent working there.
- Prof J Thevelein of the Laboratory of Molecular Cell Biology, Department of Biology, Catholic University of Leuven in Belgium allowing me to work within his department.
- The National Research Foundation for financial support.
- The Flemish Community for Bilateral Scientific and Technological Cooperation between Flanders and South Africa (Project BIL96/09) for financial support during my work overseas.

Published results

Czérnic P, B Visser, W Sun, A Savouré, L Deslandes, Y Marco, M Van Montagu and N Verbruggen, 1999. Characterization of an *Arabidopsis thaliana* receptor-like kinase gene activated by oxidative stress and pathogen attack. Plant Journal 18: 321 - 328.

Chapter 1

Introduction

Introduction

Since plants form the basis of the food chain, man, beast and pathogen depend on them for their own existence. To survive, plants developed elaborate and effective defense strategies to overcome predation and utilization by animals. In addition, the continual changes in environmental conditions forced plants to develop strategies to adapt to both short term fluctuations and longer term climatic changes. Since plants experience stressful conditions during the adaptation to both herbivores and changing environmental conditions, it is not surprising that the plant's response during the two different stresses overlaps significantly.

The first area of similarity between biotic and abiotic stresses is the need to sense either the presence of potential pathogens and predators or any changes in environmental conditions. This function is performed by a large variety of plant receptor proteins that are located either on the outside of the cell as part of the plasma membrane (Walker, 1994) or in the cytosol as a soluble protein (Hammond-Kosack and Jones, 1996). Their primary function is to bind ligands that are produced by the primary stimulus. An example is when a plant carrying a resistance gene is attacked by a virulent pathogen expressing an avirulent gene (Flor, 1971). Resistance is obtained when the avirulence gene product (the ligand) is bound by the virulence gene product (the receptor) (Zhou *et al.*, 1995). Upon binding, a very specific signal is transferred into the cell that activates an effective defense system allowing the plant to survive (Zhou *et al.*, 1997).

This interaction between receptor protein and ligand is very specific since it developed over thousands of years. This implies that for each ligand, a unique receptor protein is present in the plant cell to initiate the defense response.

One class of receptor proteins is the receptor-like protein kinases that have a very characteristic structure (Walker, 1994; Torii, 2000). The proteins are anchored to the

plasma membrane through a hydrophobic transmembrane domain. Attached to this is an intracellular region that contains eleven conserved subdomains characteristic of serine/threonine specific protein kinases (Hanks and Quinn, 1991). A third region is located on the outside of cell where it is ideally situated to bind ligands that are produced on the cell surface (Dietz, 2001).

The sequence diversity of the extracellular regions allowed the classification of the proteins into different groups (Torii, 2000). It also indicates the large variety of different ligands that can potentially be bound by the receptors. These receptors have thus far been shown to be involved in a variety of different cellular processes. Some were shown to act as resistance proteins involved in plant defense (Song *et al.*, 1995; Feuillet *et al.*, 1997), others have the ability to bind plant hormones such as brassinosteroids (Li and Chory, 1997) while others play important roles in plant development (Becraft *et al.*, 1996; Clark *et al.*, 1997) and pollination (Stein *et al.*, 1996). Finally, others appear to be involved in the recognition of secondary defense products such as salicylic acid (Pastuglia *et al.*, 1997; Komjanc *et al.*, 1999).

Several other proteins interact with these receptor proteins. Included are extracellular receptor-like proteins that share sequence homology with the extracellular domains of receptor-like protein kinases (Giranton *et al.*, 2000), protein phosphatases (Stone *et al.*, 1994), other protein kinases (Zhou *et al.*, 1995) and transcription factors (Zhou *et al.*, 1997). The binding of the downstream proteins is dependent on the activation of the receptor through phosphorylation (Zhou *et al.*, 1995; Stone *et al.*, 1999). The receptor is activated when the ligand is bound via either the homo- or heterodimerization of the receptor protein leading to autophosphorylation on both serine and threonine amino acids within the kinase domain (Wang *et al.*, 1998; Trotochaud *et al.*, 1999).

A second area of similarity found between signaling events activated by different stresses is the usage of shared signaling compounds. Even though the ligand bound by receptors may differ between two different stresses such as a pathogen attack and dehydration, the downstream signaling events occurring inside the cell may overlap since several components such as salicylic acid, hydrogen peroxide and Ca^{2+} are involved in several unrelated stress conditions (Jenkins, 1999; Bowler and Fluhr,

2000). These compounds are implicated to control cross-tolerance between different stresses. This extensive cross-talk between different signal transduction pathways has become the norm rather than the exception.

In addition to the primary ligand, several other molecules are also implicated to act as secondary messengers that are involved in both the activation and the amplification of the initial signal. One of these is reactive oxygen species that includes hydrogen peroxide (Wobbe and Klessig, 1996). The reactive oxygen species cause changes in the cellular redox potential, thereby activating different proteins through either reduction or oxidation events (Després *et al.*, 2003).

It is thus clear that the detection of changing extracellular conditions by receptors and the subsequent activation of the defense responses inside the cell are key events important for the survival of the plant cell. In future, more research will be directed towards understanding the complex interaction between different signaling pathways that provides cross-tolerance when plants are stressed.

This study forms part of the research drive to understand the role of receptor proteins in the plant cell. During this project, an attempt will be made to assign a putative function for At-RLK3, a receptor-like protein kinase from *Arabidopsis*. This will be done by studying the gene and protein structure, by doing a detailed expression and biochemical analysis of the gene and encoded protein respectively and finally by producing transgenic plants expressing an antisense copy of the gene. Any subsequent altered phenotype of the transgenic plants will be helpful to state where and when At-RLK3 is active within *Arabidopsis*.

Chapter 2

Literature review

Literature review

2.1 Introduction

Plants are sessile organisms unable to move from one location to another in order to escape unfavorable conditions. With a bombardment of changing conditions with which the plant has to cope, plants have developed specialized mechanisms allowing it to adapt. Stimuli, to which the plant must be able to respond, can be either due to the environment or due to internally generated signals. The former includes light, pathogens, wounding, the availability of water and temperature extremes, while the latter includes hormones, steroids and products from both the primary and secondary metabolism of the plant.

Broadly seen, the ability of the plant to react to the various stimuli relies on three major events within the cell. The first is the ability to detect the particular stimulus very early after the change has taken place. This requires a specific receptor protein that would probably bind a specific ligand that is produced. It is thought that this binding leads to the activation of the receptor protein, usually through reversible phosphorylation.

The next step is the transfer of the signal across the plasma membrane into the cell nucleus. This involves a subset of proteins constituting a signaling cascade that can be phosphorylated and dephosphorylated. Proteins involved in this signaling cascade include both protein kinases and phosphatases that are responsible for the reversible phosphorylation events.

The final step is the reaction of the plant towards the changing conditions with the induced synthesis of relevant proteins. This is presumably achieved by the activation of certain transcription factors that recognize and bind specific sequence motifs on the promoter regions of a particular subset of genes (Jenkins, 1999). These encoded proteins will then direct and facilitate the adaptation of the plant.

Several such subsets of proteins have already been described. Low temperature leads to the synthesis of over 30 different proteins that are involved in the adaptation of the plant (Hughes and Dunn, 1996). On the other hand, a large number of heat-shock proteins are produced once a plant is subjected to elevated temperatures (Wang *et al.*,

2003). Pathogen attack also leads to the expression of the pathogenesis related (*PR*) genes that encode a diverse set of proteins that allows the plant to adapt to the presence of the pathogen (van Loon, 1997).

Even though each subset of proteins is specific for each particular stimulus, different subsets do share some common proteins. Therefore, in order to ensure that the reaction to each stimulus is optimal, it is important that each response must be appropriate in context to each other. This implicates the coordination and integration of responses through "cross-talk" between relevant signal transduction pathways.

This information on how plant signaling mediates the effects of the stimuli is acquired through physiological, pharmacological and genetic approaches. During this literature study, some light will be shed on how plants respond to changes in the environment through receptor mediated signaling and cross-talk between different signaling pathways.

2.2 The extracellular matrix of the plant cell

The extracellular matrix (ECM) of a plant cell is a dynamic component where several crucial events of a plant's life take place. Together with the plasma membrane and apoplastic space, these three cellular components play an important role in the way that the cell will respond to changes occurring on the outside of the cell.

The plant cell wall is a combination of polysaccharides, proteins, lignin and other minor compounds that forms a rigid network around the cell (Showalter, 1993). Cellulose micro fibrils are interconnected with arabino- and xyloglucan molecules that are imbedded in a polygalacturonic acid matrix that are salt-bridged by Ca^{2+}. The cell wall is further attached to the plasma membrane and the cytoskeleton via attachment sites formed by arabinogalactan proteins with glycosylphosphatidyl inositol anchors (Youl *et al.*, 1998), cell-wall associated protein kinases (He *et al.*, 1996) and integrin-like proteins with arginine-glycine-aspartic acid binding sites (Reuzeau and Pont-Lezica, 1995). Such a structure produces an ECM-cytoplasm continuum similar to that found in animal cells.

The cavity between the plant cell wall and the plasma membrane contain in addition to the cell wall bound proteins, several other types of soluble proteins, including secreted PR proteins that act as anti-fungal and anti-microbial compounds (Dietz, 2001). Also present in the apoplast are inhibitors of various enzymes involved in metabolic regulation and defense (Weil *et al.*, 1994; Leckie *et al.*, 1999). The apoplast also contains reactive oxygen species (ROS) in the form of hydrogen peroxide (H_2O_2) and O_2^-, which together with ascorbic acid define the redox state of the apoplast. The ROS are involved in the cross-linking of the cell-wall components and play an important role in the defense reaction (Baker *et al.*, 1997).

The pH of the apoplastic fluids is slightly acidic (Dietz, 1997), but can change due to environmental factors (Mühling *et al.*, 1995) and the developmental state of the plant (Taylor *et al.*, 1996). The pH is important for effective receptor binding and signal transduction to occur from the ECM (Dietz, 2001).

Both the plasma membrane and the ECM are prime sites for signal perception which activates several downstream signal-transduction pathways (Dietz, 2001). Signal perception of changing environmental conditions and pathogenic attack is often perceived in the ECM which then leads to the activation of an internal signaling cascade. The important function of the detection of changes and the initiation of a signaling cascade is thought to be fulfilled by receptor proteins and other cell wall associated proteins (Satterlee and Sussman, 1998; Morris and Walker, 2003). The signals indicating a change could either be a chemical or a physical stimulus. The perception of the former might take place either extracellularly via transmembrane receptor proteins or the stimulus could be transported into the cell via a transporter protein with the recognition event taking place within the cell.

2.3 The plant's response to changing conditions

In response to changing conditions on the outside of the cell, plants have developed a complicated, but very effective signaling mechanism that allows the plant to adapt. This signaling mechanism employs both enzymes and metabolites that are both preformed, as well as inducibly produced, in response to the change.

2.3.1 Plant metabolites involved in the adaptation process

Central to the response of plants to a variety of different stimuli, is a number of natural compounds that play important roles in its adaptation. These include jasmonic acid (JA), ethylene, abscisic acid (ABA), salicylic acid (SA), nitric oxide (NO) and H_2O_2.

2.3.1.1 Salicylic acid

SA is synthesized via the phenylpropanoid pathway when phenylalanine is converted to cinnamic acid via phenylalanine ammonialyase (Verberne *et al.*, 1999). A second pathway for SA synthesis is proposed to occur within the chloroplast where isochorismate is converted to SA via isochorismate synthase and isochorismate pyruvate lyase (Wildermuth *et al.*, 2001). SA biosynthesis is subjected to both positive and negative feedback regulation (Shah *et al.*, 1997; Verberne *et al.*, 2000; Feys *et al.*, 2001; Wildermuth *et al.*, 2001).

SA has a number of physiological effects within the plant cell. These include the induced expression of a number of defense-related genes, including nine PR genes and members of the TIR-NBS-LRR class of disease resistance (*R*)-genes (Shirasu *et al.*, 1997; Shirano *et al.*, 2002) as well as a number of genes encoding receptor proteins (Ohtake *et al.*, 2000). SA is also involved in the activation of both systemic acquired resistance (SAR) (Uknes *et al.* 1993) and the localized hypersensitive reaction (HR) to avirulent pathogens (Mauch-Mani and Slusarenko, 1996) by mediating the oxidative burst leading to cell death (Shirasu *et al.*, 1997). SA is also implicated in responses to several abiotic stresses, including temperature (Dat *et al.*, 1998), ozone and UV radiation (Yalpani *et al.*, 1994; Rao and Davis, 1999; Senaratna *et al.*, 2000) and salt and osmotic stress (Borsani *et al.*, 2001).

Both the levels of SA in the cell (Uknes *et al.* 1993), as well as the sensitivity of the plant towards SA (Yu *et al.*, 1997), play a role in the induction of an appropriate response. When challenged with a pathogen, the intracellular SA levels of plants increase within the infected tissues, as well as in distant uninfected tissues (Wobbe and Klessig, 1996). SA is implicated to act as a transportable signal molecule that activates the defense response in these distal parts (Shulaev *et al.*, 1995). This signal might be in the form of normal SA or methyl-salicylate (Shulaev *et al.*, 1997). When SA synthesis is either inhibited (Mauch-Mani and Slusarenko, 1996) or broken down within the plant (Gaffney *et al.*, 1993; Vernooij *et al.*, 1994), the plants fail to express the PR genes or to activate the defense responses.

Both protein phosphorylation and dephosphorylation are part of this SA-induced *PR*-gene expression (Conrath *et al.*, 1997). This was demonstrated by the cloning of a gene encoding a very unique mitogen-activated protein kinase (MAPK) from tobacco that is phosphorylated upon SA application (Zhang and Klessig, 1997).

The way that SA activates the defense reaction is still somewhat unclear. It was found that SA inhibits both the plasma membrane bound catalase (Conrath *et al.*, 1995) and ascorbate peroxidase (Durner and Klessig, 1995), enzymes responsible for H_2O_2 degradation. The elevated H_2O_2 levels could then act as second messengers to activate the defense responses via the induced expression of several genes (Wobbe and Klessig, 1996). It was however later found that H_2O_2 functions upstream of SA, making such a mechanism unlikely (Du and Klessig, 1997). Another mechanism likely to activate defense responses is the production of SA radicals which then could initiate lipid peroxidation (Neuenschwander *et al.*, 1995).

Recent evidence has indicated the role that NPR1 plays in the SA-induced activation of the plant defense. The gene was isolated from *Arabidopsis* using mutants (Cao *et al.*, 1997). SA and pathogen inoculation activates the expression of *NPR1* (Yu *et al.*, 2001). This activation is dependent on a SA-inducible protein complex binding to a W-box element present on the promoter region of the gene. Upon SA treatment, NPR1 is translocated into the nucleus by means of a nuclear localization signal (NLS) (Kinkema *et al.*, 2000). The protein then physically interacts with TGA2 which is a member of the TGA-element binding protein family of bZIP DNA binding proteins

(Fan and Dong, 2002). Several members of the TGA-element binding protein family was shown to bind to a SA-responsive element located on the promoter region of *PR-1* (Zhang *et al.*, 1999). This binding is enhanced by SA, indicating a clear involvement of SA in this signaling event.

In addition, SA activates the expression of Pti4, a transcription factor binding to a GCC box-present of the promoter regions of many *PR*-genes (Gu *et al.*, 2000). This induction however did not lead to enhanced expression of the PR-genes, suggesting that SA acts as a negative regulator of Pti4 functioning.

Together with the NPR1 dependent SA signaling pathway, an additional NPR1-independent pathway was also found (Shah *et al.*, 2001) utilizing SSI2 that involves a lipid-derived signal (Kachroo *et al.*, 2001). This protein is thought to act as a molecular switch that modulates cross-talk between the SA/NPR1-mediated signal pathway for PR-1 induction and the JA/Ethylene mediated pathway leading to defensin gene expression (Shah *et al.*, 1999).

2.3.1.2 Jasmonic acid

JA is a fatty acid plant hormone derived from linoleic acid via the octadecanoid pathway (Doares *et al.*, 1995). It is involved in plant growth and development, the resistance of the plant against insects (Li *et al.*, 2002), pathogens (Thomma *et al.*, 1999), wounding (León *et al.*, 2001) and various other stress factors (Creelman and Mullet, 1997).

JA and ethylene appear to be mutual stimulators of each other's biosynthesis (Laudert and Weiler, 1998). In addition, JA and ethylene co-regulate the induced expression of *PR-3*, *PR-4* and *PR-12* genes (Penninckx *et al.*, 1998). This regulation is completely independent from that of the SA-dependent regulation of the *PR-1*, *PR-2* and *PR-5* genes, indicating two separate signaling pathways operating in the plant.

Some interaction between the SA- and JA-dependent signaling pathways has recently been found (Shah *et al.*, 1999). One common regulatory point is NPR1 that controls SA-mediated SAR. This gene was also found to be essential for both JA- and ethylene- dependent activation of SAR induced by *Pseudomonas fluorescens* (Pieterse

et al., 1998). A possible molecular switch modulating the two independent pathways was identified in SSI1 (Shah *et al.*, 1999). Furthermore, the interplay between the two pathways was shown during ozone stress (Rao *et al.*, 2000) when it was proposed that JA signaling pathways reduce cell death due to ozone exposure by attenuating the oxidative burst and HR that is controlled by SA. This interaction might then modulate the relative amount of SA- and JA-inducible defenses to obtain an appropriate reaction depending on the pathogen.

2.3.1.3 Ethylene

Ethylene is a gaseous plant hormone that influences a very wide array of different cellular processes, including plant defense and general stress responses (Bleecker and Kende, 2000). While ethylene and JA are positive regulators of each other's biosynthesis and function in similar signaling pathways (2.3.1.2), SA acts in an antagonistic manner towards both JA and ethylene biosynthesis and a signaling step downstream of JA and ethylene (O'Donnell *et al.*, 1996).

2.3.1.4 Abscisic acid

ABA is an important plant compound that influences several physiological and developmental events (Busk and Pages, 1998; Leung and Giraudat, 1998). It plays an important role in the adaptation of plants during various abiotic stresses, including dehydration, salt stress and low temperatures (Trewavas and Jones, 1991; Jensen *et al*., 1996). ABA is also a putative signal molecule that links heavy metal stress to the increased expression of lipid-transfer proteins, since heavy metals affect the water status of the plant.

ABA application leads to the induction of H_2O_2 synthesis (Lin and Kao, 2001), but in other systems to a decrease in H_2O_2 levels (Schopfer *et al.*, 2001). H_2O_2 responses to ABA therefore seem to be tissue specific and may depend on many factors. ABA signaling in guard cells depends on both NO and H_2O_2, indicating a coordinated effect by both the compounds (Neill *et al.*, 2002). H_2O_2 and NO also play an important role in drought induced ABA synthesis (Zhao *et al.*, 2001; Neil *et al.*, 2002). This clearly indicates the very complex signaling system that is regulated by ABA.

2.3.1.5 Reactive oxygen species

ROS in plants has gained importance over the last few years as both effector and signaling molecules in response to a wide variety of different biotic and abiotic conditions (Bartosz, 1997). Included in the group of ROS, are the free radicals O_2^- and the highly reactive $^{\cdot}OH$, as well as H_2O_2. O_2^- can also react with NO to produce the very toxic $ONOO^-$ anion. ROS causes damage to all biomolecules and inhibits the plasma membrane Ca^{2+} ATPase leading to elevated Ca^{2+} levels (Price *et al.*, 1996). Various abiotic stresses, including dehydration, salt stress, temperature extremes and irradiation disturb the redox balance, leading to ROS accumulation. ROS accumulation is also due to enhanced enzymatic synthesis during pathogen attack, wounding, anoxia and in response to elicitors, ABA and gibberellic acid (GA) treatment, ozone and UV light (Bolwell, 1999; Pei *et al.*, 2000; Fath *et al.*, 2001; Rao and Davis, 2001; Baxter-Burrell *et al.*, 2002).

Several enzymes are implicated in the synthesis of H_2O_2 (Bartosz, 1997; Bolwell, 1999), including NADPH oxidase (Sagi and Fluhr, 2001) and peroxidase (Bolwell *et al.*, 2002). Also involved in H_2O_2 production, are the Rop proteins that regulate production via NADPH oxidase (Baxter-Burrell *et al.*, 2002). It is however proposed that different stimuli activate specific H_2O_2 generating enzymes (Bolwell *et al.*, 2002). The accumulation of H_2O_2 can also be dependent on the reduced activity of antioxidant enzymes, e.g. in the case of GA treatment (Fath *et al.*, 2001) and the SA-dependent inhibition of catalase (Durner and Klessig, 1995).

H_2O_2 is a key molecule in plant cellular adaptation and it is thought that it is involved in downstream signaling. The most noticeable role of H_2O_2 is programmed cell death (PCD) that occurs during the induced HR after pathogen attack which is mediated by NO (Delledonne *et al.*, 2001). H_2O_2 modulates Ca^{2+} channels in plasma membranes (Murata *et al.*, 2001), activates a MAPK signaling pathway (Samuel *et al.*, 2000) leading to cross-tolerance to various environmental stresses (Kovtun *et al.*, 2000) and induces the expression of defense genes (Lamb and Dixon, 1997). Finally, H_2O_2 also activates PAL, a key enzyme needed for the synthesis of SA, thereby indicating the involvement of ROS during SAR (Shirasu *et al.*, 1997).

The primary target for H_2O_2 signaling remains elusive and doubts exist whether a H_2O_2 specific receptor do exist (Neill *et al.*, 2002). H_2O_2 does however activate the induced expression of various genes, including protein kinases and transcription factors (TF) (Desikan *et al.*, 2001). No specific H_2O_2 regulatory sequences present on the promoter regions of these genes have however been described.

2.3.1.6 Nitric oxide

Apart from H_2O_2 and O_2^-, an additional reactive molecule was proposed to play an important role in PCD. This was done, since the half-life of O_2^- is too short to be effective, while H_2O_2 on its own is ineffective in causing cell death. Thus, the involvement of NO in plant defense reactions was shown (Dangl, 1998; Durner *et al.*, 1998). NO was also found to be involved in other cellular processes, such as photomorphogenesis and plant development (Noritake *et al.*, 1996; Beligni and Lamattina, 2000).

NO is synthesized by nitric oxide synthase (Ninnemann and Maier, 1996). The active synthesis of NO was found in the nucleus, in the chloroplasts and peroxisomes as well as along the plasma membrane (Huang *et al.*, 2002). Target proteins of NO-mediated signaling include those involved in pathogen attack, oxidative stress and SA signaling (Huang *et al.*, 2002). As was the case for SA, two different signaling pathways, one dependent and one independent of NO, was identified in plants to activate the defense response (Klessig *et al.*, 2000). In addition, NO appears to regulate the expression of various defense genes through both SA-dependent and SA-independent pathways.

2.3.2 Receptor-like protein kinases and adaptation

Reversible phosphorylation is a key mechanism for the transfer and amplification of cellular signals in plants (Ranjeva and Boudet, 1987). Protein phosphorylation was shown to occur in response to very diverse conditions. These range from the regulation of leaf and flower development, the transduction of calcium mediated responses, disease resistance up to the self-incompatibility (SI) response. This clearly indicates the major role that protein kinases play in these adaptation processes.

Recent evidence has shown that plants have a similar system as mammals to detect and transfer signals across the cell wall into the nucleus where adaptations could be initiated. For the detection and transfer of an external message, mammalian systems utilize receptor protein kinases (RPKs). These proteins are membrane bound and their function is to perceive the external environmental signals (Lemmon and Schlessinger, 1994). The RPKs contain glycosylated amino terminal domains, which allow the recognition and binding of ligands (Ullrich and Schlessinger, 1990). The proteins are anchored to the cell membrane by a single hydrophobic transmembrane domain. Linked to the extracellular domain is a protein kinase domain. The majority of RPKs are phosphorylated on tyrosine residues within the kinase domain (Ullrich and Schlessinger, 1990), but a few were discovered which are phosphorylated on serine and threonine residues (Lin *et al.*, 1992). RPKs show a preference for Mn^{2+} above Mg^{2+} as co-factor (Yardin and Ullrich, 1988).

The action of RPK proteins in mammalian cells can be summarized as follows (Hardie, 1999). The proteins are thought to be monomers in the absence of ligands. When a ligand binds to the extracellular domain, a dimer is formed which leads to tyrosine phosphorylation of each other. This creates docking sites for other proteins with high affinity for phospho-tyrosine residues. Once bound, large signaling complexes are assembled. Components of these complexes include enzymes which produce second messengers, factors that promote the activation of the RAS-family of proteins, protein-tyrosine phosphatases and protein-tyrosine kinases.

During the last decade, plant genes were identified that encode proteins similar in structure and function to RPKs (Table 2.1). These proteins typically contain a signal peptide, an extracellular domain, a single hydrophobic transmembrane domain and a carboxyterminal kinase domain. They presumably function in a way similar to RPKs. Because of the structural similarity between the plant proteins and mammalian RPKs, these proteins were called receptor-like protein kinases (RLKs).

RLKs varies in size with the largest being SR160 (160 kilodalton [kDa] with an open reading frame of 3621 base pairs [bp]) (Scheer and Ryan, 2002) and the smallest being RPK1 (59.7 kDa with an open reading frame of 1623 bp) (Hong *et al*., 1997). The proteins all contain several conserved N-linked glycosylation sites with a

consensus sequence of N-X-S/T. These motifs were found on both the extracellular and intracellular domains. Active glycosylation was proven for SRK (Stein *et al.*, 1991; Stein *et al.*, 1996) and TMK1 (Schaller and Bleecker, 1993).

A detailed description of all published RLK genes and their encoded proteins until March 2004 will now follow. References to each RLK are presented within the table and will only be included in the text when a peculiarity of the gene or protein is referred to. In addition, where applicable, the spatial and inducible expression patterns, as well as putative functions of the RLKs, are indicated. Where the inherent serine/threonine kinase activity of the RLKs was proven, it was also indicated in the table.

Table 2.1. A summary of all cloned and characterized plant *RLK* genes and its encoded proteins up to March 2004. The different RLKs are categorized according to the class it belongs to.

Name of gene	Place of expression	Inducers of expression	Activation of enzyme activity		Putative function	Reference
			Proven	Ser/Thr		
S-class						
SRK_6	Stigmas	Induction of expression during stigma maturation	X	X	Self-incompatibility response of plants	Stein *et al*., 1991 Stein and Nasrallah, 1993 Stein *et al*., 1996
SRK-910	Anther, pistil, stigma		X	X	Self-incompatibility response of plants	Goring and Rothstein, 1992
SRK_2	Pistils and anthers	Pollen development				Stein *et al.,* 1991
SRK_3						Delorme *et al.,* 1995
SFR2	Vegetative tissue, flower, anthers, stigmas, seed	Flower development, wounding, SA, pathogenic (*Xanthomonas campestris*, *Ralstonia solanacearum*) and non-pathogenic (*Escherichia coli*) bacterial infection			Plant defense	Pastuglia *et al.,* 1997
ZmPK1	Whole plant					Walker and Zhang, 1990

ARK1	Leaves, stem, floral buds	SA	X	X	Plant development	Tobias *et al.,* 1992 Tobias and Nasrallah, 1996
ARK2	Leaves and sepals, styles of mature flowers				Development of the sporophyte	Dwyer *et al.,* 1994
ARK3	Leaves, roots and inflorescences	Temporal and tissue-specific regulation SA			Development of the sporophyte	Dwyer *et al.,* 1994
OsPK10	Both shoots and roots with more in the former		X	X		Zhao *et al.,* 1994
RLK1	Rosettes	SA				Walker, 1993
RLK4	Shoot apex, base of lateral roots, lateral root primordial	Auxin	X	X	Active during the initiation of organs	Walker, 1993 Coello *et al.,* 1999
RKC1						Ohtake *et al.*, 2000
KIK1	Shoots and husk leaves		X	X		Braun *et al.,* 1997
BcRK1	Leaves, stigma, floral buds					Suzuki *et al.,* 1997

LRR-class						
OsLRK1	Young panicles, shoots and seeds, above ground meristems	Panicle development			Plant development by controlling the number of floral organs	Kim *et al.,* 2000a
Xa21			X	X	Disease resistance against *Xanthomonas oryzae*	Song *et al*., 1995 Wang *et al*., 1998
TMK1	Vegetative and reproductive tissues		X	X		Chang *et al.,* 1992 Schaller and Bleecker, 1993
RPK1	Vegetative and reproductive tissues	ABA, dehydration, salt and cold stress, inhibited by GA			Osmotic stress response	Hong *et al.,* 1997
SbRLK1	Highest in mesophyll and lower in vascular cells with very low expression in the roots					Annen and Stockhaus, 1999
RKF1	Stamens, floral meristems, anthers, pollen	SA	X	X	Stamen development	Takahashi *et al.,* 1998
PRK1	Pollen grains		X	X	Post-meiotic development of pollen	Mu *et al.,* 1994 Lee *et al.,* 1996a Lee *et al.*, 1997

BRI1	The whole plant		X	X	Brassinosteroid signal transduction	Li and Chory, 1997 He *et al.,* 2000 Friedrichsen *et al.,* 2000 Wang *et al.,* 2001
BAK1	The whole plant		X		Brassinosteroid signal transduction	Nam and Li, 2002
OsBRI1					Brassinosteroid signal transduction	Yamamuro *et al.,* 2000
tBRI1					Brassinosteroid signal transduction	Montoya *et al.*, 2002
HAESA (RLK5)	Abscission zones of floral and vegetative tissues	Stage of floral development, SA	X	X	Controls floral abscission	Walker, 1993 Horn and Walker, 1994 Jinn *et al.,* 2000
TMKL1						Valon *et al.,* 1993
CLAVATA1	Centre of shoot meristem		X	X	Shoot apical and floral meristem development	Clark *et al.,* 1997 Williams *et al.,* 1997 Stone *et al.*, 1998
ERECTA	Young floral buds, flowers, siliques, stems and leaves				Regulate organ-shape in the shoot apical meristem	Torii *et al.,* 1996
FLS2	Whole plant				Pathogen recognition	Gómez-Gómez and Boller, 2000
LTK1	Root, leaves, embryo, endosperm	Development of the endosperm			Endosperm development	Li and Wurtzel, 1998
LTK2	Endosperm	Development of the endosperm				Li and Wurtzel, 1998

LTK3	Endosperm					Li and Wurtzel, 1998
SERK	Early embryogenic tissues, zygotes after pollination	Presence of competent single cells	X			Schmidt *et al.,* 1997
LePRK1	Mature and germinated pollen		X		Adhesive and signaling roles	Muschietti *et al.,* 1998
LePRK2	Germinated pollen	Pollen germination	X			Muschietti *et al.,* 1998
LRPKm1		High induced expression during the incompatible and lower induced expression during the compatible interaction with *Venturia inaequalis*, SA			Plant-pathogen interaction signaling	Komjanc *et al.,* 1999
OsTMK	Internodes, region with dividing and elongating cells	Gibberellin	X	X	Plant growth	Van der Knaap *et al.,* 1999
INRPK1	Cotyledons, vegetative roots, leaves	Short-day treatment of plants			Photo-induced flowering	Bassett *et al.,* 2000
SARK	Older leaves and flowers, epicotyls, roots	Senescent tissues, BA, ACC, darkness			Senescence of leaves and flowers	Hajouj *et al*., 2000
SYMRK	Roots				Bacterial symbiosis	Stracke *et al.,* 2002
RKL1						Ohtake *et al.*, 2000

HAR1	Roots, nodules, stems, leaves, cotyledons				Regulates root growth and nodule number	Krusell *et al.*, 2002
SYM29	Roots, nodules and leaves				Probably similar to *HAR1*	Krusell *et al.*, 2002
PSK receptor					Binding of phytosulfokine hormone peptide	Matsubayashi *et al.*, 2002
GmNARK	Nodulated roots and shoots				Long-distance control of nodulation	Searle *et al.*, 2003
NORK1					Nodulation and mycorrhizal association	Endre *et al.*, 2002
SR160					Systemin perception during wounding	Scheer and Ryan, 2002
LeNIK					Binding to nuclear shuttle protein	Mariano *et al.,* 2004
GmNIK					Binding to nuclear shuttle protein	Mariano *et al.,* 2004
Chitinase-like class						
CHRK1	Flowers, leaves, open flowers	Tobacco mosaic virus, *Phytophthora parasitica*, flower development	X			Kim *et al.,* 2000b
Pathogenesis related class						
PR5K	Rosette leaves, siliques, stems, inflorescence and roots		X		Plant defense responses	Wang *et al.,* 1996

Epidermal growth factor class						
Wak1	High mRNA levels in leaves and stems and low in dark-adapted leaves, flowers, siliques and roots	*Pseudomonas syringae* (compatible), SA, INA, repressed by wounding				Kohorn *et al.,* 1992 He *et al.,* 1996 He *et al.,* 1998
Lectin-like class						
Ath.lecRK1	Whole plant except the roots	Growth phase of the cells	X	X		Hervé *et al.,* 1996
lecRK-a1	Cotyledons, leaves abscission zones and stipules	Oligogalacturonic acid, senescence and wounding			Plant development and adaptation	Riou *et al.,* 2002
lecRK-a3	Roots and aerial rosettes					Riou *et al.,* 2002
PnLPK	Strong expression in roots, older leaves and dedifferentiated callus with lower expression in other tissue	Wounding	X	X		Nishiguchi *et al.,* 2002
Tumor necrosis factor-like class						
CRINKLY4					Leaf epidermal and aleurone differentiation	Becraft *et al.,* 1996

Receptors lacking a kinase domain						
EILP		Hyphal wall component elicitor, *Pseudomonas syringae* pv. *glycinae*, *Pseudomonas syringae* pv. *tabaci*, SA			Disease resistance	Takemoto *et al.,* 2000
Cf-2					Resistance against *Cladosporium fulvum*	Dixon *et al.,* 1996
Cf-9					Resistance against *Cladosporium fulvum*	Jones *et al.,* 1994
AtELP					Chlathrin-dependent protein trafficking	Ahmed *et al.,* 1997
Tumors lacking an extracellular domain						
NTS16	Full length - stigma and style Shorter form – style, sepal, leaf and filament	Pollination, ethylene				Li and Gray, 1997
Pto			X	X	Disease resistance gene	Martin *et al.,* 1993 Loh and Martin, 1995
Fen			X	X	Response protein against fenthion	Loh and Martin, 1995 Martin *et al.,* 1994

C-X8-C-X2-C class						
RKF2	Whole plants					Takahashi *et al*., 1998
PvRK20-1	Flower buds, roots	Root development, wounding, *Fusarium solani*, nodulation factors, down-regulated by symbionts			Plant defense at defined developmental stages	Lange *et al.,* 1999
RKC1	Young seedlings, rosettes, lower expression in other tissues	SA, Zn and BA				Ohtake *et al.,* 2000
Miscellaneous RLKs						
LRK10	Leaves, culm and spikelets				Disease resistance	Feuillet *et al.,* 1997 Feuillet *et al.,* 1998
CrRLK1			X	X		Schulze-Muth *et al.,* 1996
LRRPK	Roots, cotyledons	Darkness, any light repressed expression			Light signal transduction	Deeken and Kaldenhoff, 1997
RKF3	Whole plant					Takahashi *et al*., 1998
PERK1	Whole plant, with strong expression in stem, petal and pistil	Wounding, *Sclerotinia sclerotiorum*	X	X	Sensing of cell wall damage due to wounding by pathogens	Silva and Goring, 2002
IRK1						Kowyama *et al.,* 1996

2.3.2.1 RLK structure

2.3.2.1.1 The signal peptide

All the described RLKs share a characteristic signal peptide located on the N-terminal side of the encoded polypeptide (Table 2.1). These signal peptides are characteristic of proteins that are translocated to the ER for secretion (von Heijne, 1990). Typically, the average signal peptide consists of 20 hydrophobic amino acids followed by 3 positively charged amino acids. In the case of RKF1, the signal peptide consists of 15 hydrophilic amino acids that were immediately followed by 14 hydrophobic amino acids (Takahashi *et al.*, 1998). This secretion motif supports the localization of the RLKs in the plasma membrane of the cell as predicted by the presence of the transmembrane (TM) domain (2.3.2.1.3).

A putative signal peptide cleavage site was also identified for RKF1 (Takahashi *et al.*, 1998) and SbRLK1 (Annen and Stockhaus, 1999). This site is located right at the end of the signal peptide and is proposed to act as the site where the protein is processed after being transported to the plasma membrane. Despite LTK1 and LTK2 being both RLKs, only LTK2 had a signal peptide (Li and Wurtzel, 1998). SERK (Schmidt *et al*., 1997) and PERK1 (Silva and Goring, 2002) are two RLKs that also lack signal peptides. It was however proposed that Type 1b integral membrane proteins that lack a signal peptide are still inserted into the plasma membrane via the usual endoplasmic reticulum (ER) translocator protein machinery (Singer, 1990).

2.3.2.1.2 The extracellular domain

The defining portion of an RLK protein is the extracellular domain that is proposed to function as a ligand binding entity. It is therefore not strange to find tremendous variety in the amino acid sequence of the extracellular domains of RLKs. This immediately led to the suggestion that the huge amino acid sequence variety allows RLKs to recognize and bind different ligands. Due to this variety, attempts were made to place RLKs in different classes based on consensus sequences present within the extracellular domain.

2.3.2.1.2.1 The S-class

The S-class of RLKs was the first to be described (Table 2.1). The extracellular domain of this class share extensive homology with the self-incompatibility-locus glycoproteins (SLG) from *Brassica* (Nasrallah *et al*., 1988). Characteristic of this extracellular domain is the presence of 12 conserved cysteines with 10 being absolutely conserved in the following consensus sequence: $CX_5CX_5CX_7CXCX_NCX_7CX_NCX_3CX_3CXCX_NC$ (Torii and Clark, 2000). In addition, a second box, the conserved PTDT box with a sequence of WQSFDXPTDTΦL where Φ is any aliphatic amino acid, was also described (Walker, 1994).

Huge sequence diversity occurs within this group with several members sharing little homology with the SRK receptors. While ARK1 shares 60% identity to SRK, it only shares 23% identity with ZmPK1 (Tobias *et al.*, 1992). ZmPK1 on the other hand shares only 22% identity with RLK1 and RLK4 (Walker, 1993).

2.3.2.1.2.2 The leucine-rich repeat class

The leucine-rich repeat (LRR) class currently contains the largest number of RLKs (Table 2.1) (Torii and Clark, 2000). This receptor class is almost exclusively found in plants (Li and Wurtzel, 1998). The exception is the trk tyrosine kinase receptors of animals (Schneider and Schweiger, 1991). Proteins having LRRs are thought to be involved in protein-protein or protein-ligand interactions (Kobe and Deisenhofer, 1994). Variations in LRR structure, length and number in RLKs were reported (Chang *et al.*, 1992; Muschietti *et al*., 1998), indicating the possibility that each RLK plays a role in a different signaling pathway. The LRRs can be arranged in either one continuous block, or in two or more separate blocks (Zhang, 1998).

LRR RLKs contain between 3 (Stracke *et al.*, 2002) and 28 (Takemoto *et al*., 2000) tandem LRR repeats on the extracellular domain of the protein. The consensus LRR motif ($LX_2LX_2LX_2LXLX_2NXLX\mathbf{G}XIPX_2$) is 24 amino acids in length with conserved leucines and a characteristic glycine which is absent from the intracellular 23 amino acid LRR motif (Jones *et al.,* 1994*)*. The LRR regions are surrounded by paired cysteines that

are 6 – 8 amino acids apart (Kobe and Deisenhofer, 1994). In most RLKs, the whole of the LRR region is surrounded by the paired cysteines. In the case of OsTMK, the first 10 of the 13 LRRs are surrounded by two paired cysteines while the last 3 LRRs are only preceded by a third pair (van der Knaap *et al.*, 1999).

Other unique motifs were also reported to be part of the extracellular domain of the LRR RLKs. These include a proline rich region that is conserved in extensins (Schmidt *et al.*, 1997; Li and Wurtzel, 1998), a serine rich region, a protein degradation target sequence (Li and Wurtzel, 1998) and a leucine zipper (LZ) (Walker, 1993; Li and Chory, 1997; Komjanc *et al.*, 1999; Scheer and Ryan, 2002). While the presence of the first region implicates the interaction between the RLK and cell wall components, the second is implicated in the formation of both hetero and homo-dimers (Landschulz *et al.*, 1988).

In the case of BRI1 (Li and Chory, 1997; Friedrichsen *et al.*, 2000), SR160 (Scheer and Ryan, 2002), GmNARK (Searle *et al.*, 2003) and the PSK receptor (Matsubayashi *et al.*, 2002), a short island of between 38 - 70 amino acids interrupts the LRR regions close to the TM domain. FLS2 also has a 23 amino acid island located between the 16th and 17th LRR motifs. This island is indicative of a leucine zipper (Gómez-Gómez and Boller, 2000). These islands share organizational homology with Cf-2, -4 and -9 (Jones and Jones, 1997). Within the BRI1 island, three conserved cysteines were shown to be essential for brassinolide (BL) binding (He *et al.*, 2000), indicating that the conserved nature of these islands is important for their functioning.

2.3.2.1.2.3 The lectin-like class

A plant lectin is a protein that has at least one non-catalytic domain that binds reversibly to a specific mono- or oligosaccharide (Peumans and van Damme, 1995). The class of lectin-like RLKs in *Arabidopsis* is part of an extended family of 38 putative RLKs (Shiu and Bleecker, 2003) whose extracellular domains share homology with lectin proteins. This presents the possibility that these RLKs might bind oligosaccharides generated during cell wall degradation as ligands.

The extracellular domain of PnLPK shares four essential Mn^{2+}-binding amino acids that are also present in legume lectins (Nishiguchi *et al*., 2002). Two of these amino acids are also able to bind Ca^{2+}. These two cations are necessary for tertiary lectin stability and carbohydrate binding. In contrast, despite the similarity between the Ath.lecRK1 extracellular domain and plant lectins, most of the key amino acids that form part of the putative monosaccharide binding domain differ from that of the legume lectin (Hervé *et al.*, 1996).

2.3.2.1.2.4 The epidermal growth factor class

The epidermal growth factor (EGF) class of RLKs is represented by a small gene family containing both Wak1 (Kohorn *et al*., 1992) and Wak4 (He *et al*., 1996). The extracellular domain of these RLKs shares similarity to the 10^{th} and 11^{th} EGF repeats of the Crumbs protein from *Caenorhabditis elegans* (Tepass *et al*., 1990). Characteristic of the EGF motifs is the presence of a conserved arrangement of 6 cysteines that have been implicated in protein-protein interactions in animals (Rebay *et al.*, 1991). This class of RLKs is unique since in addition to being plasma membrane bound, it is also cell wall associated.

2.3.2.1.2.5 The tumor necrosis factor receptor class

CRINKLY4 is the only member of this group (Becraft *et al.*, 1996). The extracellular domain of CRINKLY4 has seven copies of a 39 amino acid repeat motif with a proximal cysteine rich motif that resembles the extracellular domain of the mammalian tumor necrosis factor receptor (TNFR). The TNFR motif has a conserved arrangement of six cysteines located within a 26 amino acid stretch that shares a striking similarity to the second cysteine repeat of mammalian TNFRs (Loetscher *et al.*, 1990).

2.3.2.1.2.6 The pathogenesis related class

The only member of this class is PR5K isolated from *A. thaliana* (Wang *et al*., 1996). The extracellular domain of the protein contains all 16 cysteine amino acids that are conserved among the PR5 antimicrobial proteins. It also has a very unique C-terminal

region of 60 amino acids which is rich in both serine and threonine residues of which the significance still have to be established.

2.3.2.1.2.7 The chitinase-like class

There is only one protein that fall within this class, namely CHRK1 (Kim *et al.*, 2000b). The extracellular domain of this RLK is homologous to both class V tobacco and *Bacillus* WL-12 A chitinases. The extracellular domain did not show any chitinase activity, despite the high percentage identity between the two proteins. It was speculated that the change of a highly conserved glutamic acid to valine disabled the chitinase activity, but retained the binding ability of the extracellular domain. Thus, when a pathogen attacks a plant, chitinases might degrade the cell walls of the pathogen with the production of chitin elicitors that might be bound by CHRK1 that will then activate a defense response.

2.3.2.1.2.8 The cysteine-rich repeat class

The cysteine-rich repeat (CRR) class is characterized by the presence of one or more repeats of the C-X8-C-X2-C motif (Chen, 2001). The class is closely related to the S-class of RLKs (Ohtake *et al.*, 2000). In *Arabidopsis*, a large cluster of 20 genes located on chromosome 4 all have two copies of the conserved motif, while their expression is induced by SA (Ohtake *et al.*, 2000). In total, 44 RLKs and at least 60 other proteins from *A. thaliana* all have CRR repeats. Proteins functioning in the apoplast region often carry unique motifs with conserved cysteines that are thought to participate in the formation of disulfide bonds in the three-dimentional structure of the protein. It was thus proposed that proteins within this class may play important roles in SA-mediated defense responses.

2.3.2.1.2.9 Miscellaneous RLK proteins

Although the majority of RLKs can be placed into the above-mentioned classes, several RLKs were cloned that cannot be placed into any of the groups. The reasons for this are that their extracellular domains do not share any homology with other proteins and that they contain very unique motifs.

Included within this grouping is RKF3 (Takahashi *et al*., 1998), LRRPK (Deeken and Kaldenhoff, 1997), PERK1 (Silva and Goring, 2002) and LRK10 (Feuillet *et al.*, 1997, 1998). LRRPK contains a leucine rich extracellular domain that is framed by basic amino acids (Deeken en Kaldenhoff, 1997). This region resembles an interrupted leucine zipper with an almost completely conserved four repeats of L-X-L-4X-L-16X, but a similar structure has not been described before.

The Lrk10 RLK was identified from wheat as part of a small family of the *wrlk* genes (Feuillet *et al.*, 1997). The wrlk proteins have three conserved and three variable domains within the extracellular domain, with no clear homology to any other RLK (Feuillet *et al.*, 1998). All encoded wrlk proteins did have 12 invariant cysteine residues that is a hallmark of S-domain containing proteins from *Brassica* (Nasrallah *et al.*, 1991). In one short region surrounding an N-linked glycosylation site, the spacing of the cysteines was comparable with SLG and SRK.

The extracellular region of PERK1 is very rich in proline and share similarities with the hydroxyl-proline rich glycoproteins that are cell wall associated (Silva and Goring, 2002). One of the key elements of the extracellular domain is the presence of several $S(P)_{2-3}$ motifs with a single $S(P)_4$ motif that is characteristic of plant extensin proteins (Cassab, 1998).

2.3.2.1.2.10 Receptor proteins with alternative structures

Several receptor proteins have been identified whose structures differ from that of classical RLKs. Three groups are recognized, namely those that share homology to prokaryotic two-component signal transduction systems, those receptors that lack the characteristic RLK kinase domain and proteins that are functional protein kinases, but lack the extracellular ligand binding domains.

The first group is those that show similarity to members of the prokaryotic two-component signal transduction systems that act as histidine kinases (Urao *et al.*, 2000). This type of signaling is mediated by phosphotransfer between two types of signal

transducers, namely a sensory histidine kinase and a signal transducer. The former has an N-terminal input domain with a C-terminal kinase domain, while the latter has an N-terminal receiver domain with a C-terminal output domain. Upon activation, the sensor is phosphorylated on an invariant histidine within the kinase domain, where after this phosphoryl group is transferred to an aspartate residue within the receiver domain of the signal transducer. This leads to activation of the DNA binding activity of the signal transducer with a certain subset of genes being expressed.

The plant two-component receptors identified include ethylene receptors (ETR1 - Chang *et al.*, 1993), a cytokinin receptor (CKI1 – Kakimoto, 1996) and an osmosensor (ATHK1 – Urao *et al.*, 1999). Although the former two are plasma membrane bound, no extracellular domain is present. It was proposed that in the absence of ethylene, ETR1 is active and blocks CTR1, a downstream regulator of the ethylene response. The ethylene response is then switched off. When ethylene is present, ETR1 is inactivated, the control on CTR1 is released and the cell is able to respond to the presence of the ethylene.

CKI1 allows the plant to respond to applied cytokinin (Kakimoto, 1996). When overexpressed, CKI1 confer cytokinin-independent growth of *Arabidopsis*. CKI1 also contains two transmembrane domains, suggesting CKI1 to be a cytokinin receptor with the extracellular domain acting as the binding site for cytokinin.

AtHK1 also has two transmembrane regions with a putative extracellular domain (Urao *et al.*, 1999). The gene transcripts accumulated under both high and low osmolarity conditions. In addition to osmolarity, high salinity and drought also leads to the induced expression of the gene in roots. The enzyme is inactivated upon the onset of high salinity.

The second group of receptors consists of RLKs that have both an extracellular and transmembrane domain, but no kinase domain. Usually, the extracellular domains share homology with the extracellular domain of some of the above mentioned RLKs. These proteins most likely act as receptor-like proteins (RLPs) that have the ability to interact *in*

vivo with RLKs. The absence of a kinase domain in the above mentioned proteins indicates that for the proteins to function as receptors, they must in some way interact with an intracellular protein kinase that will be able to transfer an extracellularly generated signal to the nucleus. This protein might be in the form of a complete RLK, or in the form of a kinase that lacks an extracellular domain.

Included in this group are AtELP that has 3 EGF repeats in the extracellular domain (Ahmed *et al.*, 1997), Cf-9 that has 28 imperfect copies of a 24 amino acid LRR motif (Jones *et al*., 1994), Cf-2 that has 38 LRRs (Dixon *et al.*, 1996) and EILP that has 28 LRRs (Takemoto *et al.*, 2000). Also present is a TM domain anchor that ensures that the proteins are plasma membrane bound. Cf-9 share very high homology with RLK5, TMK1, TMKL1, antifungal poly-galacturonase-inhibiting proteins (PGIPs) and Cf-2. The size of these proteins suggests that they might interact with another protein during binding of the ligand which would then generate a cytoplasmic signal.

The third group consists of proteins that have an intracellular kinase domain and a TM domain, but only a very short extracellular domain. Included in this group is NTS16 (Li and Gray, 1997), Pto (Martin *et al*., 1993) and Fen (Martin *et al.*, 1994). It is thought that these proteins can interact with extracellular ligand binding molecules to transduce the signal across the plasma membrane. The latter two proteins are functional serine/threonine protein kinases that render tomatoes resistant to *Pseudomonas syringae* pv. tomato and sensitive to fenthion.

2.3.2.1.3 The transmembrane domain

All RLKs contain a central hydrophobic region characteristic of a type 1a integral membrane protein (Weinstein *et al*., 1982). The TM domain acts as the physical connection between the extracellular ligand binding domain and the catalytic kinase domain and spans the plasma membrane. A short region of acidic amino acids usually resides on the extracellular side of the TM domain, while several basic amino acids immediately follow the TM domain. These two regions acts as very efficient stop signals to ensure efficient incorporation of the RLK within the plasma membrane. The average

size of the TM domain is 23 amino acids with the two flanking regions being 6 amino acids each.

Using different purification techniques, several RLKs were proven to be located within either the TM domain (Chang *et al.*, 1992; He *et al.*, 1996; Stein *et al.*, 1996; Ahmed *et al.*, 1997; Feuillet *et al.*, 1998; Jinn *et al.*, 2000; Kim *et al.*, 2000b; Friedrichsen *et al.*, 2000; Nam and Li, 2002) or the microsomal fraction of pollen (Mu *et al.*, 1994; Muschietti *et al.*, 1998). Wak1 is unique since despite being plasma membrane bound, it was also found to be cell wall associated (He *et al*., 1996).

2.3.2.1.4 The kinase domain

Despite their structural diversity and varied substrate specificity, most eukaryotic protein kinases have 11 blocks of conserved amino acid sequences present in their catalytic domains (Hanks and Quinn, 1991). Detailed studies of plant protein kinases have shown that they form a distinct family. In contrast to mammalian protein kinases with tyrosine specificity, plant RLKs exhibit serine/threonine specificity within their catalytic domain. This is due to the amino acid differences of sub-domain VIb (DLKPEN vs. DLAARN) and VIII (G[T/S]XX[Y/F]IAPE vs. FPIKWMAPE) between plant and mammalian protein kinases.

All RLK genes thus far cloned, except one, code for putative active protein kinases as was deduced from their amino acid sequences (Table 2.1). The exception was TMKL1 whose coding sequence indicated that several key amino acids responsible for kinase activity were absent suggesting that it might be an inactive protein kinase *in vivo* (Valon *et al.*, 1993). Several mutated RLK alleles with single amino acid substitutions were also found (Li and Chory, 1997; Clark *et al*., 1997; Gómez-Gómez and Boller, 2000). The kinase activity of several RLKs was proven when they were expressed as recombinant proteins in *Escherichia coli* (Table 2.1) or when immunoprecipitated (Muschietti *et al*., 1998; Jinn *et al.*, 2000).

The amino acid sequences of all cloned RLK genes implicate serine/threonine specificity, most probably on multiple target sites within the kinase domain (Horn and Walker, 1994; Sessa *et al.*, 1998; Stone *et al.*, 1998; Coello *et al.*, 1999). Serine/threonine specificity was proven for several RLKs (Table 2.1). The ratio of phosphorylation events between serine and threonine residues also differed. An exception was PRK1 which displayed dual serine and tyrosine kinase specificity (Mu *et al.*, 1994). The kinase domain of PRK1 showed alterations to a number of key amino acids needed for serine/threonine specificity.

The same changes were also found in LePRK1 and LePRK2 (Muschietti *et al.*, 1998), even though they exhibited only serine/threonine specificity. This indicates a possible pollen specific alteration of protein kinase activity, since all three genes are expressed in pollen. INPRK1 has two overlapping consensus recognition sites for tyrosine-specific protein kinases (R/KX2-3D/EX2-3Y) within the highly conserved subdomain IX (Bassett *et al.*, 2000). This led the authors to suggest that kinase activity could be modulated by various other proteins kinases and may therefore function in concert with more than one signal transduction pathway.

Phosphorylation can occur via both an inter- and intra-molecular mechanism. In addition to autophosphorylation activity, several RLKs were capable of trans-phosphorylation implicating the importance of protein dimerization upon activation (Horn and Walker, 1994; Schulze-Muth *et al.*, 1996; Williams *et al.*, 1997; Coello *et al.*, 1999; Friedrichsen *et al.*, 2000; Nishiguchi *et al.*, 2002). In contrast, CrRLK1 predominantly autophosphorylates, indicating that dimerization might in this case not be important (Schulze-Muth *et al.*, 1996). When tested, a number of RLKs showed significantly higher kinase activity in the presence Mn^{2+} than with Mg^{2+} (Schaller and Bleecker, 1993; Horn and Walker, 1994; Schulze-Muth *et al.*, 1996; Takahashi *et al.*, 1998; Nishiguchi *et al.*, 2002).

SRK autophosphorylation was intermolecular in nature and involved dimerization or the binding of SRK with SLG or a differentially transcribed form of SRK (Giranton *et al.*,

2000). It was therefore concluded that an active kinase domain is essential for the successful initiation of signal transduction (Clark *et al.*, 1997; Li and Chory, 1997). A very interesting feature of the intracellular kinase domain of SERK is the presence of a LRR region (Schmidt *et al*., 1997). Since LRRs are implicated in protein-protein interactions (Kobe and Deisenhofer, 1994), this might mean that this region can act as an anchoring site for another protein involved in downstream signaling.

Another very important region of protein kinases is an activation loop that is located within the kinase domain (Johnson *et al.*, 1996). Changes of certain amino acids within this loop was shown to be crucial for CLV1 (Clark *et al.*, 1997), BRI1 (Li and Chory, 1997) and ERECTA function (Torii *et al.*, 1996).

2.3.2.2 Gene copy number and structure

When analyzed, all RLKs showed that they are either present as a single copy within the genome, or as part of a small gene family. In several cases, the genes are present as single copies, but they do share a very high degree of similarity with the other family members (Li and Wurtzel, 1998).

In the case of RLK genes implicated in plant defense, such as the *Wak* genes (He *et al*., 1996; He *et al.*, 1998) and *Xa21* and its relatives (Song *et al.*, 1995), the whole gene family is located as a unit on a particular chromosomal region. In contrast, LRR RLKs involved in plant development, differentiation processes and hormone detection are usually single copy genes that are distributed throughout the genome (Komjanc *et al.*, 1999).

The number and organization of introns within RLK genes vary considerably. It is however possible to see distinct patterns of relatedness in both the number, as well as the position of these introns. Several *RLK* genes have no introns (Zhang and Walker, 1993; Schulze-Muth *et al.*, 1996; Li and Chory, 1997) while others have up to 26 (Torii *et al*., 1996).

In the case of genes containing one intron, two groups are formed. *INPRK1* has a single 92 bp intronic sequence located within subdomain VIII of the kinase domain (Bassett *et al.*, 2000) which is similar in position to the single intron of *Xa21* (Song *et al*., 1995), *FLS2* (Gómez-Gómez and Boller, 2000), *TMK1* (Chang *et al.*, 1992), *CLV1* (Clark *et al*., 1997), *HAR1* (Krusell *et al.*, 2002) and *GmNARK* (Searle *et al.*, 2003). The second grouping is *EILP* that contains a single intron in a position similar to that in the *Cf-2*, *-4*, *-5* and *-9* genes (Jones *et al*., 1994; Dixon *et al*., 1996; Thomas *et al*., 1997). *PRK1* (Mu *et al.*, 1994) and *Lrk10* each contain 2 introns with the whole of the extracellular domain being encoded within the first exon (Feuillet *et al.*, 1997).

The next group of RLKs has 5 to 6 introns (Stein *et al.*, 1991; Tobias *et al.*, 1992; Dwyer *et al.*, 1994; Wang *et al.*, 1996; Suzuki *et al*., 1997). In all these genes, the whole of the extracellular domain is encoded by the first exon. In the case of *SRK2*, *SRK6* (Stein *et al*., 1991) and *PR5K* (Wang *et al.*, 1996), the kinase domain is also encoded by a single exon while for the *ARK* genes the kinase domain is encoded by a number of exons (Dwyer *et al.*, 1994).

RKF1 (Takahashi *et al.*, 1998) and *ERECTA* (Torii *et al.*, 1996) has 21 and 26 introns respectively. What was very characteristic of these two genes was the fact that each individual LRR was encoded by a separate intron giving rise to the suggestion that the LRR region developed via exon duplication (Torii *et al.*, 1996).

Another peculiarity of two RLK genes is that in the 5' untranslated regions of the mRNAs, a short open reading frame (ORF) occurs (Deeken and Kaldenhoff, 1997; Bassett *et al*., 2000). The two ORFs are respectively 47 and 29 amino acids in length, but the importance of these two encoded polypeptides is currently unknown. In addition, within the *LRPKm1* gene, a second ORF is present that codes for a putative proline-rich protein (Komjanc *et al.*, 1999).

2.3.2.3 RLK expression

Plant RLKs show marked differences in their expression patterns (Table 2.1). While some genes are expressed only in specific reproductive tissues, others are expressed predominantly in vegetative tissues. Some genes are expressed constitutively, while others are inducible. This induction can be stimulated by hormones, pathogens, chemicals that are produced during the plant defense response and several abiotic stresses. The expression pattern of a particular RLK gene can therefore be a good indication of where and under what conditions the protein may play a role.

The majority of *RLKs* codes for a single mRNA transcript. However, in several cases, Northern blot analysis indicated the presence of multiple mRNA transcripts. In the simplest cases, in addition to the full length transcript, 1 to 3 additional shorter transcripts were present (*ARK1* - Tobias *et al*., 1992, Tobias and Nasrallah, 1996; SRK_6 – Stein *et al.*, 1991; *SFR2* - Pastuglia *et al*., 1997; SRK_3 - Delorme *et al.*, 1995; *SRK* - Giranton *et al*., 1995; *PR5K* – Wang *et al.*, 1996; *NTS16* - Li and Gray, 1997; *CHRK1* - Kim *et al*., 2000b). By using DNA sequencing and Southern blots with different probes, the smaller transcripts were shown to code for the extracellular portion of the protein (Tobias *et al*., 1992; Tobias and Nasrallah, 1996; Kim *et al*., 2000b). An exception was SRK_6 where one of the shorter transcripts appeared to encode a polypeptide consisting of the TM and kinase domain (Stein *et al.*, 1991).

The shorter transcripts arise due to an in-frame stop codon present within the first intron of the above mentioned genes (Stein *et al.*, 1991; Tobias *et al*., 1992; Chang *et al.*, 1992; Dwyer *et al.*, 1994; Delorme *et al.*, 1995; Suzuki *et al.*, 1997). This stop codon is located 2 amino acids downstream of the exon/intron splice site and could therefore be used as an alternative stop codon during alternative mRNA splicing. A poly-adenylation signal is present within the first intron that will allow for the successful processing of the polypeptides. Since the first exon of these *RLK* genes codes for the full length extracellular domain, such an alternative transcript would give rise to a soluble extracellular protein that might help shuttle ligands across the cell wall to bind to the cognate receptors on the plasma membrane (Dzelzkalns *et al*., 1992).

The most complex RLK expression pattern yet found was for INPRK1 that showed at least six different transcription products on a Northern blot (Bassett *et al.*, 2000). The full length mRNA (INPRK1) was 4.4 kb in length. A second 2.6 kb mRNA (INPRK1b) was produced due to alternative splicing of a cryptic intron and the use of a secondary poly-A attachment site. The 17 kD INPRK1b polypeptide is identical to the first 129 amino acids of INPRK1 and is thought to act as a secreted extracellular soluble polypeptide. INPRK1a was also produced due to alternative splicing leading to a polypeptide exactly the same as INPRK1, but lacking 21 of the LRRs. This transcript was localized specifically in the roots. The synthesis of the 49 kD INPRK1c transcript was due to an alternative transcription initiation site located to the 5' end of the portion of gene encoding the TM domain. This mRNA therefore encodes a polypeptide consisting of the TM domain and the catalytically active protein kinase domain, similar to SRK_6 (Stein *et al.*, 1991).

Although not found in all RLKs, alternative splicing can function to increase the coding capabilities of genes. This phenomenon was also found in mammalian RPKs where some genes were alternatively expressed with both the normal and a truncated form of the receptor being produced (Petch *et al.*, 1990).

2.3.2.4 Mechanisms of RLK functioning

The study of human receptor protein kinases has led to a general model for receptor-mediated cellular signaling (Heldin and Ostman, 1996). In this model, a ligand is bound by an RLK that induces the receptor to form a dimer with the concomitant activation of the intracellular kinase domain. The binding of the ligand is specified by the amino acid sequence of the extracellular domain of the receptor protein. Each phosphorylated site acts as a binding site for various proteins all containing an SH2 domain. These proteins can be divided into two classes, namely those with catalytic activity and those that act as molecular adaptors. This binding of proteins then results in the activation of several cellular events such as the production of second messengers and the initiation of a protein kinase cascade (Hunter, 1995).

Since RLKs are plasma membrane bound and implicated in signal transduction, it is only reasonable to assume that a similar mechanism functions in plants. This section will discuss interacting proteins and ligands that regulate RLK function in plants, as well as the manner in which RLKs function *in vivo*.

2.3.2.4.1 RLK-interacting proteins

Since RLKs are membrane-spanning proteins, both secreted extracellular proteins and intracellular proteins could potentially interact with it. The function of the extracellular proteins could be to facilitate the binding of the ligand to the extracellular domain of the RLK that is mediated through either homo- or heterodimerization of the RLK (Torii, 2000). Intracellular proteins on the other hand could be responsible to transfer the phosphoryl group onto other secondary proteins.

All the more, the importance of the so-called RLPs is becoming clearer. The amino acid sequence of these RLPs is nearly identical to that of the extracellular domain of the cognate RLKs. RLPs can be generated in two ways. The first is that an *RLP* encoding gene is present on the genome as was found in the *Arabidopsis* genome (Shiu and Bleecker, 2003). Another possibility is the occurrence of alternative splicing as was previously described for some RLKs (2.3.2.3). This leads to the production of multiple mRNAs of which the alternative transcript encodes a secreted truncated protein that might act as an RLP.

Several such RLK and RLP combinations were described. The first was the SRK/SLG combination that is involved in the SI response in *Brassica* (Nasrallah and Nasrallah, 1993). This combination represents the female component of the SI response with the pollen providing the male component. A physical interaction between the two proteins were proposed (Giranton *et al.*, 2000) based on the sizes of protein complexes formed *in vivo*. Another described RLK/RLP complex is that of CLV1 and CLV2, proteins involved in the regulation of the shoot apical and floral meristem development (Jeong *et al.*, 1999). It was shown that the sequence of CLV2 closely resembled that of CLV1 and that these two proteins physically interact.

BRI1 was identified as a possible receptor involved in brassinosteroid binding (Li and Chory, 1997). Treatment of plants with BL activates BRI1 via autophosphorylation (Wang *et al.*, 2001). It has been proposed that the receptor must first interact with a second secreted protein before being able to bind BL, since its LRR regions are implicated in protein-protein interactions, and not steroid binding. One such candidate is BRS1, a secreted serine carboxypeptidase that was shown to have BL binding capacity (Li *et al.*, 2001).

Another BRI1 binding protein is BAK1, an RLK that was identified based on its ability to physically interact with BRI1 leading to transphosphorylation of the two proteins (Nam and Li, 2002). Even though also an LRR RLK, BAK1 is much smaller than BRI1 with only 5 extracellular LRRs present and both the second cysteine pair and the 70 amino acid island characteristic of BRI1 being absent.

Similar to RPKs, phosphorylated plant RLKs could function as docking sites for a number of downstream proteins (Hardie, 1999). Several such proteins have been found and include protein phosphatases (Stone *et al*., 1994; Braun *et al*., 1997; Li *et al.*, 1999; van der Knaap *et al.*, 1999), thioredoxins (Bower *et al.,* 1996), transcription factors (Zhou *et al.*, 1997) and ARC1 from *Brassica* (Stone *et al.,* 1999).

Kinase-associated protein phosphatase (KAPP) was identified in *Arabidopsis*, maize and rice (Stone *et al*., 1994; Braun *et al.*, 1997; van der Knaap *et al.*, 1999). All three proteins consist of a type one signal anchor, a central kinase interaction domain (KID) and a carboxyterminal type 2C protein phosphatase. The KID physically interacts in a phosphorylation-dependent manner with the kinase domains of TMK1, SRK-A14, RLK4, KIK, RLK5 (Stone *et al*., 1994; Braun *et al.*, 1997; van der Knaap *et al.*, 1999) and CLV1 (Williams *et al.*, 1997; Stone *et al.*, 1998). Three other RLKs, CRINKLY4, Xa21 and ZmPK1 were however found not interact with KAPP (Braun *et al.*, 1997). These results indicate that KAPP can bind to multiple RLKs, but it is not a common component of all RLK signaling pathways.

OsTMK1 (van der Knaap *et al.*, 1999) and CLV1 (Williams *et al.*, 1997; Stone *et al.*, 1998) are able to transphosphorylate OsKAPP. The expression pattern of *KAPP* also encompasses that of *KIK* (Braun *et al.*, 1997) and *CLV1* (Williams *et al.*, 1997; Stone *et al.*, 1998), suggesting an *in vivo* interaction between the proteins. Finally, KAPP also actively dephosphorylates CLV1, indicating that KAPP is a negative regulator of CLV1 signaling (Williams *et al.*, 1997; Stone *et al.*, 1998).

Prf is a LRR-protein whose locus is closely linked to that of Pto (Salmeron *et al.*, 1996). Although not needed for the specificity of Pto, it is needed for anchoring of Pto to the plasma membrane, since interaction between the two proteins was shown (Zhou *et al*., 1995) leading to the activation of the kinase activity of Pto (Rathjen *et al.*, 1999). Pti1 is a target protein kinase of Pto phosphorylation that binds to Pto in a phosphorylation-dependent manner (Zhou *et al.*, 1995). The fact that a protein kinase acts as a substrate for RLK phosphorylation is indicative of a signal-amplifying kinase cascade (Johnson *et al.*, 1996).

Another downstream phosphorylation target of Pto is Pti4, a transcription factor that physically interacts with Pto (Zhou *et al.*, 1997). Pti4 is able to bind the GCC-box *cis*-element present on the promoter regions of PR genes, indicating a very direct manner of transfer of phosphorylation signals.

The cytoplasmic kinase domain of SRK was shown to bind ARC1 in a phosphorylation dependent manner (Stone *et al.*, 1999). When the ARC1 protein was absent, the SI phenotype was partially compromised, indicating that this protein is an essential interacting protein of SRK that acts immediately downstream of SRK.

The two thioredoxins were shown to interact with the catalytic domain of SRK from *Brassica*, but not with two RLKs from *Arabidopsis* (Bower *et al*., 1996). This interaction led to the phosphorylation of the thioredoxins, suggesting that the thioredoxins play a specific role in the self-incompatibility response of *Brassica*, since SRK was shown to be directly involved.

2.3.2.4.2 Ligands bound by RLKs

All RLKs are thought to bind a ligand with their extracellular domains (Ullrich and Schlessinger, 1990). LRRs are thought to provide variable scaffolds to accommodate the evolution of perception of a vast variety of polypeptide signals (Kobe and Deisenhofer, 1994). The specificity of binding is most probably provided by the non-consensus residues present between the LRR regions. Ligands bound by LRRs are generally small peptides or glycoproteins, but might also include larger molecules such as the BL steroid hormone (Li and Chory, 1997). Ligands can include proteins, glycoproteins, oligosaccharides and lipids.

In the SI response, the SCR polypeptide was identified as the male component of the response that act as the ligand (Schopfer *et al.*, 1999). SCRs comprise a novel class of small positively charged secreted polypeptides that share eight conserved cysteine residues. The polymorphic nature of SCRs allows very high *S*-haplotype specificity, thereby preventing self-pollination.

One of the best studied ligand-receptor interactions is that of CLV1 and CLV3 (Fletcher *et al.*, 1999). CLV3 is a 96 amino acid protein containing a signal peptide indicating that it is secreted. It acts as a ligand for binding to CLV1and the physical interaction between the two polypeptides was shown (Trotochaud *et al.*, 1999, 2000).

Systemin is an 18 amino acid compound that is released in tomatoes and potatoes when the plants are attacked by insects and other mechanical wounding (Ryan and Pearce, 1998). The released systemin activates plant defenses by increasing proteinase inhibitors and the expression of polyphenol oxidase genes. Systemin functions as a long-distance signaling molecule by moving within the phloem. As it moves through the plant, systemin is recognized by SR160 (Scheer and Ryan, 1999) which then leads to the induction of JA synthesis and elevated H_2O_2 levels. The SR160 receptor shares high homology with BRI1 (Scheer and Ryan, 2002) and tBRI1 (Montoya *et al.*, 2002). Furthermore, systemin also binds to tBRI1 (Montoya *et al.*, 2002). This indicated the

possibility that a tBRI1/SR160 dual receptor combination is able to bind two different ligands, namely both systemin and BL (Szekeres, 2003).

Phytosulfokine (PSK) is a 5 amino acid hormonal peptide that plays an important role in cell dedifferentiation as an intercellular signal (Matsubayashi and Sakagami, 1996). PSK is thought to function in conjunction with auxin and cytokinin. A receptor protein was identified that is able to bind PSK at plasma membrane level, which when overexpressed, lead to accelerated cellular growth (Matsubayashi *et al.*, 2002).

Elicitors are either plant or pathogen derived compounds that fall into a very wide and diverse group of molecules (Boller, 1995). Some elicitors are race-specific to a particular pathogen, while others are general elicitors (Sessa and Martin, 2000). Race-specific elicitors play a very specialized role in plant-pathogen interactions. In the gene-for-gene interaction that mediates plant resistance to pathogens, the encoded products of the *R*-gene and a pathogenic avirulence (*avr*) gene interacts with each other (Scofield *et al.*, 1996; Tang *et al.*, 1996). Such a recognition event leads to the activation of the HR with the characteristic localized cell death (Hammond-Kosack and Jones, 1996) and later on the SAR that provides long-lasting and nonspecific resistance (Ryals *et al.*, 1996).

Tomato Cf-4 and Cf-9 proteins are LRR-proteins involved in pathogen resistance and share very high amino acid identity (Thomas *et al.,* 1997). Both proteins are extracellular with several LRR regions that are interrupted by short amino acid islands. They interact with Avr-4 (Thomas *et al.*, 1997) and Avr-9 (Jones *et al.*, 1994) avirulence proteins respectively. The interaction of the latter is however not directly, but most probably with an RLK as part of a receptor complex (Carrington *et al.*, 1999). The *Avr-9* polypeptide from *Cladosporium fulvum* is a 28 amino acid secreted peptide that binds to the extracellular region of the Cf-9 protein (van der Ackerveken *et al.*, 1992; Jones *et al.*, 1994). This peptide produced a necrotic response in tomatoes carrying the *Cf-9* resistance gene. Avr-4 on the other hand is 84 amino acids in length (Jones and Jones, 1997).

AvrPto is a race-specific elicitor encoded by the *avrPto* avirulence gene that binds autophosphorylated Pto in a gene-for-gene dependent manner (Scofield *et al*., 1996; Tang *et al*., 1996). Two amino acids, Thr38 and Ser198, are essential for this binding (Sessa *et al.*, 2000). Thr38, the main autophosphorylation site, is located outside the catalytic domain.

General elicitors are compounds that are common to a whole class of organisms, such as flagellin from bacteria (Felix *et al*., 1999). These elicitors activate a defense response by ROS and ethylene production, as well as PR-gene expression (Gómez-Gómez and Boller, 2002). This defense reaction however does not represent the all-out defense response that is usually activated in a gene-for-gene interaction.

Bacteria use flagella composed of flagellin subunits to move around (Gómez-Gómez and Boller, 2002). A conserved 15 amino acid portion of the N-terminal region of flagellin called flg22 is a potent elicitor of plant defense responses (Felix *et al.*, 1999). In *A. thaliana*, the elicitor causes the oxidative burst, callose deposition, expression of *PR-1* and *PR-5* and a strong inhibition of growth (Gómez-Gómez *et al*., 1999). In contrast, flagellin from symbiotic bacteria like *Rhizobium* and *Agrobacterium* does not induce the defense responses (Felix *et al*., 1999; Gómez-Gómez *et al*., 1999). It was proposed that FLS2 acts as the receptor to bind flg22 as a ligand thereby activating the plant defense response towards the bacteria present (Gómez-Gómez and Boller, 2002).

2.3.2.4.3 *In vivo* modes of RLK action

Research into the way in which plant RLKs bind to ligands is still in its infancy. The well documented CLV system however does give us an insight into this mechanism of binding (Trotochaud *et al.*, 1999; Torii, 2000). CLV1 forms a disulfide-linked core oligomer, most probably via the two paired cysteine motifs located on either side of the LRR regions. CLV2, the extracellular RLP involved in CLV signaling, most probably helps with the binding of CLV3 to the extracellular domain of CLV1. Upon binding of CLV3, both the negative regulator KAPP and Rop proteins are recruited. The latter is a GTPase, but the activation thereof by CLV1 is still not proven. In addition to forming a

heterodimer with CLV2, it is possible that CLV1 can also form a homodimer with itself, or a heterodimer with another RLK.

A similar mechanism was proposed for Xa21 signaling (Wang *et al.*, 1998; Torii, 2000) where Xa21D, an RLP, will bind to Xa21, the transmembrane RLK. Upon binding of the ligand (currently unknown), the kinase domain autophosphorylates and the signal is transferred. Heterodimerization of Xa21D with another RLK is proposed, since Xa21D on its own also leads to a weak activation of the defenses (Wang *et al.*, 1998).

When AvrPto bind to Pto intracellularly, the latter is phosphorylated when Pti1, a cytoplasmic kinase, also binds (Sessa and Martin, 2000). Pti1 is then phosphorylated by Pto on Thr233 that is located in the Pti1 kinase activation domain (Sessa *et al.*, 2000). This phosphorylated amino acid can then be the target of a type 2A phosphatase.

Two putative models for BL signaling via BRI1 were proposed (Li, 2003). In the first, BL is bound by BRI1 which then autophosphorylates. The activated BRI1 subsequently phosphorylates BAK1 to initiate BL signaling. In the second, BL binding leads to heterodimerization of BRI1 and BAK1 that in turn leads to the activation of both RLKs via transphosphorylation.

2.3.2.4.4 Downstream activated proteins

Two downstream components of a signal transduction cascade possibly initiated by RLKs are MAPKs and TFs. MAPKs were shown to be involved in plant defense and development and the detection of several other biotic and abiotic factors, including SA and JA treatments, osmotic and oxidative stress and ozone. Since the available information on MAPKs is extensive and falls outside of the scope of this review, the reader is referred to an excellent review concerning the subject (Morris, 2001). It is important to note that as yet, the direct involvement of a MAPK signaling pathway with an activated RLK still has to be proven.

The final stage of a signal transduction cascade is the activation of TFs that will bind to the promoter region of a subset of genes whose expression may help the plant to respond appropriately to a particular stimulus. A few candidate TF genes have been identified and include *BPF1* (da Costa e Silva *et al.*, 1993) and *WRKY* (Rushton *et al.*, 1996) that are induced during the plant defense response. The WRKY proteins bind to *cis*-acting elements (W-boxes with a TGAC central motif) that are present on the promoter regions of plant defense genes. These include genes encoding PR proteins (Rushton *et al.*, 1996) and RLKs (Du and Chen, 2000). Thus, the pathogen induced WRKY proteins may serve as common transcriptional activators that act downstream of a MAPK signaling pathway (Eulgem *et al.*, 2000) to activate the expression of a large set of pathogen-responsive genes throughout the plant kingdom.

Similar to MAPKs, the information on the direct relationship between receptors and TFs is still very scarce. The only direct linkage thus far described is between Pto and Pti4, 5 and 6, three transcriptional activators (Zhou *et al.*, 1997). The phosphorylation of Pti4 by Pto was also shown (Gu *et al.*, 2000). All three transcriptional activators are able to bind to a GCC box present on the promoter region of many *PR*-genes, indicating the direct influence of activated Pto on the transcription of defense genes.

Even though research on plant signal transduction is still in its infancy, the future holds great promise for the description of complete signaling pathways that start at a plasma membrane bound receptor that is activated by a particular stimulus. The receptor then relays the message via a well defined signaling cascade with the final activation of a specific TF. This activation will then lead to the induced expression of several genes whose encoded products will then allow the plant to respond.

2.3.2.5 RLK function

When one considers the structural, amino acid and expression diversity of the identified and cloned RLKs (Table 2.1), it is only logical to assume that RLKs have very diverse functions. While the functions of some RLKs have been established beyond doubt, others still only have a putative function.

2.3.2.5.1 RLKs and plant development

Several RLKs are implicated to be involved in plant development, be it during the SI reaction, pollen development, the regulation of meristem development or the regulation of organ shape.

2.3.2.5.1.1 Regulation of organ shape

Erecta is an RLK that plays a very important role in the coordination of cell growth in shoot apical meristems (Torii *et al.*, 1996). Mutant plants lacking expression of the gene have an upright stature, compact inflorescences with flower clustering at the top, short pedicels and short, blunt siliques. Since this phenotype is the result of a decrease in cell number, it is therefore proposed that *Erecta* is likely to be involved in a process that regulates the number of cells constituting mature organs, and therefore also organ shape.

2.3.2.5.1.2 Regulation of meristem development

The shoot apical meristem of plants is a population of undifferentiated cells that give rise to the above-ground portions of the plant (Clark, 1997). The position of the cell within the meristem determines its fate in plant development. This development depends on the communication of cells located within the same layer of cells within the meristem. Mutants for the *CLV1* gene accumulates undifferentiated cells in the whole of the meristem, leading to the formation of enlarged meristems, fasciated stems and increased floral organ whorls and organ numbers (Clark *et al.*, 1997). The wild-type *CLV* genes are therefore thought to restrict cell proliferation within the meristem, or to facilitate the transformation of the undifferentiated cells.

2.3.2.5.1.3 Hormone signaling

Brassinosteroids are plant growth regulators involved in many plant developmental processes. Mutant plants that are defective in brassinosteroid synthesis are dwarfs that display a light-grown habit when grown in the dark (Li and Chory, 1997). Several searches were launched to isolate brassinosteroid receptors, but all the identified mutants belonged to one single complementation group. This indicates that BRI1 is probably the

only non-redundant component of a brassinosteroid signaling pathway. *BRI1* homologues were recently identified from several other plant species (Table 2.1).

2.3.2.5.1.4 Epidermal differentiation

The epidermis of plants is the outermost cell layer of plant organs and is important for their separation. Epidermal cell fate is partly determined by positional cues of the cells, indicating the importance of cell-cell communication. The CRINKLY4 gene is needed for proper epidermal development, since *cr4* mutants have crinkly leaves with a rough surface due to the loss of proper epidermal cell patterning (Becraft *et al.*, 1996). The epidermal cells are enlarged and disorganized, with areas where the epidermis is connected to organs with interconnecting bridges that are several cells thick. The function of the epidermis to restrict cell division patterns to anticlinal planes and prevent surface dedifferentiation is therefore severely compromised in cr4 mutants.

2.3.2.5.1.5 Pollen development

Pollen development in plants involves a mitotic event to produce two haploid microspores. The expression of *PRK1* is confined to pollen and pollen tubes, with maximal expression found at pollen maturation (Mu *et al.*, 1994). Three lines of plants expressing an antisense copy of the gene produced normal and aborted pollen in a 1:1 ratio. While all pollen looked normal at the uninucleate stage, half of the pollen did not undergo mitosis and were therefore aborted (Lee *et al.*, 1996a). This indicated that PRK1 is needed for post-mitotic development of microspores. In addition, RPK1 also influences female gametogenesis (Lee *et al.*, 1997), suggesting that it may represent a signaling component needed for normal gametogenesis.

2.3.2.5.1.6 Self-incompatibility

SI occurs within hermaphroditic plants in an effort to prevent self-fertilization. The reaction is highly specific and involves genes occurring on a single genetic locus, the *S*-locus complex (Goring and Rothstein, 1992; Stein and Nasrallah, 1993).

The SI reaction is initiated when parents of a cross carry an identical haplotype, causing the germination of the pollen to abort. The recognition of self-pollen is achieved by SLG and SRK, both of which are expressed in the stigma (Stein *et al*., 1996). It has been proposed (Giranton *et al*., 2000) that SRK interacts with the self-pollen borne ligand SCR (Schopfer *et al.*, 1999). This interaction then leads to the activation of the kinase domain leading to the recruitment of cytoplasmic targets that could mediate the SI response.

2.3.2.5.1.7 Nodulation

The importance of nitrogen metabolism in plants is slowly coming to the fore, with the recent cloning and characterization of four RLKs implicated in root nodulation in legumes. Two of these LRR RLKs were found to play a role in the non-nodulation phenotype of *Medicago* (Endre *et al.*, 2002) and pea (Stracke *et al.*, 2002). Both proteins have a huge 400 amino acid extracellular domain followed by 3 LRR regions. A third LRR-RLK involved in hyper-nodulation, HAR1, was also recently cloned and share high homology with CLV1 (Krusell *et al.*, 2002). An equivalent gene from soybean, GmNARK, was also recently cloned (Searle *et al.*, 2003). The latter two RLKs are thought to be important for regulation of the later stages of nodulation. Using mutants of these two genes, it was shown that the root does not autoregulate nodulation, but that a regulation signal travels from the root to the stem.

2.3.2.5.1.8 RLKs implied to function in plant development

Somatic embryogenesis is the process where somatic cells develop into plantlets. SERK was identified to play a role in somatic embryogenesis, since its expression marked embryogenic competence and thus somatic embryo formation (Schmidt *et al.*, 1997). Once the somatic embryo reached a particular stage of development, *SERK* expression was terminated. SERK therefore seems to play a role in determining embryogenic fates.

Two RLK genes, *LePRK1* and *LePRK2* were isolated from mature pollen (Muschietti *et al.*, 1998). The expression of both genes was confined to mature and germinating pollen, but not in immature pollen, clearly implicating a different function to that of PRK1 (Mu *et al.*, 1994). The two proteins were localized to the plasma membrane of germinating

pollen tubes. LePRK2 was phosphorylated in pollen membrane fractions, while LePRK2 was partially dephosphorylated by a style-extract. All these data led the authors to predict a role for both RLKs in the interaction between germinating pollen and the pistil.

2.3.2.5.2 RLKs and plant defense

Most incompatible plant/pathogen interactions lead to the HR that includes an oxidative burst, cell wall reinforcement, the activation of the defense related genes and localized cell death (Hammond-Kosack and Jones, 1996). Plants involved in compatible interactions also display the typical defense reaction, but at a lower level and usually at a later stage with no cell death taking place (Sessa and Martin, 2000). It was proposed that most of the *R*-genes in plants encode LRR proteins that can act as receptors that are able to trigger the defense response upon recognition of pathogen-derived elicitors (Hammond-Kosack and Jones, 1996).

RLKs involved in plant defense can broadly be divided into three groups, the actual disease resistance RLK proteins that bind race-specific elicitors, RLKs that bind non-race specific elicitors and those that are in some way involved in the defense response, most probably as a secondary signal transducer (Romeis, 2001).

All disease resistance genes contain characteristic LRR motifs. The predominant class of predicted R gene products in plants possesses a nucleotide binding site (NBS) and LRRs (Hammond-Kosack and Jones, 1997). Others such as Xa21 (Song *et al*., 1995) and Lrk10 (Feuillet *et al.*, 1997), have both an extracellular LRR domain and an intracellular kinase domain, Pto consists of only an intracellular kinase domain (Martin *et al*., 1993) while Cf-2 and Cf-9 are transmembrane proteins with extracellular LRR domains (Thomas *et al.*, 1997).

Pto encodes an intracellular protein kinase that confers race-specific resistance to tomato against strains of *Pseudomonas syringae* pv. tomato expressing the *avrPto* avirulence gene (Martin *et al.*, 1993). Interaction between Pto and AvrPto leads to the activation of the hypersensitive response (Zhou *et al.*, 1995). Infection of transgenic tomato plants

overexpressing *Pto* leads to a greater resistance of tomatoes against *P. syringae* pv tomato (Tang *et al*, 1999). Resistance was also found against *Cladosporium fulvum* and *Xanthomonas campestris* pv *vesicatoria*, indicating that in the absence of AvrPto, Pto is able to render plants resistant against a wide array of pathogens.

This cross-resistance caused by Pto is probably due to cross-phosphorylation of Pti1 (Zhou *et al*., 1995), suggesting that Pto on its own has a basal ability to trigger downstream defense responses. Normal Pto kinase activity is proposed to be low, while interaction with avrPto may lead to activation with subsequent increases in the phosphorylation of Pto-interacting proteins leading to various resistance responses.

In addition to cross-resistance, *Pto* overexpression also leads to spontaneous localized cell death on true leaves accompanied by callose deposition and lignification with intense light being essential (Tang *et al.*, 1999). This indicates that Pto on its own is not able to initiate cell death, but needs intense light for H_2O_2 accumulation through either enhanced photosynthesis or the inactivation of H_2O_2 scavenging enzymes. These plants also accumulate SA while having induced PR and Pti4 gene expression (Zhou *et al*., 1997). All the above indicate that Pto acts in two ways, the first in a gene-for-gene manner with AvrPto to give specialized disease resistance and secondly in a general resistance response in an AvrPto-independent manner.

Another Avr-R gene interaction is represented by the Cf-2/Avr2 and Cf-9/Avr9 interactions (Dixon *et al.*, 1996). Activation of the resistance genes by binding of the avirulence proteins leads to the activation of the plant defense systems.

Xa21, a plasma membrane bound RLK confers resistance to rice against *Xanthomonas oryzae* pv *oryzae* race 6 containing an as yet unidentified avirulence gene (Song *et al.*, 1995). Successful recognition by the plant leads to the activation of the characteristic defense reactions.

LRK10 is a disease-resistance gene against leaf rust which was isolated from wheat (Feuillet *et al*., 1997). The gene demonstrated the typical RLK structures and co-segregated with the LR10 disease resistance locus. LRK10 shows limited homology in the extracellular domain with the S-class of RLKs, indicating a very distant relationship between these proteins (Feuillet *et al*., 1997).

Plants must be able to distinguish pathogenic from symbiotic bacteria. Some receptors might therefore be specifically directed towards the recognition of common bacterial characteristics, such as the flg22 peptide that is a component of bacterial flagella (Gómez-Gómez and Boller, 2002). When the peptide is presented to the plant, the defense response with all the characteristic events is activated. Flagellin peptides from symbiotic bacteria however do not activate the defense response (Gómez-Gómez *et al.*, 1999). The RLK responsible for the detection of flg22 is FLS2 (Gómez-Gómez and Boller, 2000) that physically binds to flg22 (Bauer *et al.*, 2001).

Pr5K is an RLK of which the extracellular domain shares very high similarity with the Pr-5 family of PR proteins (Wang *et al.*, 1996). It has been proposed that this receptor is involved in plant defense by interacting with a PR5 target protein in the recognition of microbes. The gene expression is however not induced by SA or viruses, thus a clear function still has to be established.

SFR2 is implicated in the defense response of *Brassica* during pathogen infestation since its expression is induced by SA, bacterial infection and wounding (Pastuglia *et al*., 1997). The long time lapse between application and the *de novo* protein synthesis suggests that this protein might only play a role in the later stage defense responses.

Wak1 was also implicated in plant defense against pathogens (He *et al*., 1998). The role of Wak1 is however not directly related to the pathogen itself, but rather in response to the activated defense response. Wak1 is postulated to be a PR protein that needs the expression of *NPR1* for synthesis, also acting as a positive regulator of *NPR1* expression.

The survival of a plant under attack by pathogens seems to depend on WAK1, since the absence of the gene rendered the plant susceptible to higher levels of SA and INA.

An RLK that responds to physical changes in the cell wall is PERK1 (Silva and Goring, 2002). This RLK has a proline-rich extracellular domain similar to extensins and its expression is induced upon wounding or fungal infection, but not SA and JA. This indicates that the gene is induced upon the physical wounding of the plant due to fungal growth and penetration, and not due to the nature of the pathogen itself. It is thus thought that PERK1 is important to determine physical changes to the cell wall caused by fungal infection and herbivory.

The expression of PnLPK is induced upon mechanical wounding (Nishiguchi *et al*., 2002). SA and JA treatments did however not lead to an induced expression of the gene, suggesting that the expression of the gene is regulated by a SA and JA independent signal transduction pathway.

2.3.2.5.3 RLKs and abiotic stresses

Several RLKs are implicated to be involved in signaling as a result of environmental cues, since their own expression was induced under these changing conditions.

2.3.2.5.3.1 Osmotic stress

The response of plants to osmotic stress is very complicated, since osmotic stress can be caused by salt through the reduction of water potential, by dehydration through loss of water as well as cold that reduces the amount of water reaching the leaves from the roots (Yamaguchi-Shinozaki and Shinozaki, 1993). The signal for osmotic stress can be one of several, including changes in water activity, changes in cell wall-plasma membrane interactions and the loss of membrane "stretch". The plant response can be divided into early and late responses.

Forming part of the shorter interval response, is the activation of a number of phospholipid-based and MAPK signaling cascades. One of these pathways is the

phospholipase C (PLC) signaling pathway (Munnik, 2001). This activated pathway leads to the production of two second messengers, inositol 1, 4, 5-triphosphate (IP_3) and diacylglycerol (DAG). DAG is rapidly converted to phosphatidic acid (PA) that is thought to act as a lipid signal activating various enzymes (Munnik, 2001). IP_3 on the other hand rapidly releases Ca^{2+} from the intracellular stores, thereby maintaining the integrity of both osmotic and turgor pressures (Knight *et al.*, 1997). PA is also produced via another lipid signaling pathway, the phospholipase D (PLD) pathway (Munnik, 2001). It is proposed that the ability of the plant to distinguish between and react to osmotic and salt stresses is located within the IP_3 production and Ca^{2+} signal transduction pathways (Knight *et al*., 1997).

The longer term response to osmotic stress includes the production of proteins and osmolytes such as sugars and amino acid derivatives that prevent protein denaturation and oxidative damage (Zhu, 2001). One component is ABA that plays an important role in the osmotic stress response, since it induces the expression of several genes, including those encoding PLC (Lee *et al*., 1996b) and PLD (Frank *et al*., 2000). In contrast, some genes are induced by osmotic stress, but not by ABA indicating the presence of both an ABA-dependent and ABA-independent signaling pathway (Shinozaki and Yamaguchi-Shinozaki, 1997). It is however possible that the two pathways are overlapping and interacting (Bray, 2002).

Genes that are ABA regulated contain within their promoter region a *cis*-acting element called an ABA-responsive element (ABRE) with an ACGT core sequence. An ABRE-binding protein (bZIP) containing a basic region adjacent to a leucine-zipper motif binds to the ABRE (Busk and Pages, 1998). Several bZIP genes are responsive to cold, ABA and drought, indicating their importance in ABA-signaling (Nakagawa *et al*., 1996). In contrast, the drought-responsive element (DRE) from *A. thaliana* mediates transcription in response to desiccation, cold and salinity, but not to ABA (Shinozaki and Yamaguchi-Shinozaki, 1997). This distinction is however not clear-cut, since in tobacco isolated DRE-elements are regulated by ABA (Busk *et al*., 1997). Two additional motifs, MYB

and MYC, are also implicated to regulate ABA-induced genes in response to severe water-deficit (Abe *et al.*, 2003).

In addition to ABA, several other key signaling compounds (2.3.1) are involved in water stress response. In *Chlamydomonas*, osmotic stress leads to a 6 – 10 fold increase in JA levels (Munnik and Meijer, 2001). Osmotic stress also leads to oxidative stress conditions causing damage to the plant (Smirnoff, 1993). SA was further shown to be directly involved in changes taking place in plants subjected to osmotic and salt stress conditions (Borsani *et al.*, 2001).

The role for SA under salt stress conditions is proposed to be as a signaling molecule forming a feedback amplification cycle in concert with ROS, since SA production is not induced (Borsani *et al.*, 2001). SA increases the oxidative damage generated by NaCl and osmotic stress which in turn is responsible for the cell lethality.

Protein phosphorylation is a key component of the osmotic response. As in any plant adaptation process, the recognition by a receptor of the changing conditions is crucial for a fast response. Up to date, only two receptors responsive to osmotic stress have been identified. The first is AtHK1, a protein that forms part of a two-component histidine kinase family (Urao *et al.*, 1999). This protein was identified as an osmoreceptor based on the fact that the protein complemented a yeast osmosensor deletion mutant.

The second putative receptor is RPK1, an RLK from *Arabidopsis* that was shown to be induced by ABA, water-, salt- and cold stress (Hong *et al*., 1997). The induction of gene expression by the various osmotic conditions clearly indicates some involvement of RPK1 in the plant response to external osmotic conditions. Although *RPK1* expression was induced by ABA application, the use of ABA-deficient mutants revealed that the induction is ABA-independent.

2.3.2.5.3.2 Light

LRRPK was isolated from *Arabidopsis* and its expression was found to be light-regulated (Deeken and Kaldenhoff, 1997). The expression of the gene is down-regulated in light, while darkness leads to the activation of expression. This correlated very well with the expression pattern of the gene where maximal expression was detected in the roots. In addition, the promoter region of the gene had four *PhyA* repressor elements, motifs that are present in the upstream regions of the Phytochrome A gene that confer light repression of the phytochrome genes.

2.3.2.5.3.3 Steroid detection

Many plant steroids have been described, with brassinosteroids being the only class that influences plant growth when applied to the plant exogenously. Of these, BL is the most active and influences plant development (Chory *et al.*, 1991). Loss-of-function mutations of two genes, DET2 and CPD, had pleiotrophic effects, suggesting the involvement of BL in several processes in the plant including the expression of light and stress-regulated genes, the promotion of cell elongation, normal leaf and chloroplast senescence and flowering (Li *et al.*, 1996).

BRI1 was initially identified as a BL responsive RLK (Li and Chory, 1997) that is able to bind BL (Friedrichsen *et al.*, 2000) which leads to activation of the protein (Wang *et al.*, 2001). A second RLK, BAK1, was identified as a partner for BRI1 for the detection of BL (Nam and Li, 2002). In addition to BRI1, several genes homologous to BRI1 was identified in other plants (Yamamuro *et al.*, 2000), indicating that the detection mechanism for BL was conserved in the plant kingdom.

2.3.2.5.3.4 Oxidative stress

Plants are continuously subjected to oxidative stress, be it as part of the normal biochemical functioning of the plant or synthesized as part of the defense response to both biotic and abiotic stresses (Bowler and Fluhr, 2000).

As yet, a receptor for oxidative stress in plants has not been found. Since H_2O_2 is a simple molecule and a mild oxidant, it is possible that the molecule can interact with cysteine residues within proteins (Finkel, 2000). In yeast, SLN1 acts as a peroxisensor (Singh, 2000). The *Arabidopsis* genome has several genes with homology to SLN1 making it possible that one of these encoded proteins can act as a peroxisensor. Although several H_2O_2 responsive promoters have been described, no conserved motif conferring H_2O_2 mediated gene expression has been described.

2.4 Plant signal transduction pathways

When one considers the term signaling pathway, immediately a single linear succession of events comes to mind. However, research has shown that pathways are very complex and more often than not, involve components from various other related pathways (Jenkins, 1999). The response of plants to different stimuli must be appropriate in the context of other responses where the necessary coordination and integration is achieved through interaction and cross-talk between relevant signal transduction pathways. Currently, this cross-talk between different pathways is poorly understood.

Shared signaling components include Ca^{2+} and H_2O_2 that are involved in the adaptation to various biotic and abiotic stresses, implying that both might be central components controlling cross-tolerance (Bowler and Fluhr, 2000).

Other shared components include catalytic enzymes and transcription factors. The regulation of shared components by two different signaling pathways is illustrated by the enzymes, PAL and CHS (Dixon and Paiva, 1995; Mol *et al*., 1996). PAL is a key enzyme in the phenylpropanoid pathway leading to the synthesis of several antimicrobial compounds. CHS on the other hand is important for the synthesis of flavonoids and anthocyanins from a branch in the phenylpropanoid pathway which enhances the plant's defense against UV light. When parsley is illuminated with UV light, expression of both genes is induced. In contrast, only *PAL* expression is induced when the plants are treated with an elicitor derived from *Phytophthora* (Lozoya *et al*., 1991), indicating the

repression of CHS expression by the elicitor. This differential regulation is most probably located at the level of transcription factors. It is therefore thought that when the plant is attacked by the pathogen, *CHS* expression is repressed, allowing the cell to channel all the energy into the production of compounds with antimicrobial activity, since pathogen attack needs a more urgent response than protection against uv light.

As indicated in 2.3.1, plant metabolites such as SA can be involved in more than one response towards changing environmental conditions. Furthermore, more than one metabolite might be simultaneously involved in a single response. A good example of this was illustrated in tobacco (Vidal *et al.*, 1997). When treated with an extract from a pathogen, treated tobacco leaves showed a rapid increase in the expression of several *PR* genes, characteristic of SAR. The same *PR*-genes were also induced by treating the plant with SA, but the expression was slower and weaker. In addition, the *PR-1a* gene was induced by SA, but not the pathogen extract.

The net effect of signaling pathways is the induced expression of subsets of proteins that allow the plant to either adapt to or withstand unfavorable conditions such as osmotic stress or pathogen attack. Wounding and pathogen infection both lead to SAR in distal parts of the plant (Maleck and Dietrich, 1999). Furthermore, systemic signaling also occurs during abiotic stresses (Bowler and Fluhr, 2000) such as during ozone stress where the expressed genes overlap significantly with those expressed during the hypersensitive response (Sharma *et al*., 1996). The systemic response is dependent on SA and H_2O_2 accumulation for both pathogen infection (Yalpani *et al*., 1994) and ozone stress (Sharma *et al*., 1996) indicating that similar signaling pathways must be employed.

Synergistic interactions were also found where a combination of two signaling pathways leads to a stronger response than the individual transduction. When plants are treated with either ethylene or methyl-jasmonate (MeJA), osmotin expression was stimulated by ethylene, but not with MeJA (Xu *et al*., 1994). However, when given as a combination, the induced expression of osmotin was much greater than with ethylene alone.

This synergistic effect may be of great help to the plant defense responses. When a gene is regulated by various signals that are seemingly unrelated, a particular combination of signals might be the key to a particular inductive stimulus. Such combinations might therefore play an important role to define the particular reaction, as well as the intensity of the reaction, that will allow the plant to survive.

This point was illustrated when soybean was treated with physiological concentrations of SA, which is much lower than that normally used in studies (Shirazu *et al*., 1997). On their own, the physiological SA concentration and *Pseudomonas syringae* infection produced moderate levels of H_2O_2 accumulation. When the two were however combined, the hydrogen peroxide levels were significantly higher with accelerated cell death associated with the HR and the induction of *PAL* expression, again indicating the importance of synergism in the signaling pathways.

During osmotic stress, it is thought that the stress is translated into different signals in a dose-dependent manner (Munnik and Meijer, 2001). Protein phosphorylation patterns were shown to differ in alfalfa at different salt concentrations, with two different kinases being activated (Munnik *et al*., 1999). This means that each stress level produces its own unique combination of signals that activates the appropriate response. This was also found in phospholipid signaling (Munnik and Meijer, 2001). PA is produced as the result of the activation of both the PLC and PLD phospholipid signaling pathways. These two pathways are however activated at different levels of osmotic stress, yet a specific MAPK pathway is activated by PA (Lee *et al*., 2001). Although cross-talk between the pathways is implicated, it might be that PA represents a common down-stream component for the two pathways. This idea is further strengthened by the fact that some of the constituents of MAPK cascades are activated by cold, drought, salinity, H_2O_2, heat, shaking, wounding, pathogens, elicitors, ABA, SA and ethylene, suggesting that these molecules might function as promiscuous networking molecules (Bowler and Fluhr, 2000).

Increasingly, the importance of cross-talk among different signal transduction pathways is becoming clearer. This cross-talk could lead to cross-tolerance, rendering the plant

tolerant to various other stresses after being subjected to a particular stress. The diversified role of receptor proteins is also coming to the fore. It was proposed that a single receptor might be able to bind several different ligands, most probably via heterodimerization between different RLKs. This was proposed for a heterodimer consisting of tBRI1 and SR160 that is most likely able to bind two different ligands, systemin and BL (Szekeres, 2003).

On the other hand, it is also possible that a particular RLK can initiate a single signaling pathway when treated with different external stimuli by binding to a ligand that is common to the different stimuli. Although it still has to be proven, good examples might include SFR2 (Pastuglia *et al.*, 1997), LRPKm1 (Komjanc *et al.*, 1999), Wak1 (He *et al.*, 1998) and EILP (Takemoto *et al.*, 2000). Expression of these RLKs is induced by several different stimuli, most probably via a common component of the different stimuli.

Thus, while the identification and characterization of individual components of plant signal transduction pathways are continually taking place, the most laborious and time-consuming research in future will focus on fitting these individual components into integrated and interactive signaling pathways. It is here that the greatest challenges lie for those studying plant signal transduction in an effort to understand how plants respond to changing environmental conditions.

2.5 Summary

In summary, even though plant signal transduction research is still in its infancy, clear pictures of very efficient interconnected pathways are emerging. Central to these pathways are receptor proteins located on the plasma membrane of the cell, be they RLKs or two-component histidine kinases. Presumed to play a key role in the transfer of signals across the plasma membrane, these receptors are ideally structured and situated to facilitate this signaling.

RLK signaling complexes reveal several similar signaling mechanisms. A good example is the similarity that exists between race/cultivar specific disease resistance and SI (Wang *et al*., 1996). During race-specific plant-pathogen interactions, the products of the pathogenic *avr* and plant *R* genes interact causing incompatibility with the plant being resistant to the pathogen (Flor, 1971). Should the *R* gene be absent, the interaction is compatible and disease ensues. During SI, male and female plants carrying the same haplotype at the S-locus will express incompatibility and the germinating pollen will be rejected (Stein *et al*., 1996). In the absence of recognition, pollination will proceed.

The future thus holds a lot of promise for fascinating research into plant signaling. By learning from already described signaling complexes such as the above mentioned, the way that plants react to changing environments will be elucidated in the not to distant future.

Chapter 3

Expression analysis of *At-RLK3*

Expression analysis of *At-RLK3*

3.1. Introduction

Signal transduction in mammals has received a lot of attention in the last two decades. It was shown that a special class of receptor proteins forms a key component of mammalian signal transduction pathways. These proteins, called RPKs, have a very characteristic structure with an extracellular ligand binding and intracellular kinase domain that are linked to each other via a typical hydrophobic type 1b integral transmembrane domain. The presence of this region meant that these proteins are located within the plasma membrane of the cell, an ideal site where they can act as ligand binding and signal transduction entities (Lemmon and Schlessinger, 1994).

In an attempt to determine whether structurally similar proteins exist in plants, Walker and Zhang (1990) cloned and identified *ZmPK1* from maize, the first plant equivalent of mammalian RPKs. Since then, a large number of plant genes that share structural similarities to *RPKs* were identified (Table 2.1). During the early years, several of these plant genes were identified, but at that stage, the ability of the encoded proteins to bind ligands was still unproven. These proteins were therefore called receptor-like protein kinases. Since then, the number of identified RLKs increased in leaps and bounds, implicating a similar role to mammalian RPKs.

Similar to RPKs, the structure of RLKs is very characteristic (Walker, 1994). Located on the amino end of the protein is a predicted signal peptide that ensures translocation of the protein to the plasma membrane via the ER. A characteristic peptide cleavage site ensures efficient processing of the RLK after its arrival at the plasma membrane (Takahashi *et al.*, 1998; Annen and Stockhaus, 1999). Several RLKs do not have signal peptides (Schmidt *et al*., 1997; Li and Wurtzel, 1998; Silva and Goring, 2002), but since it was previously found that some proteins can still be translocated through the ER even though they lack a signal peptide (Singer, 1990), the absence of this motif should not prevent the insertion of the RLK into the plasma membrane.

The extracellular domain of the RLK family is very diverse. In an attempt to organize the RLKs, an artificial grouping system was devised. The placement of RLKs into these groups is based on characteristic motifs or amino acid sequences located on their extracellular domains. Up to date, 8 such classes were identified. Several RLKs

however have unique extracellular domains with no characteristic motifs, making it impossible to place them into a particular grouping at this stage.

The biggest of these RLK classes is the LRR class. RLKs belonging to this group contain LRR regions (Torii and Clark, 2000), motifs that are proposed to be important for protein-protein interactions (Kobe and Deisenhofer, 1994). These LRR regions are typically 24 amino acids in length with a conserved sequence of $LX_2LX_2LX_2LXLX_2NXLX\mathbf{G}XIPX_2$. A conserved glycine is present as part of the LRR motif, that is absent from the characteristic intracellular motifs (Jones *et al.,* 1994*)*. Also typical of this class are two paired cysteines that surround the LRR regions. In some cases, the LRR regions are present as a single continuous block, but in others, the LRR motifs are split into two by a short unrelated island (Li and Chory, 1997; Scheer and Ryan, 2002; Searle *et al.*, 2003; Matsubayashi *et al.*, 2002). The importance of this island for ligand binding was shown for BRI1 (He *et al.*, 2000).

The second largest class contains RLKs whose extracellular domains share homology with the SLG proteins from *Brassica* (Nasrallah *et al*., 1988). Characteristic of this class is the presence of 12 conserved cysteines with 10 that are absolutely conserved in the following motif: $CX_5CX_5CX_7CXCX_NCX_7CX_NCX_3CX_3CXCX_NC$ (Torii and Clark, 2000). In addition, a second PTDT motif with a conserved sequence of WQSFDXPTDTΦL was also described (Walker, 1994). Despite belonging to the same class, huge sequence diversity exists among the members.

A newly described RLK class is the cysteine-rich class of which a large number is clustered on chromosome 4 of *Arabidopsis*. RLKs included in this class all have either one or two copies of a C-X8-C-X2-C motif (Chen, 2001) that is proposed to act as important motifs to facilitate the three-dimentional structure of the protein in the apoplast.

The other RLK classes all contain between 1 and 4 members. In the case of CRINKLY4 (Becraft *et al.*, 1996) and Wak1 (Kohorn *et al.*, 1992), the extracellular domains share homology to previously described mammalian RPKs. CHRK1 (Kim *et al.*, 2000b) and PR5K (Wang *et al.*, 1996) are unique in that their extracellular domains share high homology with plant catalytic enzymes, but these domains lack

the enzymatic activity characteristic of the normal catalytic enzymes, suggesting a binding rather than catalytic function for the extracellular domain. Lectin binding RLKs represent a class which is thought to be able to bind mono- or polysaccharides as the native lectin proteins do (Hervé *et al.*, 1996; Nishiguchi *et al.*, 2002).

Finally, several RLKs identified at this stage cannot be placed in a particular grouping. These RLKs however also have unique features such as similarity to hydroxyl-proline rich glycoproteins (Silva and Goring, 2002), a leucine-rich region flanked by basic amino acids indicative of a leucine–zipper (Deeken and Kaldenhoff, 1997) and extracellular regions that share no homology with any other RLK (Feuillet *et al.*, 1997).

Linking the extracellular domain to the intracellular kinase domain is a hydrophobic transmembrane domain that is thought to anchor the RLK to the plasma membrane (Weinstein *et al*., 1982). These domains are typically followed by a short stretch of basic amino acids that acts as a stop signal ensuring efficient incorporation of the protein into the membrane.

The kinase domains of RLKs all have the characteristic 11 conserved subdomains present in eukaryotic protein kinases (Hanks and Quinn, 1991), but in contrast to RPKs that target tyrosine residues for phosphorylation, all plant RLKs target either serine or threonine residues for phosphorylation. The difference in specificity is due to the amino acid sequences of subdomains VIb and VIII. Only one RLK, PRK1, was shown to have tyrosine specificity in addition to serine specificity (Mu *et al.*, 1994) which is thought to be essential for the role that it plays in pollen (Muschietti *et al.*, 1998). Several authors have however also speculated that the tyrosine specificity was most probably due to mutations that occurred during the expression of the protein in *E. coli*.

All the *RLK* genes thus far cloned and identified are either present as a single copy or forms part of a very small gene family. On the genomic level, the number of introns present ranges from 0 (Zhang and Walker, 1993; Schulze-Muth *et al.*, 1996; Li and Chory, 1997) to 26 (Torii *et al*., 1996) where in the latter, each intron code for one LRR region. The group of RLKs that has 5 or 6 introns, shares a novel in-frame stop

codon located within the first intron (Stein *et al.*, 1991; Tobias *et al.*, 1992; Dwyer *et al.*, 1994; Wang *et al.*, 1996; Suzuki *et al*., 1997). In all these cases, the whole of the extracellular domain is encoded by the first intron. The presence of this stop codon led to the formation of multiple mRNA transcripts as detected with Northern blotting. In several cases, the second smaller transcript was shown to encode the extracellular domain of the RLK (Tobias *et al*., 1992; Tobias and Nasrallah, 1996; Kim *et al*., 2000b), while in the case of SRK_6 one of the shorter transcripts appeared to encode a polypeptide consisting of the TM and kinase domain (Stein *et al.*, 1991).

The expression of the *RLK* genes differed widely (Table 2.1). In some cases, the expression was ubiquitous (Takahashi *et al.*, 1998; Gómez-Gómez and Boller, 2000), while in others the expression was highly specific both in timing (Jinn *et al.*, 2000) and place (Stone *et al.*, 1998). While the induction of expression of some genes were highly specific (Matsubayashi *et al.*, 2002), others were induced by several diverse factors (Pastuglia *et al.*, 1997; Hajouj *et al.*, 2000). In one case, several factors all relating to osmotic stress were shown to induce expression of the gene (Hong *et al.*, 1997). The diversity in expression patterns and locations are indicative of the wide variety of roles RLKs are proposed to play in plants.

3.2 Aim

Since *At-RLK3* was cloned without any prior knowledge of the function or characteristics of the gene and its encoded protein, the aim of this chapter was to characterize the gene on genomic DNA and expression levels.

3.3 Materials and methods

3.3.1 Materials

3.3.1.1 Plant material

When intact plants were needed for experiments, *A. thaliana* Columbia and Landsberg erecta ecotype seed were used. The expression experiments were done using an *A. thaliana* Landsberg cell suspension culture (Axelos *et al*., 1992).

3.3.1.2 Chemicals

Gamborg's B5 medium (Duchefa, Haarlem, The Netherlands) was used for the cultivation of cell suspension cultures. TRIzol reagent (Invitrogen) was used for all RNA extractions from both cell suspension cultures and intact plants. All solutions used for RNA extraction were treated with diethylpyrocarbonate (DEPC) (Sigma). All modification enzymes, as well as the Taq DNA polymerase and deoxyribonucleotide triphosphates (dNTPs), were obtained from Roche. For both Southern and Northern blot analysis, a Hybond N^+ nylon membrane (Amersham) was used. For the purification of DNA fragments from agarose gel pieces, the JetSorb system (Promega) was used. DNA probes were prepared using the *Redi*prime^{TM}II system (Amersham) and the labeled probe purified using Bio-Spin columns (Biorad). The pGem-T vector was obtained from Promega. The 0.24 – 9.5 kb RNA ladder was obtained from Invitrogen. All other reagents and chemicals used were of highest purity.

3.3.2 Methods

3.3.2.1 Determination of the copy-number of *At-RLK3* in *A. thaliana*

3.3.2.1.1 Cultivation of *A. thaliana* ecotypes

To determine the copy number of the *At-RLK3* gene in *Arabidopsis*, seed from both Columbia and Landsberg erecta ecotypes was planted (Weigel and Glazebrook, 2002). Before planting, the seed was sterilized by immersing it in 70% (v/v) ethanol for 30 sec and then in 1% (w/v) sodium hypochlorite, 0.1% (v/v) polyoxyethylenesorbitan monolaurate (Tween 20) for 25 min with vigorous shaking. The seed was rinsed six times in sterile water, resuspended in sterile 0.1% (w/v) agarose at 30^{o}C and sown on K1-medium (0.5x Murashige and Skoog [MS] salts, 0.1

mM myo-inositol, 2.3 mM 2-(N-morpholino) ethanesulfonic acid [MES] pH 5.7, 1% (w/v) sucrose, 0.5 $\mu g.ml^{-1}$ nicotinic acid, 0.5 $\mu g.ml^{-1}$ pyridoxine, 1 $\mu g.ml^{-1}$ thiamine, 0.4% (w/v) plant tissue culture agar). The sown seed was incubated in dimmed light for three days to stimulate germination and then placed in a growth chamber at 23°C with a 16 h day en 8 h night cycle. The light intensity of 120 $\mu E.m^{-2}.s^{-1}$ was produced using cool-white fluorescent tubes. Ten day old seedlings were used for all the experiments. The harvested tissue was stored at –80^{o}C until used.

3.3.2.1.2 Extraction of genomic DNA from *A. thaliana* ecotypes

Genomic DNA was isolated from *A. thaliana* Columbia and Landsberg erecta plants as described (Verbruggen *et al.*, 1993), but omitting the cesium chloride purification. The concentration of the extracted genomic DNA was determined and expressed as $\mu g.ml^{-1}$ (Sambrook *et al.*, 1989). The purity of the DNA was determined by dividing the absorbency (A) value at 260 nm by the A_{280} value. DNA samples of which the purity was above 1.6 were used for further analysis. The quality of the genomic DNA was confirmed by separating 500 ng genomic DNA on a 1.0% (w/v) agarose gel (3.3.2.1.3).

3.3.2.1.3 Agarose DNA gel electrophoresis

All DNA and RNA samples were separated on 1% (w/v) agarose gels according to Sambrook *et al*. (1989). The gels were prepared by dissolving the agarose powder in 0.5x TAE (20 mM Tris (hydroxymethyl) aminomethane [Tris]-acetate pH 8.0, 0.5 mM ethylenedinitrilo tetraacetic acid [EDTA]), while 0.5 $\mu g.ml^{-1}$ ethidium bromide (EtBr) was also added. The running buffer used was 0.5x TAE. Before loading the DNA on the gel, the samples were mixed with loading buffer to a final concentration of 0.042% (w/v) bromophenol blue (BPB), 2.5% (w/v) ficoll and then separated at 10 volts per centimeter ($V.cm^{-1}$). The migrated DNA was visualized under ultraviolet illumination and photographed.

3.3.2.1.4 Restriction digestion of DNA

Following the extraction of the genomic DNA, 2 µg aliquots of Columbia and Landsberg ecotype DNA were respectively digested with 20 U *Eco*R1 and *Hinc*11 in the presence of buffer H (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 1

mM dithioerythritol [DTE]) and 20 U of *Bam*H1 in the presence of buffer B (10 mM Tris-HCl pH 8.0, 5 mM $MgCl_2$, 100 mM NaCl, 1 mM 2-mercaptoethanol). The DNA was digested for 2 h at 37°C. To verify that the digestion was complete, 100 ng of each digestion was separated on an agarose gel (3.3.2.1.3).

3.3.2.1.5 Southern transfer of genomic DNA

After complete digestion of the genomic DNA was obtained, the DNA was lyophilized in a freeze-dryer, dissolved in DNA loading buffer and separated overnight on a 0.8% (w/v) agarose gel at approximately 1 $V.cm^{-1}$ (3.3.2.1.3). After separation, the digested DNA was transferred to a Hybond N^+ nylon membrane using the downward alkaline method described by Chomczynski (1992). The genomic DNA was denatured for 30 min in a 3 M NaCl, 0.4 M NaOH solution, where after the genomic DNA was transferred to a nylon membrane for 2 h using 1.5 M NaCl, 0.4 M NaOH as transfer buffer. After transfer, the membrane was neutralized for 10 min in 0.2 M phosphate buffer pH 6.8 and then dried for 15 min at 80°C.

3.3.2.1.6 Preparation of DNA probes for hybridization

3.3.2.1.6.1 Preparation of DNA probes

During this stage of the project, the isolated cDNA fragment encoding the truncated *At-RLK3* gene was cloned in pFL61 (Minet *et al*., 1992) and was called TL3. For the Southern blot analysis, the truncated cDNA fragment was directly amplified from TL3 and used as a probe during the subsequent hybridization reactions.

The truncated cDNA fragment was amplified from TL3 using Bovis 10 and 11 as primers (Appendix 1). Each 50 µl polymerase chain reaction (PCR) contained 10 ng template DNA, 25 pmole of each primer, 200 µM dNTPs, 1.5 mM $MgCl_2$, 20 mM Tris-HCl pH 8.4, 50 mM KCl and 2 U Taq DNA polymerase. The reaction was covered with 50 µl light mineral oil. A control reaction lacking any template DNA was included to verify the success of the reaction. The cycling regime was a single denaturation step of 3 min at 94°C, 30 cycles of 1 min denaturation at 94°C, 1 min annealing of primers at 55°C and 2 min polymerization at 72°C with a final 10 min polymerization step at 72°C. After the reactions were completed, 50 µl chloroform and DNA loading buffer (3.3.2.1.3) were added to each tube. The success of the PCR

reaction was evaluated by separating a small aliquot on a 1% (w/v) agarose gel (3.3.2.1.3), after which the rest of the reaction was separated on a 1% preparative agarose gel. The amplified fragment was purified from the gel using the JetSorb system (Promega) according to the manufacturer's instructions.

3.3.2.1.6.2 Radioactive labeling and purification of DNA probes

The labeling of the probes was done using the *Redi*prime^{TM}II system according to the manufacturer's instructions. Fifty ng of the amplified DNA was denatured at 94°C for 5 min and immediately cooled on ice. The DNA was random-prime labeled using 50 µCi [α-^{32}P]-deoxyadenosine triphosphate (dATP) for 30 min at 37°C. The labeled fragments were purified from the unincorporated nucleotides using Bio-Spin columns according to the manufacturer's instructions. Just before use, the probes were denatured at 94°C for 10 min, placed on ice and then added directly to the hybridization solution.

3.3.2.1.7 DNA-DNA hybridization

Before use, the dried DNA membranes were rehydrated in 1x SSC (150 mM NaCl, 15 mM sodium citrate pH 7.0). The membranes were quenched in the prehybridization solution (5x SSC {750 mM NaCl, 75 mM sodium citrate pH 7.0}, 50% (v/v) formamide, 5x Denhardt's solution {0.1% (w/v) Ficoll, 0.1% (w/v) polyvinylpyrrolidone [PVP], 0.1% (w/v) bovine serum albumin [BSA]}, 0.25 mg.ml^{-1} herring sperm DNA, 0.5% (w/v) sodium dodecyl sulfate [SDS]) for 2 h at 37°C. The denatured labeled DNA probe (3.3.2.1.6.2) was diluted in 20 ml prehybridization solution, added to the membranes and hybridized overnight at 37°C. The following morning, the membranes were washed twice for 5 min each in solution A (1x SSC {150 mM NaCl, 15 mM sodium citrate pH 7.0}, 0.1% (w/v) SDS) at room temperature and then twice for 15 min at 37^{o}C with wash solution B (0.1x SSC {15 mM NaCl, 1.5 mM sodium citrate pH 7.0}, 0.1% (w/v) SDS). After exposure to an x-ray film, the membrane was washed twice for 15 min at 65°C and re-exposed. The sizes of the cross-hybridizing DNA fragments were determined using the 1 kilobase pair (kb) ladder from Invitrogen.

3.3.2.2 Expression analysis of *At-RLK3* during various treatments

3.3.2.2.1 Preparation of plant tissue for treatments

The *A. thaliana* Landsberg cell suspension culture was grown in a growth chamber at 23°C with a 16 h day en 8 h night cycle (Axelos *et al*., 1992). The light intensity was between 25 and 100 $\mu mol.m^{-2}.sec^{-1}$. The culture was refreshed weekly by a 10-fold dilution of the culture in 50 ml Gamborg's B5 medium containing 2% (w/v) sucrose and 0.2 $\mu g.ml^{-1}$ α-naphthaleneacetic acid (NAA). When cultures were to be treated, a 5-fold dilution of the original culture was done in new growth medium. Chemical treatments of the cultures were done three days after renewal. Samples of 5 ml cells were harvested at different time intervals, filtered through Whatman filter paper, frozen in liquid nitrogen and stored at –80°C.

Seedlings were cultivated as described (3.3.2.1.1). Ten-day-old seedlings were treated as described, the plant tissue harvested and immediately frozen away at -80°C. For the infiltration experiments, the aerial parts of five plants were harvested and pooled for each time point and frozen at -80°C.

3.3.2.2.2 Treatments of plant tissue

To evaluate the role of At-RLK3 in *Arabidopsis*, the expression of the gene was determined after cell suspension cultures were subjected to various stress conditions. In all cases, the treatments were done at a single reagent concentration according to that published in journals. Osmotic stress conditions were applied by adding 200 mM NaCl (ψ_s = -984 kPa at 23°C) directly to the cell suspension cultures, or by resuspending the cells after a brief centrifugation at 1000 *g* in Gamborg's B5 growth medium containing 20% (w/v) polyethylene glycol (PEG) 8000 (ψ_s = -61 kPa at 23°C). Hypo-osmotic stress conditions were simulated by diluting the cell suspension culture by half by adding an equal volume of sterile distilled water to the three-day old culture. Osmotic stress in seedlings was created by transferring the seedlings to a sterile growth medium solution containing either 200 mM NaCl or 20% (w/v) PEG8000. Oxidative conditions were simulated by either adding 1 mM H_2O_2 or 20 μM menadione dissolved in ethanol directly to the cell suspension cultures. Oxidative stress conditions were created in seedlings by exposing seedlings to 250 parts per billion (ppb) ozone.

The influence of two different signaling molecules was also tested. SA and JA were added to the cell suspension cultures at final concentrations of 200 µM and 50 µM respectively. The plant hormones that were tested were 0.1 mM indole acetic acid (IAA), 0.1 mM ABA, 0.5 mM GA, 0.1 mM benzylacylpurine (BAP) and 0.1 mM 1-aminocyclopropyl-1-carboxylic acid (ACC), a precursor of ethylene.

The influence of two heavy metals were tested, namely 1 µM $CuSO_4$ and 1.5 mM $CdCl_2$. The expression of *At-RLK3* was also tested after treating the cells with 10 mM proline, valine, methionine and glutamic acid. Finally, two environmental factors were tested. The first was elevated temperatures, where cultures were grown at 37°C and 25°C respectively. The second physical factor tested was hypoxia when ten-day-old seedlings were immersed beneath growth medium until harvested.

A. thaliana seedlings were also subjected to bacterial infection using both *Ralstonia solanacearum* and *Pseudomonas syringae* pv. *tomato*. Plants were vacuum infiltrated with *Ralstonia* according to Deslandes *et al.*, (1998), while the *Pseudomonas* infiltration was done according to Godiard *et al.*, (1998). For the treatments with *Ralstonia*, both the resistant Nd-1 and susceptible Col-5 plants were used. Two *Ralstonia* strains were used, namely wild-type GMI1000 with an intact *hrp* locus and a GMI1000 strain where the *hrp* locus was deleted. Three different *Pseudomonas syringae* pv *tomato* strains were used to infiltrate *A. thaliana* Col-0 plants, namely the virulent DC3000 virulent strain (Kunkel *et al.*, 1993) and the same strain expressing the *avrB* or *avrRpt2* avirulence genes respectively (Dong *et al.*, 1991; Whalen *et al.*, 1991). As control, 10 mM $MgCl_2$ was used to infiltrate the *Arabidopsis* plants.

For all the above-mentioned treatments, a sample was taken as a control before the onset of the particular treatment. The tissues were then treated and samples were taken after 2, 4, 8, 12 and 24 h respectively. For the treatment of seedlings with anoxia, only three samples were taken at 0, 6 and 12 h. Shorter time interval treatments were also done when the samples were collected at 0, 5, 15, 30, 60, 120 and 240 min after treatment.

3.3.2.2.3 Preparation of total RNA from treated plant tissue

3.3.2.2.3.1 Preparation of solutions and tools for RNA extraction

For the extraction of total RNA, all solutions were treated overnight with 0.1% (v/v) DEPC (Sambrook *et al*., 1989). The DEPC was destroyed by autoclaving the solutions the following day. All gel trays, mortars, pestles, glassware and spatulas were washed with 10% (w/v) SDS, rinsed with sterile double distilled water, rinsed with 100% (v/v) ethanol and allowed to dry. Before use, ethanol was added to the mortars and pestles and set alight.

3.3.2.2.3.2 Total RNA extraction from treated plant tissue

Frozen cell suspensions and plant tissues were ground to a fine powder in liquid nitrogen using a mortar and pestle. Total RNA was extracted from the tissue using the TRIzol reagent according to the manufacturer's instructions. The solution uses a mixture of guanidinium thiocyanate and acid phenol to destroy the plant cell structure. After the addition of chloroform, total RNA was precipitated from the water phase of the extraction using isopropanol. The pellet was finally dissolved in DEPC treated water (Sambrook *et al.*, 1989).

The concentration and purity of the extracted RNA was determined (3.3.2.1.2). The RNA concentration was expressed as $\mu g.ml^{-1}$. RNA samples of which the purity was above 1.8 was used for further analysis. To validate the quality of the RNA, 1.5 µg of each sample was separated on a 1.0% (w/v) agarose gel (3.3.2.1.3).

3.3.2.2.4 Formaldehyde gel electrophoresis

For the separation of total RNA under denaturing conditions, a formaldehyde-containing agarose gel was prepared (Sambrook *et al*., 1989). The gel was prepared by dissolving 1% (w/v) agarose in a 1x 3-(*N*-morpholino) propanesulfonic acid (MOPS) solution (20 mM MOPS pH 7.0, 3 mM sodium acetate, 1 mM EDTA, 0.41 M formaldehyde) (3.3.2.1.3). The running buffer used was a 1x MOPS solution lacking formaldehyde. Of each of the time points, 20 µg total RNA was lyophilized and dissolved in 18 µl RNA buffer (1x MOPS, 50% (v/v) formamide, 6.5% (v/v) formaldehyde, 50 $\mu g.ml^{-1}$ EtBr). To this, RNA loading buffer was added to a final concentration of 5% (v/v) glycerol, 0.1 mM EDTA pH 8.0, 0.0025% (w/v) BPB. The

RNA was then separated at 10 $V.cm^{-1}$ until the blue front migrated three quarters of the gel. The gel was photographed before transfer.

3.3.2.2.5 Northern transfer of total RNA

After separation, the RNA was transferred to Hybond N^+ membranes using the downward alkaline method as described (3.3.2.1.5) with the following modifications. The RNA was directly transferred to the nylon membrane without using a denaturation step. The transfer buffer used was 3 M NaCl, 8 mM NaOH, 2 mM sarkosyl. The transfer was allowed to proceed for 2 h. After transfer, the membranes were neutralized, dried and rehydrated as described and then stained with 0.02% (w/v) methylene blue in 0.3 M sodium acetate pH 5.5 for 3 min (Herrin and Schmidt, 1988). After partial destaining of the membrane with water, the RNA ladder was marked and the membranes completely destained in 0.2x SSC (30 mM NaCl, 3.0 mM sodium citrate pH 7.0), 1% (w/v) SDS.

3.3.2.2.6 DNA-RNA hybridization

3.3.2.2.6.1 Hybridization with *At-RLK3* DNA probes

For the Northern blot analysis, two probes were prepared. The first was the truncated cDNA fragment that was directly amplified from TL3 (3.3.2.1.6.1). The second DNA probe encoded the complete extracellular domain of *At-RLK3* and was amplified from a full length copy of the *At-RLK3* gene (5.3.2.1) using Bovis 7 and 12 as primers (Appendix 1). The probes were amplified and labeled as described (3.3.2.1.6). The membranes were probed as described for the Southern blot analysis (3.3.2.1.7) with the exception that both prehybridization and hybridization were performed at 42^oC, and the second wash step was performed at 68^oC. The membranes were first exposed to a phospho-imager, after which they were exposed to x-ray films. To determine the sizes of the cross-hybridizing RNA fragments, the 0.24 – 9.5 kb RNA ladder was used during gel electrophoresis.

3.3.2.2.6.2 Hybridization with the actin DNA probe

For the quantification of the expression of the *At-RLK3* gene, it was decided to reprobe the membranes with the actin gene that is constitutively expressed within plant tissue. The cloned actin gene was released from the pGem-T plasmid using

*Nco*1 and *Not*1 in buffer H (3.3.2.1.4). After separation on the agarose gel (3.3.2.1.3), the insert was purified from the gel (3.3.2.1.6.1) and labeled radio-active (3.3.2.1.6.2). Prehybridization, hybridization and washing of the membranes were done as described (3.3.2.2.6.1).

For quantification, the total radioactivity in each hybridized band was determined using the phospho-imager. To compensate for any differences in the amount of RNA loaded onto the formaldehyde gels, the obtained *At-RLK3* hybridization value at each time interval was divided by that of the actin gene, since actin is constitutively expressed in plant cells. To determine whether *At-RLK3* expression was either induced or repressed, the normalized value for *At-RLK3* at a given time was divided by the normalized value of time 0. That would therefore give time 0 a value of 1. Should the later time intervals show values higher than 1, it would indicate the induced expression of the gene following that particular treatment.

Where the hybridization with the actin gene was not possible, total RNA separated on a TAE gel (3.3.2.1.3) was used to confirm equal loading on the gel. In the case of the short time interval study (Fig. 3.18), the total RNA loaded on the gel was compared to ensure that equal amounts were loaded on the gel, even though it is not indicated in the figure.

3.4. Results and Discussion

3.4.1 Characterization of the *At-RLK3* gene

3.4.1.1 Characterization of the coding sequence

The complementation of characterized yeast mutations is a very effective technique to either clone a homologous plant gene or to assign a definite role to a gene of unknown function (Minet *et al.*, 1992). This technique allows one to clone a gene without any prior knowledge of the gene structure or the base sequence (Lecain *et al.*, 1996). In an attempt to identify *A. thaliana* genes involved in salt tolerance, the group of Dr Nathalie Verbruggen (VIB, Gent, Belgium) transformed a salt sensitive yeast mutant with a cDNA library prepared in a yeast expression vector. One of the isolated clones showed partial tolerance to elevated salt concentrations, but the experiment could not be repeated. The cDNA fragment was therefore given to the author of this thesis for the completion of a Ph.D. study during which he had to characterize the gene and its encoded protein to assign a putative role for the protein within *A. thaliana*.

The original isolated cDNA clone encoded a truncated protein kinase. Using 5'-RACE, the sequence of the full-length gene was identified and aligned with DNA sequences in the *A. thaliana* genome project database. The cDNA sequence showed 100% homology with a putative serine/threonine protein kinase gene (AJ011674) located on chromosome 4 (At4g23190.1). The gene had a 2004 bp open reading frame with a 51 and a 187 bp 5' and 3' untranslated region respectively. The coding region consisted of seven exons with 6 intronic regions with the whole of the extracellular domain being encoded by the first exon (Fig. 3.1). This was similar to SRK_6 (Stein *et al.*, 1991), *ARK1* (Tobias *et al.*, 1992); *ARK2* (Dwyer *et al.*, 1994), *Pr5K* (Wang *et al.*, 1996) and *BcRK1* (Suzuki *et al*., 1997). Several of these genes have in-frame stop-codons present within the first intron (Stein *et al.*, 1991; Tobias *et al*., 1992; Chang *et al.*, 1992; Dwyer *et al.*, 1994; Delorme *et al.*, 1995; Suzuki *et al.*, 1997) leading to the formation of multiple transcripts (Tobias and Nasrallah, 1996; Stein *et al.*, 1991; Giranton *et al*., 1995; Wang *et al.*, 1996). Since *At-RLK3* also has an in-frame stop codon present in the first intron (Fig. 3.1), it is possible that *At-RLK3* will also be expressed as multiple transcripts through differential transcription.

ATGAAGCAGAGGAGTTTGTTTTCAGTCCTCTGTTTTTTCTTCATAAGTTTTGGTGTTGCTTCAGTTTCA
GCACAAACATGCACGACGGACAAGGGGACTTTCAGACCCAACGGTACTTACGACGTAAATCGCCGTCTC
ATCCTCTCTTCTCTTCCTTCAAATGTCACGGACCAAGACGGCTTATACTACAACGGTTCCATAGGACAA
CAACCGAACCGTGTCTACGCAATAGGGATGTGCATCCCAGGATCAACTTCAGAAGACTGTTCTGATTGT
ATCAAGAAAGAGTCTGAATTTTTTTTAAAGAATTGTCCTAACCAAACAGAGGCGTATTCATGGCCAGGT
GAGCCAACGCTTTGCTATGTGCGCTACTCCAACACTTCTTTCTCAGGATCTGCTGATCTGAACCCGCGA
AATTGGCTCACCAACACTGGAGACCTAGACTCAAATCTAACAGAGTTTACGAAAATATGGGAAGGATTA
ATGGGTCGTATGATTTCTGCAGCTTCCACAGCAAAAAGCACACCTTCTTCAAGTGATAACCATTACTCA
GCTGATTCAGCAGTCTTGACACCTCTCCTGAATATATATGCATTGATGCAATGCACGCCGGATCTTTCC
TCCGGTGATTGTGAAAACTGTCTGCGACAAAGCGCAATTGACTATCAGTCGTGCTGTAGCCAGAAGCGA
GGAGGTGTTGTTATGCGGCCAAGCTGCTTTTTGCGGTGGGATTTGTATACATATTCTAACGCTTTTGAT
AATCTTACGGTGGCTTCTCCTCCTCCAGAACCTCCTGTGACTGTGCCACAACCTGCAGGTGATCAGGAC
AACCCGACCAACAATGgt**tag**tcttccacctgagaaaactatcattgtaacaaaaaaacttaaatatat
gacattgtttttctatgtgttgtgagaagATAGCAAAGGAATCTCAGCTGGAGTTGTTGTGGCGATCAC
CGTTCCCACTGTTATTGCCATCTTGATACTGCTGGTTTTAGGATTTGTTCTTTTCCGGAGAAGAAAATC
CTACCAAAGAACTAAGACTGAATgttagtttctttgtgttttacctctttattgtttcctttcgttatc
agcatgtatactcccgtagccaagacgggaaacacgaaactaatacacccattctttgcagCTGAAAGT
GATATTTCAACTACAGATTCATTGGTATACGATTTTAAGACAATTGAAGCCGCAACTAACAAGTTTTCA
ACAAGTAATAAGCTTGGTGAAGGTGGATTCGGTGCGGTTTACAAGGtaacaaggtctctgttttccatt
ttttctaagttcatattggtttatggaaataaccaacaatgtttgtttcattcattgtacgtaggGTAA
GCTTTCTAACGGAACTGATGTAGCTGTGAAGCGACTGTCGAAAAAGTCAGGACAAGGCACAAGGGAGTT
CAGGAACGAGGCTGTTCTTGTGACAAAACTTCAACATAGGAATCTGGTTAGACTTCTTGGATTCTGTTT
GGAAAGAGAGGAACAGATTCTGATCTATGAATTTGTCCACAACAAAAGCCTTGACTACTTTCTTTTCGg
tatggttcctgtcaataatttttcttattcaaaggatcctgcttcagctttatactgattgattgtcga
tatgtgcagACCCGGAAAAGCAAAGTCAGCTAGACTGGACCCGGCGATACAAGATCATTGGAGGAATTG
CTCGAGGGATTCTATATCTTCATCAAGATTCACGGCTCAAAATCATACATCGTGACCTCAAAGCCAGCA
ACATTCTCTTAGATGCAGACATGAACCCAAAAATTGCAGATTTTGGATTGGCAACTATTTTTGGAGTGG
AGCAAACTCAAGGAAACACGAACAGAATTGCTGGAACCTAgtaagttctgctcgtagtagctgaacact
aaaccattttctattgcatattaacccgtgttgtatgtttttcagCGCTTACATGTCTCCCGAGTATGC
GATGCATGGTCAATACTCCATGAAATCTGACATTTATAGCTTTGGAGTCTTAGTTCTTGAGATTATAAG
CGGCAAGAAAAACAGCGGCGTCTACCAGATGGATGAAACTAGTACTGCAGGAAACTTGGTCACTTATGt
gagcataaagtcctcaactttcaacaatttaacattttaaaacatatgaccaaggtacttaattgttca
ttgtattaggCTTCGAGGCTTTGGAGAAACAAGTCACCATTAGAGCTGGTGGATCCAACTTTTGGAAGG
AATTATCAGAGTAATGAAGTCACTAGGTGCATCCATATCGCGCTGTTATGTGTTCAAGAAAATCCAGAA
GACCGTCCAATGTTATCAACAATCATCTTAATGCTGACTAGTAACACAATTACTCTACCAGTGCCTCGC
CTACCGGGATTTTTCCCACGAAGCAGGCAACTGAAACTGGTATCTGAAGGATCAGAGTCTGATCAGTAT
ACAAGCAAGTCTTCCTCATTTAGTTCGTAA

Figure 3.1. Gene structure of *At-RLK3*. The exons are indicated in capital letters, introns in underlined small letters and the start and stop codons in bold. An in-frame stop codon in intron 1 is highlighted in a black box.

The junction site of the 74 bp intron 5 is also very close to the conserved amino acid sequence of subdomain VIII with the splice site located between the 2nd and 3rd base pair of Tyr_{516}. This amino acid forms part of the characteristic serine/threonine signature represented by subdomain VIb and VIII (Fig. 3.3).

The ORF encodes a 667 amino acid polypeptide with an estimated molecular weight of 74.1 kDa. Computational analysis of the amino acid sequence of the encoded polypeptide revealed the presence of four characteristic domains (Fig. 3.2). Using a signal peptide prediction package (http://www.cbs.dtu.dk/services/SignalP), a 24 amino acid signal peptide was identified at the amino terminal end of the polypeptide according to Nielsen *et al.*, (1997). The signal peptide can be divided into an n-region (amino acids 1-5) containing positively charged amino acids, the h-region (amino acids 6-20) where hydrophobic amino acids predominate and the c-region (amino acid 21-24) containing neutral but polar amino acids. The (-3,-1) rule stipulates that the third and first amino acids upstream of the peptide cleavage site be small and neutral, as is the case with the alanine and valine amino acids present in *At-RLK3*. The sequence of the signal peptide clearly directs At-RLK3 via the ER for transport to the plasma membrane.

Using a web-based protein sorting program (http://psort.ims.u-tokyo.ac.jp/form.html), a hydrophobic transmembrane domain (amino acids 296-312) was identified, classifying the protein as a Type 1a integral membrane protein (Fig. 3.2). Four of the six amino acids following the domain are basic, allowing for efficient anchoring of At-RLK3 to the plasma membrane. At-RLK3 will therefore most probably be anchored to the plasma membrane with one end of the protein occurring on the outside and the other on the inside of the cell. Using a motif scan program (http://hits.isb-sib.ch/cgi-bin/PFSCAN_parser), a protein kinase domain was identified in the region following the transmembrane domain (amino acids 350-629). All eleven subdomains of protein kinases were identified using the criteria set by Hanks and Quinn, (1991) (Fig. 3.3). Two major regions were identified, namely an adenosine triphosphate (ATP) binding region (amino acids 356-378) and a region characteristic of serine/threonine protein kinases (amino acids 471-483). The former region (amino acids 356 - 378) contained a glycine rich region in the vicinity of a lysine characteristic of such a region.

M K Q R S L F S V L C F F F I S F G V A S V S A*Q T C T T

D K G T F R P N G T Y D V N R R L I L S S L P S N V T D Q

D G L Y Y N G S I G Q Q P N R V Y A I G M C **I P G S T S E**

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

P T L C Y V R Y S N T S F S G S A D L N P R N W L T N T G

D L D S N L T E F T K I W E G L M G R M I S A A S T A K S

T P S S S D N H Y S A D S A V L T P L L N I Y A L M Q C **T**

P D L S S G D C E N C L R Q S A I D Y Q S C C S Q K R G G

V V M R P S C F L R W D L Y T Y S N A F D N L T V A S P P

P E S P V T V P Q P A G D Q D N P T N N D S K G I S A G V

V V A I T V P T V I A I L I L L V L G F V L F R R R K S N

Q R T K T E S E S D I S T T D S L V Y D F K T I E A A T N

K F S T S N K L G E G G F G A V Y K G K L S N G T D V A V

K R L S K K S G Q G T R E F R N D S V L V T K L Q H R N L

V R L L G F C L E R E E Q I L I Y E F V H N K S L D Y F L

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

Q D S R L K I I H R D L K A S N I L L D A D M N P K I A D

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

Y A M H G Q Y S M K S D I Y S F G V L V L E I I S G K K N

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

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

N P E D R P M L S T I I L M L T S N T I T L P V P R L P G

F F P R S R Q L K L V S E G S E S D Q Y T S K S S S F S S

Figure 3.2 Amino acid sequence of At-RLK3. The signal peptide (_) and transmembrane domain (_.._) are both underlined, the two domains of unknown function are double underlined while the two cysteine-rich repeats are double underlined and in bold. Leucine and cysteine amino acids are highlighted in black and grey boxes respectively. The predicted signal peptide cleavage site is indicated with a *.

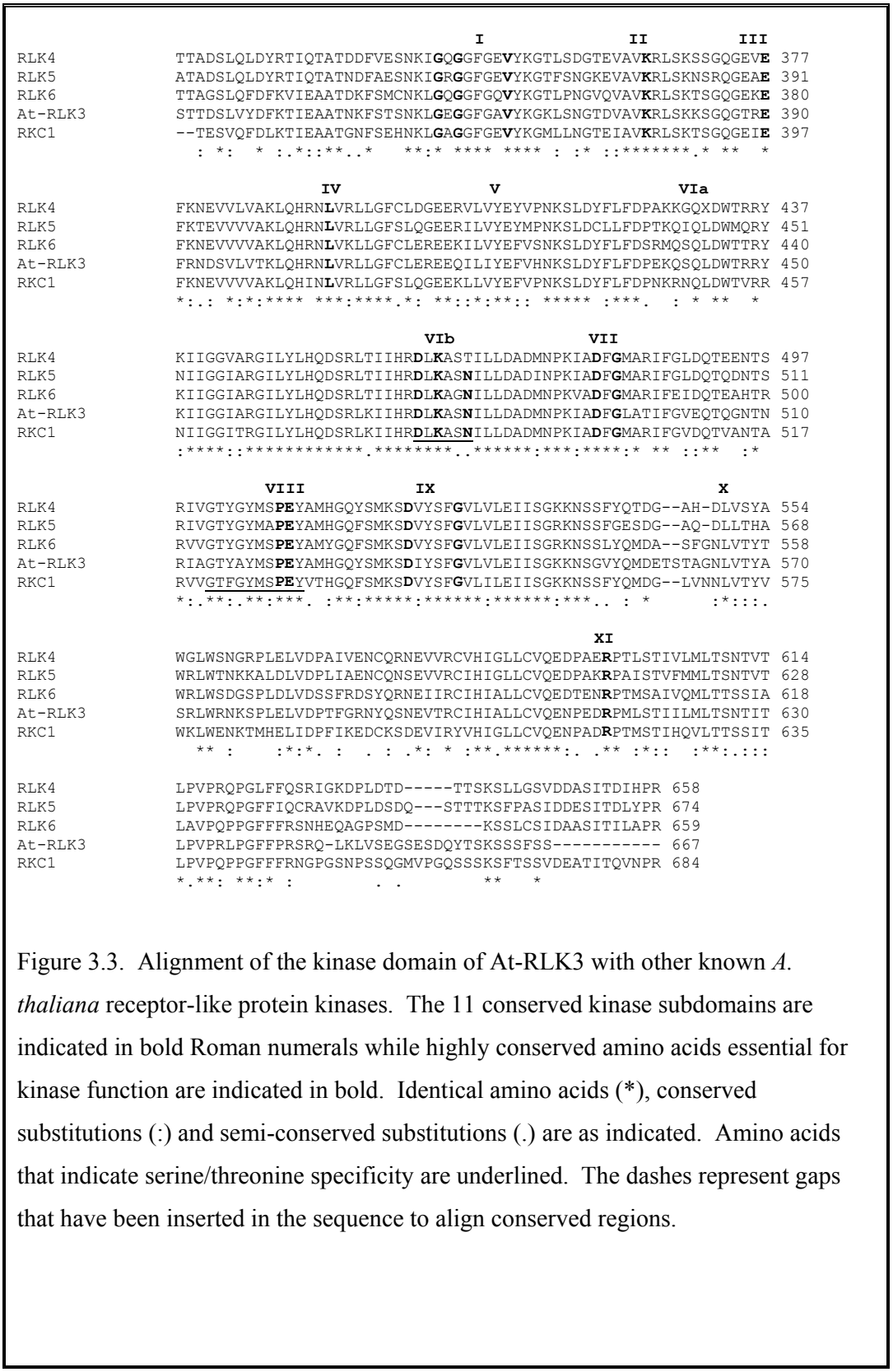


Figure 3.3. Alignment of the kinase domain of At-RLK3 with other known *A. thaliana* receptor-like protein kinases. The 11 conserved kinase subdomains are indicated in bold Roman numerals while highly conserved amino acids essential for kinase function are indicated in bold. Identical amino acids (*), conserved substitutions (:) and semi-conserved substitutions (.) are as indicated. Amino acids that indicate serine/threonine specificity are underlined. The dashes represent gaps that have been inserted in the sequence to align conserved regions.

The serine/threonine signature sequence contained the two subdomains characteristic of serine/threonine specificity, namely subdomain VIb (DLKASN) and VII (GTYAYMSPE) (Fig. 3.3). Added to this was the characteristic conserved arginine amino acid in subdomain XI. All these signatures suggested that At-RLK3 is an active protein kinase with serine/threonine specificity.

When a Blast search (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) was performed using the kinase domain of At-RLK3, a large number of homologous sequences were found. In the first alignment using ClustalW (http://www.ebi.ac.uk/clustalw/), cloned and identified RLKs from *A. thaliana* were aligned with At-RLK3 (Fig. 3.3). A second alignment was also done with all protein kinases included in the Genbank database (Fig. 3.4). In both cases, the homology among the kinase domains was striking. The highest homology with At-RLK3 was obtained with the unpublished At4g23300 polypeptide with 73% identity, while of the published sequences, the highest homology was obtained with RLK4 (66% identity). The homology was not only confined to the regions surrounding the 11 conserved subdomains, but also included areas in between the subdomains. This showed that the kinase domains of RLKs in *Arabidopsis* are highly conserved and were not influenced to a great extent by evolution.

Analysis of the extracellular domain of At-RLK3 revealed the presence of two conserved domains (http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi). The two domains shared extensive homology with the domain of unknown function (DUF) 26 that contains four conserved cysteine residues (Fig. 3.5). This region is found in serine/threonine protein kinases and is associated with the eukaryotic protein kinase domain pfam00069. The two regions stretch from amino acids 73 to 130 and amino acids 195 to 249 (Fig. 3.2). At this stage, the DUF26 domain has no definite function, but is thought to play a role on the exterior of the cell by forming two disulphide bridges with the four conserved cysteines present within the region (Fig. 3.5).

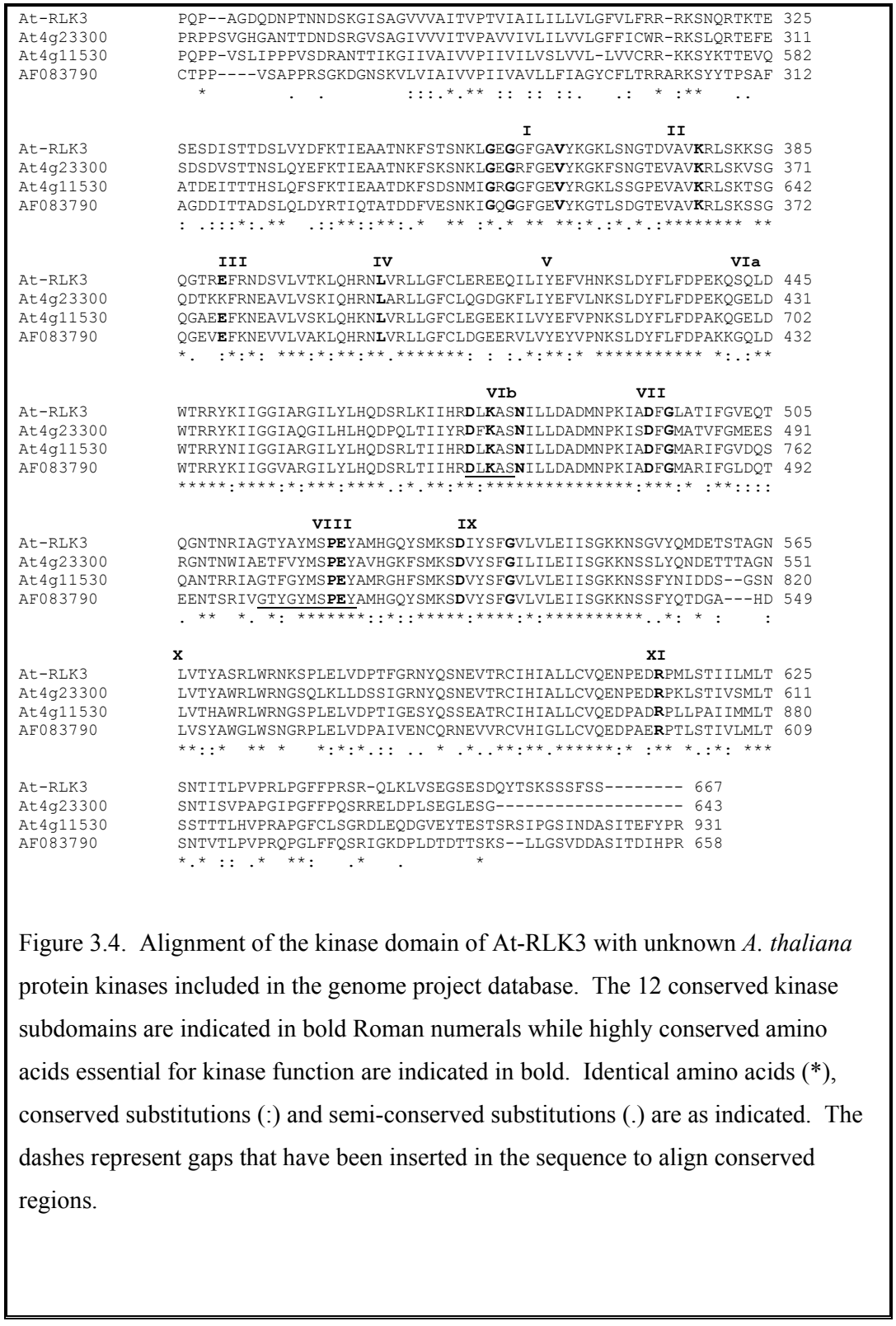


Figure 3.4. Alignment of the kinase domain of At-RLK3 with unknown *A. thaliana* protein kinases included in the genome project database. The 12 conserved kinase subdomains are indicated in bold Roman numerals while highly conserved amino acids essential for kinase function are indicated in bold. Identical amino acids (*), conserved substitutions (:) and semi-conserved substitutions (.) are as indicated. The dashes represent gaps that have been inserted in the sequence to align conserved regions.

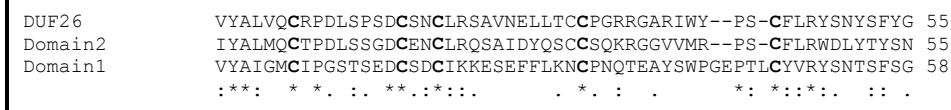


Figure 3.5. Alignment of two domains located in the extracellular domain of At-RLK3 with the DUF26 conserved sequence. Identical amino acids (*), conserved substitutions (:) and semi-conserved substitutions (.) are as indicated. The dashes represent gaps that have been inserted in the sequence to align conserved regions. The four conserved cysteine amino acids implicated in the formation of two possible disulphide bridges are indicated in bold.

When the At-RLK3 DUF26 domain 1 was aligned against the Genbank database with Blast (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi), several rice and *Arabidopsis* putative and described RLKs showed homology. All of these RLKs have either one or two DUF26 copies located on the extracellular domain. The identified RLKs included RKF1 (Takahashi *et al.*, 1998), PvRK20-1 (Lange *et al.*, 1999), RKC1 (Ohtake *et al.*, 2000) and RLK4 (Du and Chen, 2000).

When the extracellular domain of At-RLK3 was aligned with the Genbank database, the highest homology was found with two uncharacterized polypeptides from the *Arabidopsis* genome (Fig. 3.6). The homology values were 56.4% for At4g23210 and 53% for At4g23300 respectively. The first area of homology (amino acids 1 – 27) was found in the area representing the signal peptide, while the second area (amino acids 35 – 54) was very similar among all three polypeptides and fell outside the first DUF26 region (Fig. 3.6). This region does however include three leucines that might be implicated in a LRR region.

A shorter homologous region was found from amino acids 70 – 83 that forms part of the first DUF26 domain (Fig. 3.5). While the central part of the DUF26 region is less homologous, the latter part (amino acids 116 – 133) again was very homologous amongst the three polypeptides (Fig. 3.6). The last half of the first DUF26 region also showed high homology among the 3 sequences. This specific area in At-RLK3 shared a high degree of similarity with the conserved DUF26 region with 8 of the last 12 amino acids being identical and 2 being conserved substitutions. Two final regions shared extensive homology, namely amino acids 166 – 183 and 195 – 132. The latter region represents the second DUF26 region with extensive homology. The former region falls outside of any characteristic region in the extracellular domain.

When one considers the full length At-RLK3 amino acid sequence, the highest homology (64.4%) was found with the At4g23300 polypeptide (Fig. 3.7). While the extracellular domain of At-RLK3 shared the highest homology with that of At4g23210, the At4g23210 kinase domain was severely truncated. The fact that At-RLK3 and At4g23300 share such high levels of homology, might implicate some relationship or common function between the two proteins.

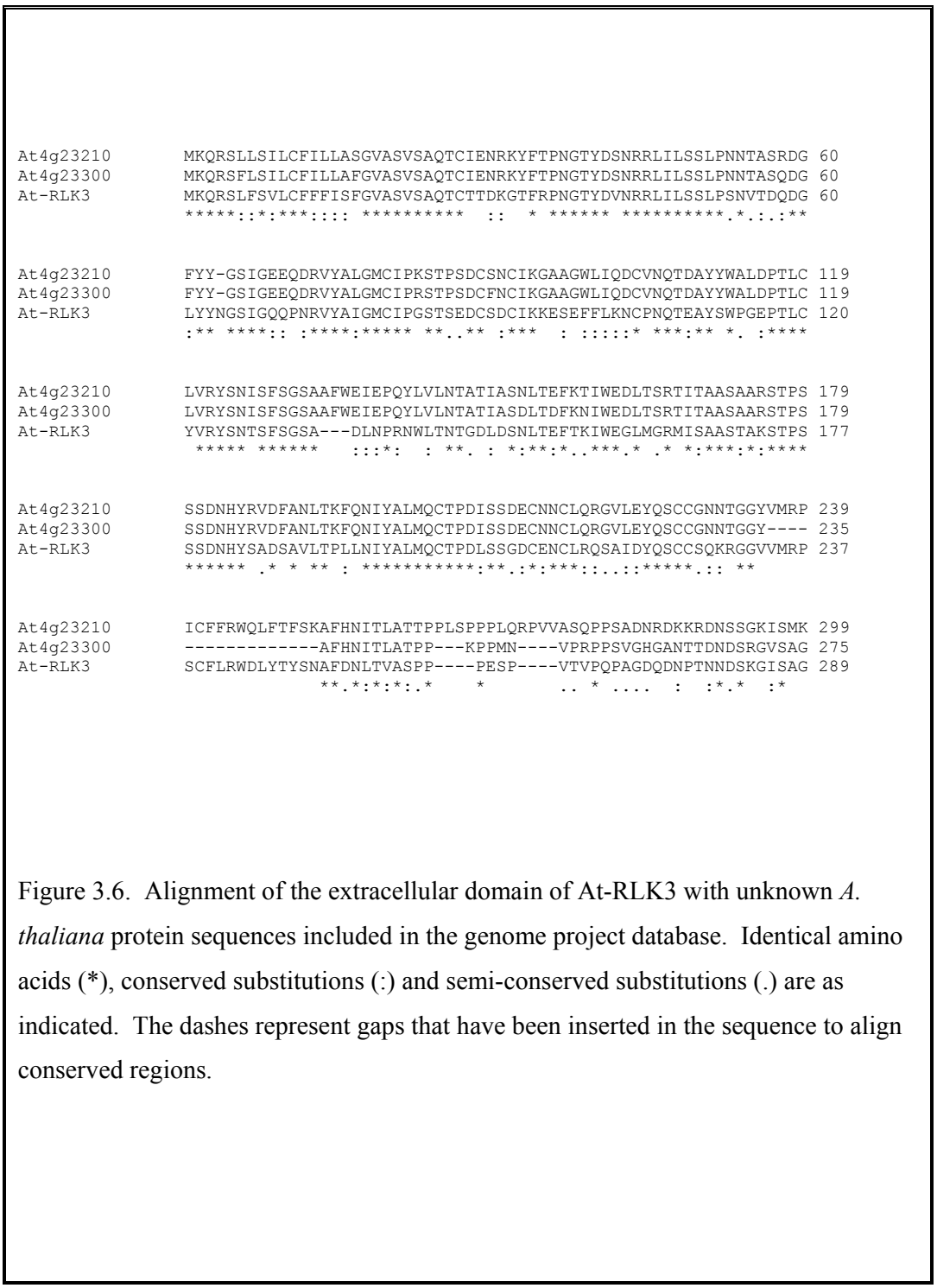


Figure 3.6. Alignment of the extracellular domain of At-RLK3 with unknown *A. thaliana* protein sequences included in the genome project database. Identical amino acids (*), conserved substitutions (:) and semi-conserved substitutions (.) are as indicated. The dashes represent gaps that have been inserted in the sequence to align conserved regions.

Since the extracellular domain of At-RLK3 is important for the putative binding of ligands, a thorough investigation of the amino acid sequence for possible characteristic motifs was undertaken. Several characteristic protein motifs were recognized using a Profile scanner (http://hits.isb-sib.ch/cgi-bin/PFSCAN). These included a single glycosaminoglycan attachment site, 8 possible N-myristoylation sites indicative of plasma membrane association, 12 protein kinase C phosphorylation sites, 4 cAMP-dependent protein kinase phosphorylation sites, 10 N-glycosylation sites and 12 casein kinase II phosphorylation sites. Of the 10 N-glycosylation sites, seven occurred on the extracellular domain and three on the intracellular kinase domain. The addition of glucose groups was implicated to play an important role in RLKs since the active glycosylation of two RLKs was found (Stein *et al.*, 1991; Schaller and Bleecker, 1993; Stein *et al.*, 1996).

Using the extracellular domain of At-RLK3, an attempt was made to place it into one of the RLK classes according to Torii (2000). The extracellular domain of At-RLK3 contains 20 leucine residues (Fig. 3.2). These residues could however not be arranged in a typical leucine rich repeat consensus motif as described by Kajava (1998). In addition to the leucine residues, 12 cysteines are also present of which 8 are found in two motifs with a C-X8-C-X2-C-X11-C consensus sequence (Fig. 3.2). These motifs form part of the DUF26 domains. The core of these two sequences was previously described by Takahashi *et al.*, (1998) as being C-X8-C-X2-C. These motifs were linked to the SA-responsive induction of gene expression for several *RLKs* (Ohtake *et al.*, 2000). Since two of these motifs were present within the extracellular domain of At-RLK3, it clearly places At-RLK3 within the cysteine-rich class of RLKs.

At-RLK3 is therefore predicted to be a plasma membrane bound receptor protein that has kinase activity. This kinase activity will be specifically directed towards serine and threonine residues. The presence of the two cysteine-rich repeats places At-RLK3 within this class. On genomic DNA level, the gene shares several characteristics with other RLKs, such as intron number and location. This is an indication that RLKs exist as families descended from a common ancestral gene. If this is the case, the characteristics of the different RLKs may also be applicable to At-RLK3, including the possibility of the gene to be expressed as multiple copies.

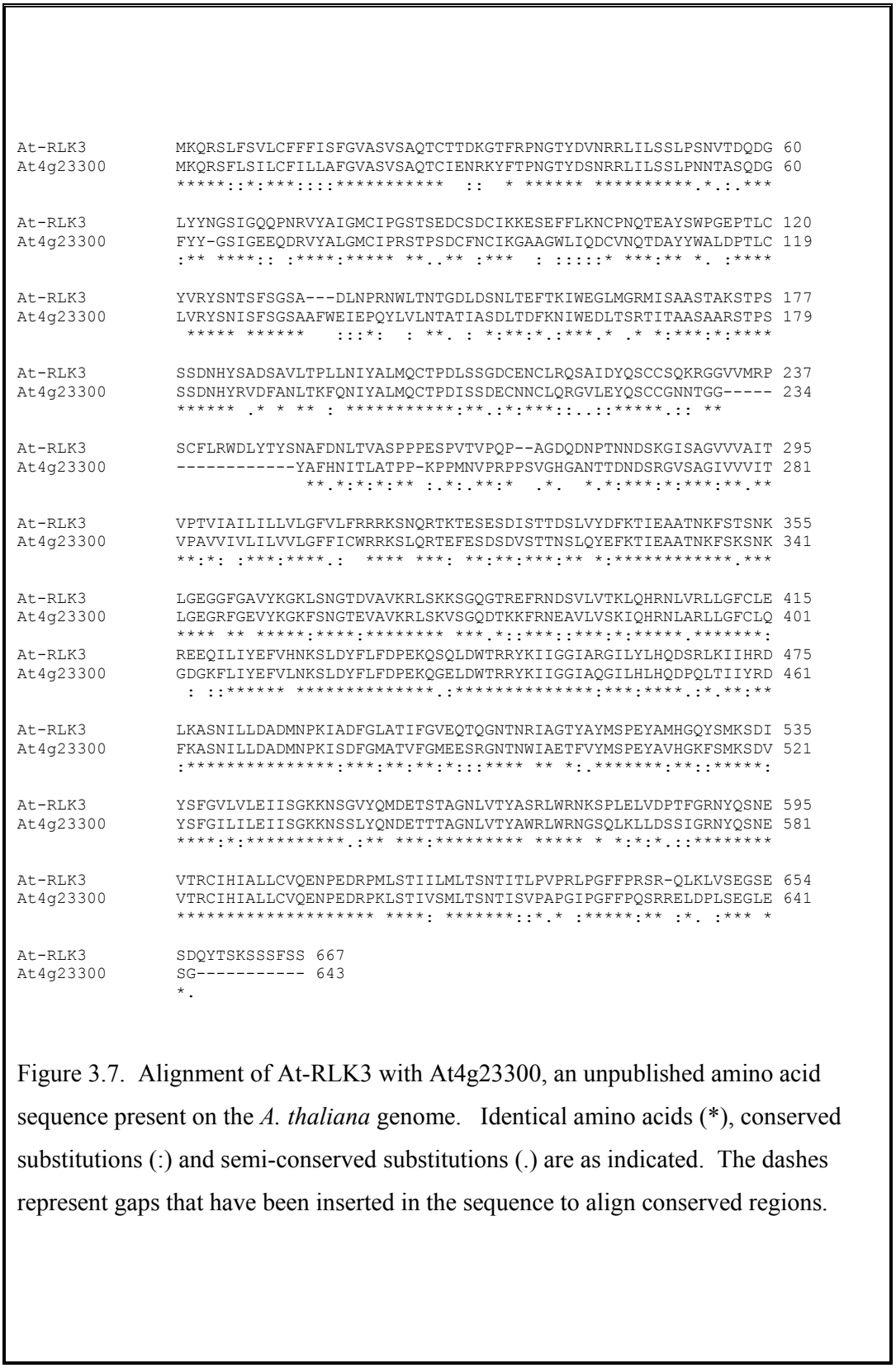


Figure 3.7. Alignment of At-RLK3 with At4g23300, an unpublished amino acid sequence present on the *A. thaliana* genome. Identical amino acids (*), conserved substitutions (:) and semi-conserved substitutions (.) are as indicated. The dashes represent gaps that have been inserted in the sequence to align conserved regions.

3.4.1.2 Characterization of the *At-RLK3* promoter region

In order to formulate a possible role for At-RLK3 in *Arabidopsis*, 1100 bp of the region upstream from the transcription initiation site was searched for *cis*-acting DNA elements using a Web-based tool (http://www.dna.affrc.go.jp/htdocs/place) (Higo *et al*., 1999). The results of the search are summarized in Table 3.1. The references in which each element is described are also indicated in the table.

The first group of elements found is common to all protein encoding genes. Three motifs were present in this grouping, namely the CCAAT and TATA boxes and a seed-specific enhancer region. All three these regions play important roles in the successful initiation of transcription.

The TATA box is the initial binding site for a complex of proteins called TFIID which includes a TATA-box-binding protein. Binding of TFIID to the TATA box facilitates the binding of RNA polymerase II to the promoter region, which leads to transcription of the gene. For successful initiation of transcription, the CAAT box is also essential. The presence of both these regulatory elements indicated that *At-RLK3* should be expressed normally within *Arabidopsis*. Enhancer regions are important to modulate the expression of genes and since the SEF3 enhancer is seed-specific, it implicates the induced expression of *At-RLK3* in seed.

Together with the normal regulatory sites, several other interesting *cis*-acting elements were identified. These motifs were characterized based on the roles they play in the expression of other plant genes and were divided into five loosely arranged groups based on their proposed function. The first group includes motifs present on the promoter regions of genes whose protein products are expressed in a tissue specific manner. Four motifs were present which render tissue-specific expression to genes. Linked to these regions were three motifs that confer endosperm specific expression, two conferring embryo and pollen specific expression and one each conferring root and seed specific expression respectively. The presence of these regions indicates that *At-RLK3* expression might play distinct roles within these tissues. Also of importance was the presence of a motif that is bound by AGL15, a protein that regulates embryonic development.

Table 3.1. *Cis*-acting elements present on the promoter region of *At-RLK3*.

Name of element	**Number of sites**	**Consensus sequence**	**Possible function of element**	**Reference**
2SSEEDPROTBANAPA	1	CAAACAC	ABRE element present on napA storage protein gene promoter that regulates ABA induced genes in response to severe water-deficit stress	Stalberg *et al.*, 1996
AACACOREOSGLUB1	1	AACAAAC	Motif conferring endosperm-specific expression of glutelin	Wu *et al.*, 2000
ACGTATERD1	1	ACGT	Motif required for dehydration-induced expression of *erd*1	Simpson *et al.*, 2003
ASF1MOTIFCAMV	1	TGACG	Motif for induced gene expression by auxin, salicylic acid and light	Redman *et al.*, 2002
C8GCARGAT	1	CWWWWWWWWG	Binding site of plant MADS-domain protein AGL15 that regulates embryonic development	Tang and Perry, 2003
CAATBOX1	12	CAAT	Consensus CAAT transcriptional motif	Shirsat *et al.*, 1989
CANBNNAPA	1	CNAACAC	Motif for embryo- and endosperm specific expression of the napin storage protein gene	Ellerstrom *et al.*, 1996
CATATGGMSAUR	1	CATATG	Motif involved in auxin responsiveness	Xu *et al.*, 1997
CCAATBOX1	3	CCAAT	Motifs present in heat shock protein gene promoter regions	Rieping and Schoffl, 1992
DOFCOREZM	3	AAAG	Binding site for maize Dof transcription factors involved in light and tissue specific expression	Yanagisawa, 2000

DPBFCOREDCDC3	2	ACACNNG	Binding site for bZIP transcription factors responsible for abscisic acid and embryo-specific expression of the maize *Dc3* gene	Kim *et al.*, 1997
EBOXBNNAPA	5	CANNTG	E-box element present on napA storage protein gene promoter	Stalberg *et al.*, 1996
GATABOX	3	GATA	Motif for light-regulated and tissue-specific expression	Lam and Chua, 1989
GCN4OSGLUB1	1	TGAGTCA	Binding site for a basic leucine zipper transcription factor that confers endosperm-specific expression	Washida *et al.*, 1999
GLUTAACAOS	1	AACAAACTCTAT	Common motif in seed storage protein gene promoter regions	Takaiwa and Oono, 1990
GT1CONSENSUS	7	GRWAAW	Consensus GT-1 binding site for light regulated genes	Zhou, 1990
GTGANTG10	4	GTGA	Motif found in tobacco g10 pollen gene	Rogers *et al.*, 2001
IBOXCORE	2	GATAA	Conserved motif for light regulated genes	Terzaghi and Cashmore, 1995
MYB1AT	1	WAACCA	MYB recognition site in dehydration responsive rd22 gene in *Arabidopsis*	Abe *et al.*, 2003
MYB2CONSENSUSAT	1	YAACKG	MYC recognition site in dehydration responsive rd22 gene in *Arabidopsis*	Abe *et al.*, 2003
MYBCORE	2	CNGTTR	ATMYB1 and 2 recognition site for regulation of water stress responsive genes in *Arabidopsis*	Urao *et al.*, 1993
MYBST1	1	GGATA	Motif for binding by a potato MYB homologue	Baranowskij *et al.*, 1994

MYCCONSENSUSAT	6	CANNTG	MYC recognition site in dehydration responsive rd22 gene in *Arabidopsis*	Abe *et al.*, 2003
NTBBF1ARROLB	2	ACTTTA	Dof protein binding site required for tissue-specific expression and auxin-regulated induction	Baumann *et al.*, 1999
POLLEN1LELAT52	2	AGAAA	Motif for pollen specific expression of tomato lat52 gene	Bate and Twell, 1998
RAV1AAT	2	CAACA	Binding site for RAV1 transcription factor	Kagaya *et al.*, 1999
ROOTMOTIFTAPOX1	5	ATATT	Motif found in promoter of rolD that is root specific	Elmayan and Tepfer, 1995
SEF3MOTIFGM	1	AACCCA	SEF3 consensus binding site found in promoter of the beta-conglycinin (7S globulin) gene that acts as a seed-specific enhancer	Allen *et al.*, 1989
SV40COREENHAN	1	GTGGWWHG	Simian virus 40 enhancer homologue found in *rbcS* gene promoter	Green *et al.*, 1987
TATABOX2	1	TATAAAT	Motif for tissue-specific expression of a pea legumin gene	Shirsat *et al.*, 1989
TATABOX4	1	TATATAA	TATA box regulatory site	-
TATABOX5	3	TTATTT	Functional TATA box element	Tjaden *et al.*, 1995
TBOXATGAPB	1	ACTTTG	TBox involved in light-regulated gene expression	Chan *et al.*, 2001
WBBOXPCWRKY1	1	TTTGACT	WB motif for WRKY transcription factor binding involved in elicitor responsiveness and early defense responses	Eulgem *et al.*, 2000
WBOXATNPR1	6	TTGAC	W-motif recognized by salicylic acid-induced WRKY DNA binding proteins	Chen *et al.*, 2002

Linked to the above mentioned were two motifs that were present in the promoter regions of genes encoding seed storage proteins. While one motif was a common regulatory element, the other was an ABRE element that regulates the expression of genes that are induced by abscisic acid during water stress conditions. This is significant, since several other motifs conferring responsiveness to dehydration are also present. These include the ABRE and E box elements, together with the MYB and MYC recognition sites that occur in several genes that are dehydration sensitive. The fact that ABA responsive elements were also found suggested that *At-RLK3* will most definitely be responsive to water stress.

The third group of motifs included those which confer expression responsiveness to several natural plant products. Among these are three motifs for auxin and two for abscisic acid responsiveness. Since the development of plants is closely linked to the presence of hormones, it might indicate a role for *At-RLK3* during plant development.

The fourth group consists of motifs that play a role in the defense reaction of the plant against potential pathogens. A key component of the defense reaction is the signaling compound salicylic acid. Present on the promoter region were two potential SA responsive motifs as was implicated by Ohtake *et al.*, (2000) and Du and Chen (2000). This TTGAC motif was previously reported to be important for the induced expression of many plant defense genes (Eulgem *et al.*, 1999). This region was absent in promoter regions of several other genes whose expression was SA unresponsive (Ohtake *et al.*, 2000). Also noteworthy was the presence of a motif that is bound by an elicitor-responsive transcription factor that is involved in early defense reactions. Together, these motifs all indicate an involvement of *At-RLK3* in plant defense reactions. Lastly, two other motif classes responsive to environmental conditions were also found. One was a heat shock element occurring in heat shock protein gene promoters. Light also seems to play a role in the expression of the gene, since five different elements involved in light regulation were present in the promoter region.

Clearly, the large number of different motifs indicates a very complex role for At-RLK3. It is however clear that if *At-RLK3* is regulated by such a large variety of different factors, it must

play a central role in each. It is therefore feasible that a common ligand produced during these diverse biotic and abiotic conditions could regulate the functionality of At-RLK3 *in vivo*.

3.4.1.3 Determination of the *At-RLK3* gene copy number

The first step in the analysis of *At-RLK3* was to determine its copy number in the *A. thaliana* genome. This was done by probing digested genomic DNA from the Columbia and Landsberg ecotypes with a DNA fragment encoding the kinase domain of At-RLK3 as probe (Fig. 3.8). The restriction enzymes were chosen on their ability to digest the *At-RLK3* gene. Both *Eco*RI and *Hinc*II do not have any internal restriction sites, while *Bam*HI has a single restriction site.

When the digested genomic DNA was probed at low stringency, both the *Eco*RI and *Hinc*II digested DNA produced a single very intense 5.7 kb hybridizing band together with several other fainter bands. The *Bam*HI digest only produced a number of faint bands. Interestingly, several of the fainter bands were polymorphic between the two ecotypes. The stringency of the hybridization reaction was increased by increasing the temperature of the final wash step to 65^{o}C to remove all partially hybridized DNA fragments. The first two digestions produced only the 5.7 kb band which correlated with the fact that no internal restriction sites for these enzymes were present within the gene. The *Bam*HI digestion produced only two small hybridizing fragments that correlated with a single internal *Bam*HI restriction site in the gene.

From these results, it was concluded that At-RLK3 exists as a single copy in *Arabidopsis*, but shares homology with several other closely related kinases, including the undescribed At4g23300 polypeptide and RLK4 (Walker, 1993) as was found during the Blast searches. This is similar to the majority of *RLKs* that are present as single copies, with homology to several other family members (Li and Wurtzel, 1998). *At-RLK3* also forms part of a large gene cluster on chromosome 4 that code for proteins all having the C-X8-C-X2-C motifs that are predicted to be SA responsive. Komjanc *et al.*, (1999) proposed that in general, genes involved in plant defense are located within a large homologous gene family located at a particular chromosomal locus, while RLKs involved in plant development is present as single copies. Since *At-RLK3* is part of such an extended family containing the cysteine-rich motif, a possible role for At-RLK3 in plant defense can be envisaged.

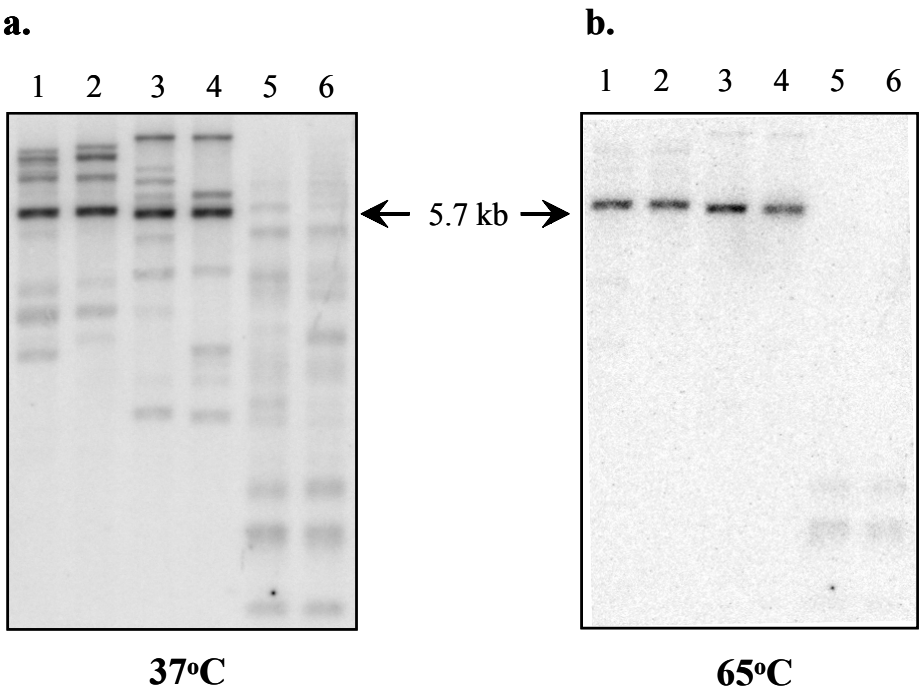


Figure 3.8. Southern blot analysis of the *At-RLK3* gene in *A. thaliana*. The membrane was probed at both (a) low and (b) high stringency conditions using the labeled DNA fragment encoding the kinase domain as probe. Lanes 1, 3 and 5 contained 2 µg genomic DNA from the *A. thaliana* Columbia ecotype digested with *Eco*RI, *Hinc*II and *Bam*HI respectively, while lanes 2, 4 and 6 contained 2 µg genomic DNA from *A. thaliana* Landsberg ecotype digested with the same enzymes.

3.4.1.4 Characterization of the expression pattern of *At-RLK3*

Since the *At-RLK3* promoter analysis indicated a very complex expression pattern, a series of Northern blots were performed to determine under which conditions *At-RLK3* is expressed. As a probe, the DNA fragment encoding the kinase domain of At-RLK3 was used, where after the blots were re-probed with the actin gene to allow for quantification.

In all cases, the kinase probe recognized a single mRNA species approximately 2.2 kb in size. This size corresponded well to the predicted size according to the cDNA sequence of the gene. The actin probe recognized a single 0.8 kb mRNA species. In all cases, the hybridization intensity for the actin gene was identical, indicating that equal amounts of total RNA was loaded on the formaldehyde gels. During quantification, the hybridization values for *At-RLK3* was standardized against that of the actin gene and used to determine whether the *At-RLK3* expression was induced, repressed or remained unaffected.

As a first step, the expression of *At-RLK3* was tested under various osmotic stress conditions (Fig. 3.9, 3.10). When challenged with high-salt conditions, expression of *At-RLK3* remained constant. Hyper-osmotic stress induced by PEG8000 on the other hand, resulted in a sharp but transient increase of expression within 2 h after treatment that returned to normal levels at 4 h. The fact that PEG8000 treated seedlings did not show a similar induction profile was most probably due to ineffective treatment of the plants. It must however be kept in mind that the PEG8000 treatment could also lead to a degree of oxygen deprivation, since oxygen solubility is reduced in such high PEG8000 concentrations. When cultured cells were treated with hypo-osmotic conditions, *At-RLK3* expression increased substantially after 4 h that was sustained for up to 24 h. When the cells were treated with an osmolyte in the form of proline, the expression was also induced within 2 h. Clearly, the expression of *At-RLK3* is responsive to changes in the water potential of the cell, irrespective of whether there is a decrease or increase in water potential. This included the treatment of the plant with an osmolyte. Both the ionic and osmotic stress components of NaCl were however unable to induce expression. These results confirmed the functionality of the dehydration responsive elements found in the promoter region of the gene, indicating that At-RLK3 must play a cardinal role in sensing of changes in osmotic pressure outside the cell.

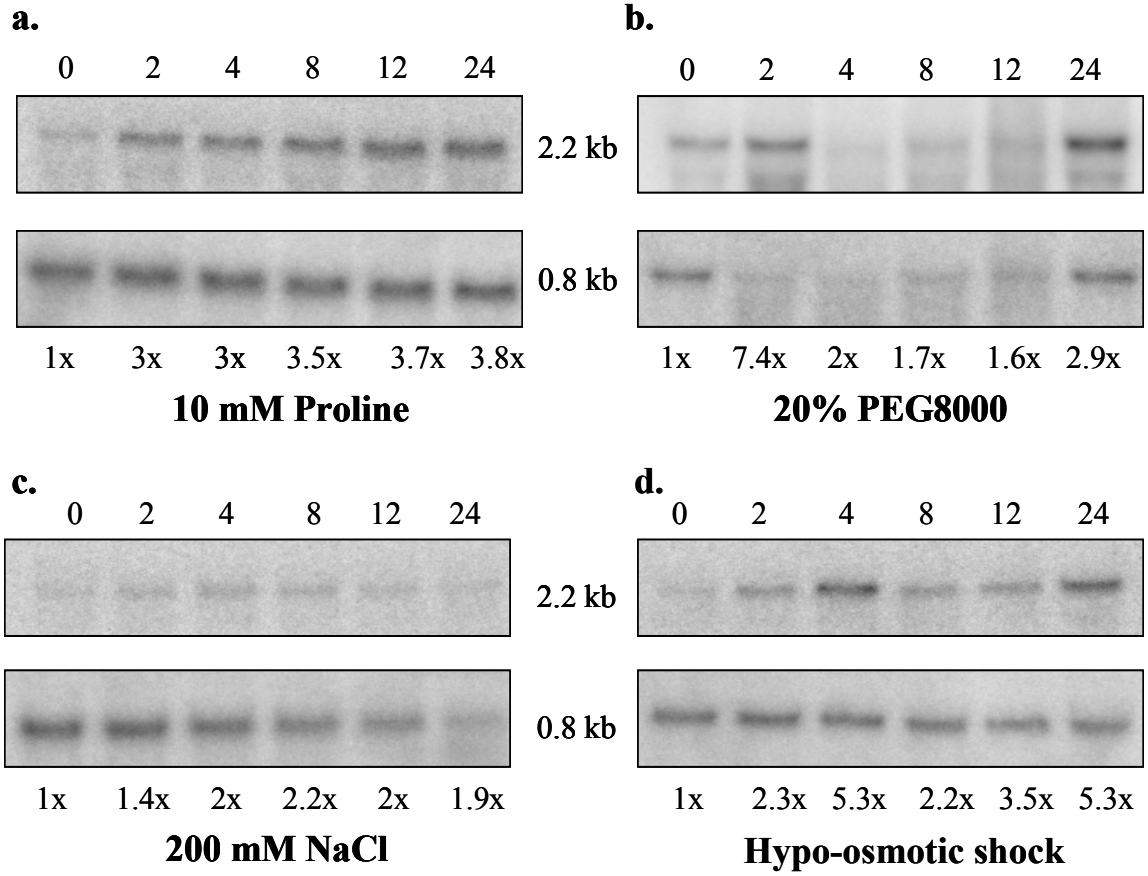


Figure 3.9. Expression of *At-RLK3* mRNA in cell suspension cultures during different stress conditions. *A. thaliana* cell suspension cultures were treated with (a) 10 mM proline, (b) 20% PEG8000, (c) 200 mM NaCl and (d) dilution of the growth medium by half with water. The time of each harvesting is indicated at the top of each panel. The top panel of each treatment represents total RNA probed with the labeled *At-RLK3* probe, while the bottom panel indicates hybridization with the actin control probe. The sizes of the hybridizing fragments are as indicated. Induction levels are indicated at the bottom of each panel.

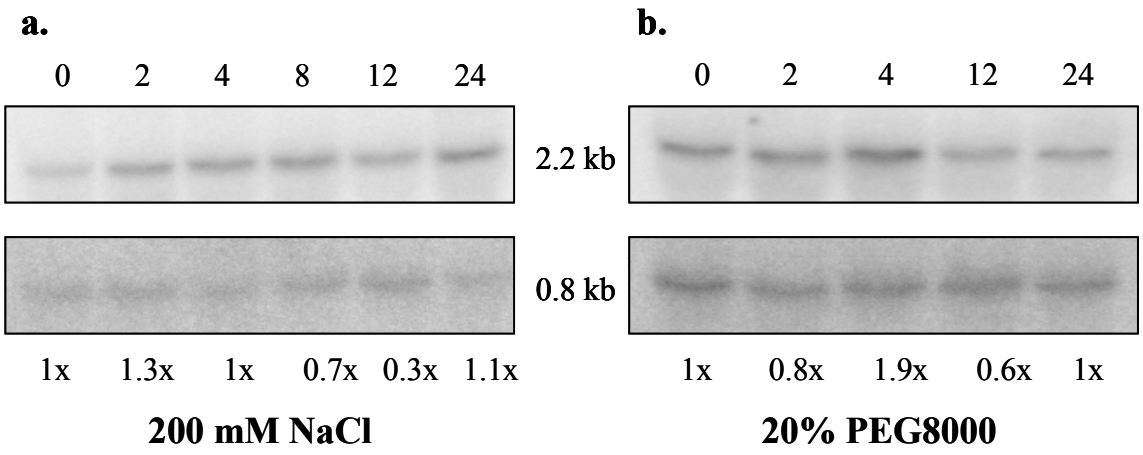


Figure 3.10. Expression of *At-RLK3* mRNA in 10 day old seedlings during different osmotic stress conditions. *A. thaliana* seedlings were treated with (a) 200 mM NaCl and (b) 20% PEG8000. The time of each harvesting is indicated at the top of each panel. The top panel of each treatment represents total RNA probed with the labeled *At-RLK3* probe, while the bottom panel indicates hybridization with the actin control probe. The sizes of the hybridizing fragments are as indicated. Induction levels are indicated at the bottom of each panel.

In addition, the expression levels of *At-RLK3* were tested when the cell suspension cultures were treated with ABA. ABA is known to accumulate upon water-deficit conditions where it plays an important role in the induced expression of other osmotic responsive genes (Bray, 1997). *At-RLK3* expression remained unaffected by exogenously applied ABA (Fig. 3.11). This was despite the fact that several ABA responsive *cis*-acting elements were present in the promoter region (Table 3.1). It is however possible that even though ABA did not activate *At-RLK3* expression on its own, ABA levels might rise due to the occurrence of dehydration stress. ABA could then in concert with dehydration responsive transcription factors, activate the expression of *At-RLK3* as seen in figure 3.9, as well as the other genes responsible for osmotic stress tolerance.

Since ABA is a plant hormone, several other hormones were also tested for their involvement in the expression of *At-RLK3* (Fig. 3.11). Plant hormones are essential for the normal development of plants, and should At-RLK3 play a role in plant development, its expression should be responsive to the presence of the different hormones. Of five hormones tested, only gibberellic acid managed to induce slightly higher *At-RLK3* expression levels. This increase was maintained for up to 24 h. Since the other hormones did not affect the expression of the gene, this might be an indication that At-RLK3 does not play a major role in the hormonally directed development of the plant.

Together with proline (Fig. 3.9), three other amino acids were also tested for their ability to regulate *At-RLK3* expression (Fig. 3.12). It was striking that two, methionine and valine, were responsible for repression of *At-RLK3* gene expression. In the case of methionine, the repression lasted for 24 h, while the transient repression effect for valine lasted for only 4 h after which expression levels recovered. In contrast, both proline and glutamic acid induced expression, with the former being more pronounced.

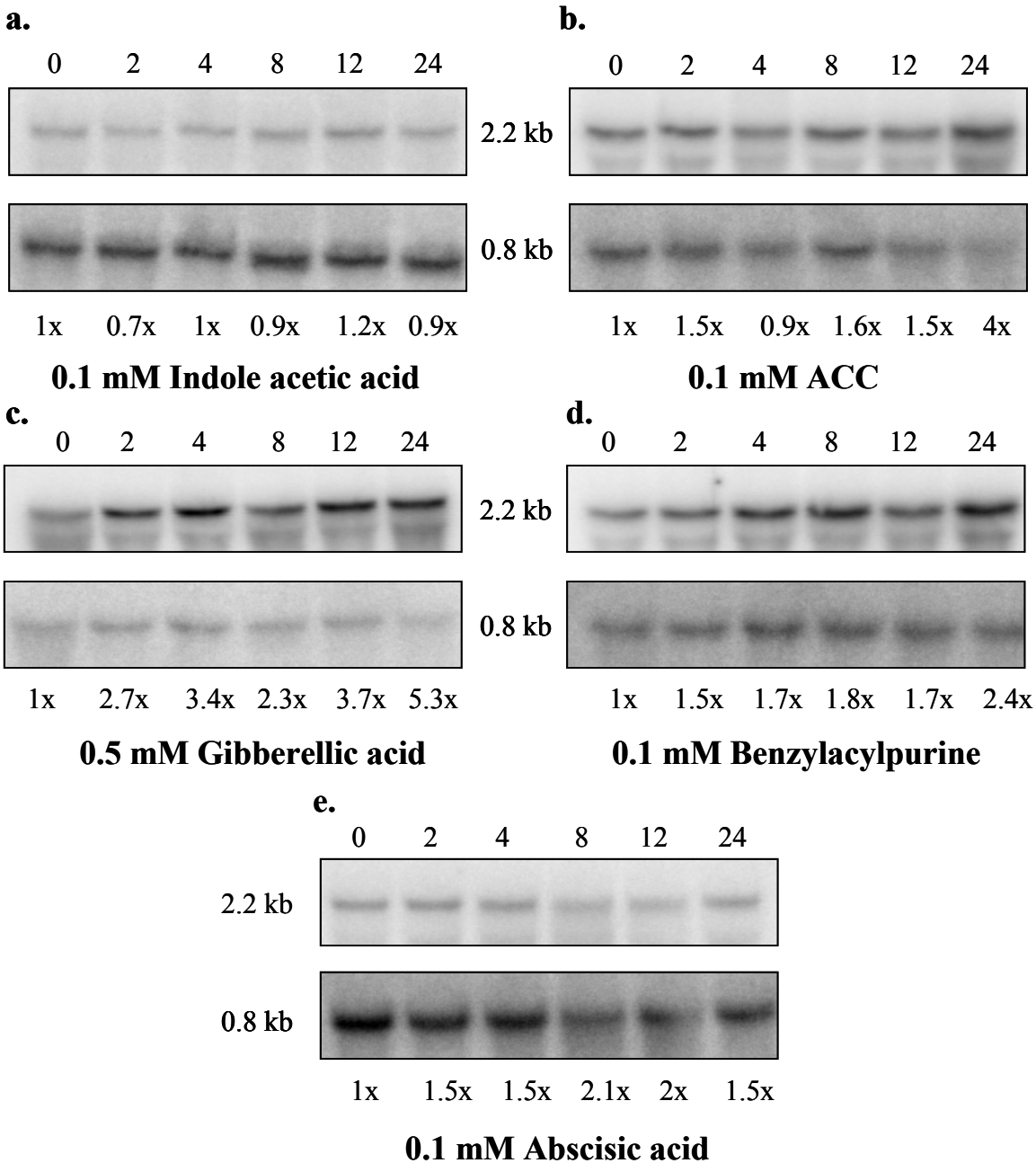


Figure 3.11. Expression of *At-RLK3* mRNA in cell suspension cultures after treatment with various plant hormones. *A. thaliana* cell suspension cultures were treated with (a) 0.1 mM IAA, (b) 0.1 mM ACC, (c) 0.1 mM GA, (d) 0.1 mM BAP and (e) 0.1 mM ABA. The time of each harvesting is indicated at the top of each panel. The top panel of each treatment represents total RNA probed with the labeled *At-RLK3* probe, while the bottom panel indicates hybridization with the actin control probe. The sizes of the hybridizing fragments are as indicated. Induction levels are indicated at the bottom of each panel.

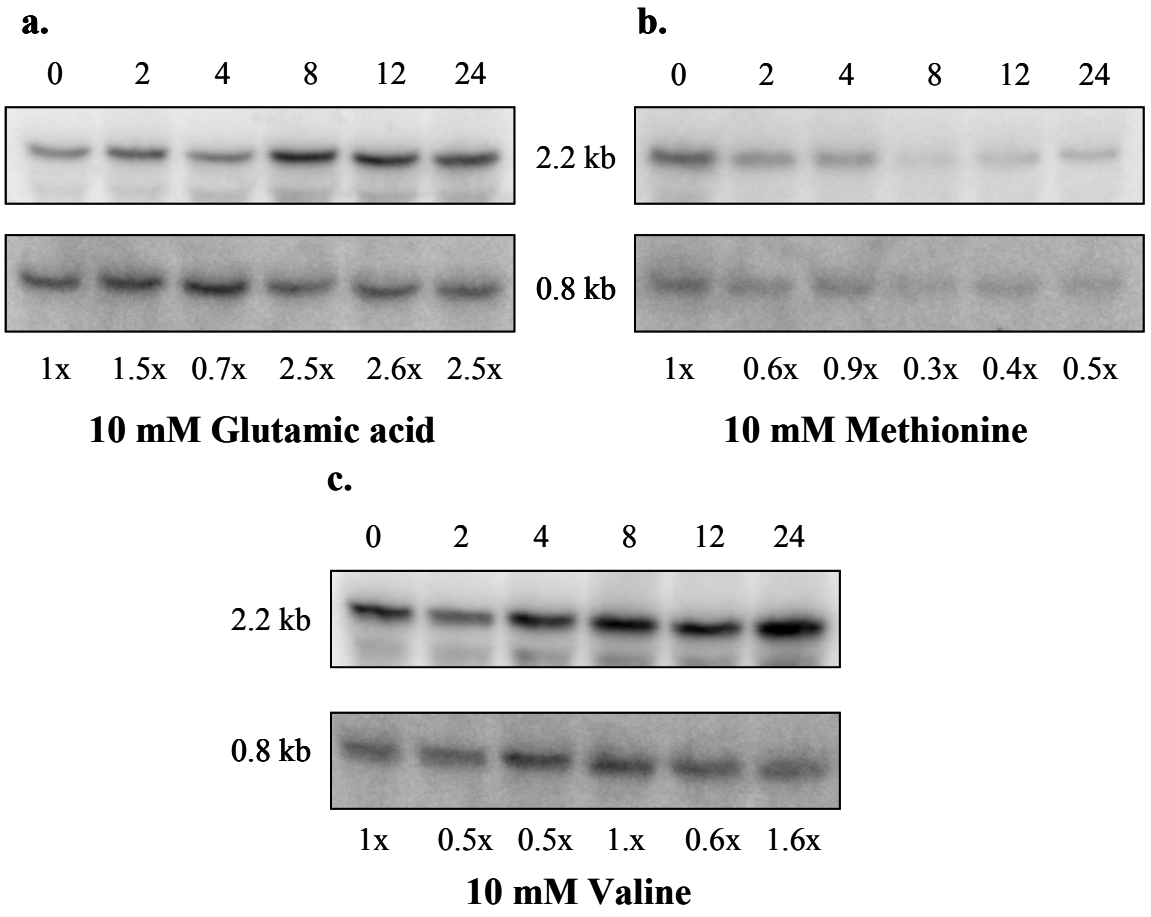


Figure 3.12. Expression of *At-RLK3* mRNA in cell suspension cultures treated with different amino acids. *A. thaliana* cell suspension cultures were treated with (a) 10 mM glutamic acid, (b) 10 mM methionine and (c) 10 mM valine. The time of each harvesting is indicated at the top of each panel. The top panel of each treatment represents total RNA probed with the labeled *At-RLK3* probe, while the bottom panel indicates hybridization with the actin control probe. The sizes of the hybridizing fragments are as indicated. Induction levels are indicated at the bottom of each panel.

The response of *At-RLK3* expression to two different heavy metals was tested (Fig. 3.13). In both cases, the expression levels were basically unaffected with only slightly higher levels being detected during the later stages of the copper treatment. Since no *cis*-acting elements giving responsiveness to heavy metals were detected in the promoter region, it was assumed that At-RLK3 plays no role in response to heavy metal contamination.

Two very interesting regions present on the *At-RLK3* promoter region prompted the next set of experiments. The two elements, WBBOXPCWRKY1 and WBOXATNPR1, are binding sites for WKRY transcription factors that are responsive to elicitors and SA respectively (Du and Chen, 2000; Ohtake *et al.*, 2000). This is clearly indicative of a function during the plant defense reaction. This involvement of At-RLK3 with the plant defense reaction was tested on three levels.

The first was to test the response of *At-RLK3* expression to the presence of two signal molecules, namely salicylic acid and jasmonic acid (Fig. 3.14). The former acts as a signal molecule in the defense reaction of the plant (Dempsey *et al.*, 1999), while jasmonic acid plays a key role in defense against insects and fungi, as well as wounding (Turner *et al.*, 2002). Salicylic acid application led to increased expression of *At-RLK3* with the maximum value reached at 4 h. Jasmonic acid application on the other hand led to decreased *At-RLK3* expression levels with a recovery at 24 h. It therefore seems as if the two signal molecules have opposite effects on the expression of *At-RLK3*, but both clearly play a role in regulation of the gene.

The role of At-RLK3 during the plant defense reaction was further analyzed when cell suspension cultures were exposed to oxidative stress conditions in the form of H_2O_2 and menadione. The production and accumulation of H_2O_2 is one of the earliest reactions that take place in the plant when subjected to pathogenic attack (Bolwell, 1999). Oxidative stress had a profound influence on the expression of *At-RLK3*, both for the rapid induction of the gene and for the very high levels of induction that was found (Fig. 3.15). H_2O_2 induced *At-RLK3* expression 13 fold within 2 h while a 17 fold induced expression for menadione was only obtained 4 h after treatment. In both cases, the sharply induced expression was transient and the expression levels soon returned to normal.

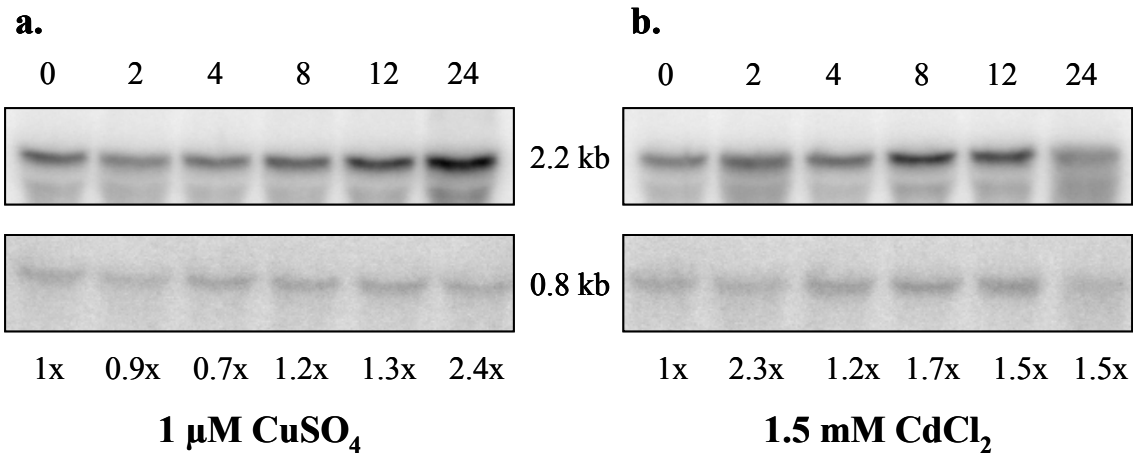


Figure 3.13. Expression of *At-RLK3* mRNA in cell suspension cultures after exposure to different metals. *A. thaliana* cell suspension cultures were treated with (a) 1 µM $CuSO_4$ and (b) 1.5 mM $CdCl_2$. The time of each harvesting is indicated at the top of each panel. The top panel of each treatment represents total RNA probed with the labeled *At-RLK3* probe, while the bottom panel indicates hybridization with the actin control probe. The sizes of the hybridizing fragments are as indicated. Induction levels are indicated at the bottom of each panel.

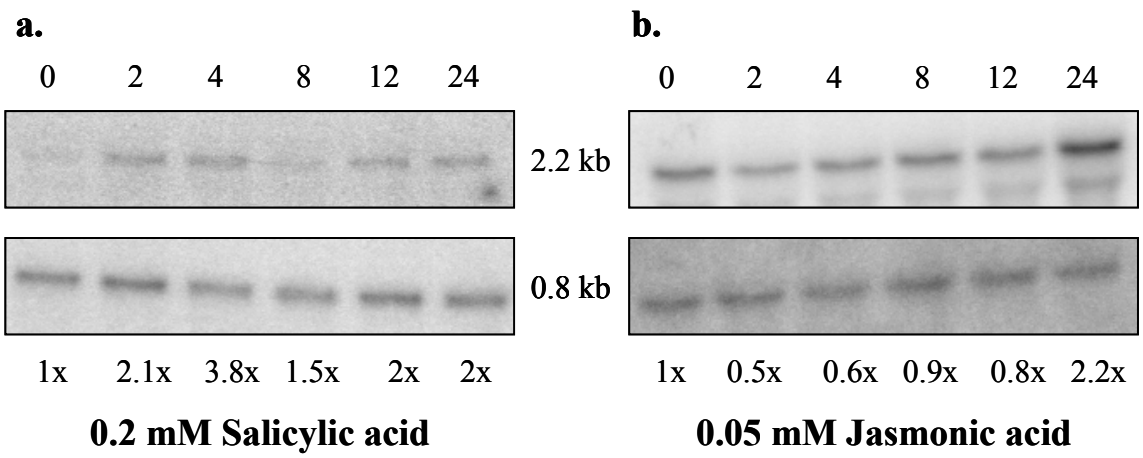


Figure 3.14. Expression of *At-RLK3* mRNA in cell suspension cultures after exposure to different plant signaling molecules. *A. thaliana* cell suspension cultures were treated with (a) 0.2 mM SA and (b) 0.05 mM JA. The time of each harvesting is indicated at the top of each panel. The top panel of each treatment represents total RNA probed with the labeled *At-RLK3* probe, while the bottom panel indicates hybridization with the actin control probe. The sizes of the hybridizing fragments are as indicated. Induction levels are indicated at the bottom of each panel.

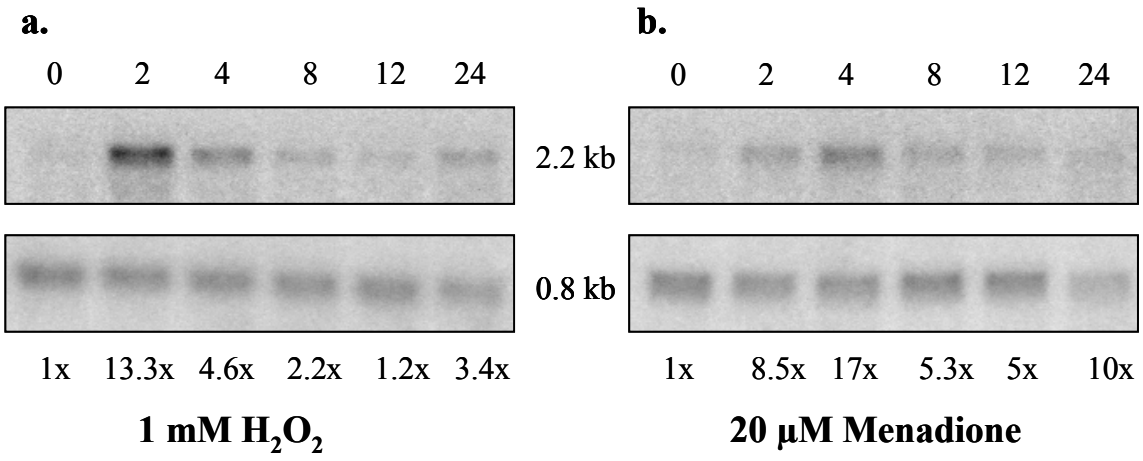


Figure 3.15. Expression of *At-RLK3* mRNA during different oxidative stress conditions. *A. thaliana* cell suspension cultures were treated with (a) 1 mM H_2O_2 and (b) 20 µM menadione. The time of each harvesting is indicated at the top of each panel. The top panel of each treatment represents total RNA probed with the labeled *At-RLK3* probe, while the bottom panel indicates hybridization with the actin control probe. The sizes of the hybridizing fragments are as indicated. Induction levels are indicated at the bottom of each panel.

This clearly indicated that At-RLK3 plays a key role in the response of the plant to oxidative stress conditions. Since oxidative stress is part of the plant defense reaction, these results acted as a second piece of evidence that the protein could play a role in the defense of the plant. *At-RLK3* expression was thus followed when the plant was challenged with different bacterial pathogens (Fig. 3.16). When the resistant Nd-1 ecotype of *A. thaliana* was challenged with the virulent GMI3000 *Ralstonia* strain, *At-RLK3* expression was induced within 6 h and reached a maximum expression level at 12 h. In contrast, the mutant strain where the *hrp*-gene cluster was removed yielded a weak and delayed induction of expression. In the susceptible Col-5 ecotype, the levels of induction were very low for both *Ralstonia* strains, indicating that the inoculation procedure could have led to the increased transcription levels.

In the Col-0 ecotype, all three *Pseudomonas* strains induced expression of *At-RLK3* within 2 h of the inoculation. This was true for both the compatible and incompatible interactions, which is different from the *Ralstonia* treatments. The reason for this discrepancy might be located in the nature of the respective pathogens, where resistance to *Ralstonia* is conferred by a single recessive locus (Deslandes *et al.*, 1998) whereas the interaction with *Pseudomonas* relies on the classical gene-for-gene interaction.

Finally, the response of *At-RLK3* expression was also tested under two environmental conditions (Fig. 3.17). Elevated temperatures repressed expression during the first 4 h, after which the expression pattern returned to normal. This was despite the presence of 3 CCAAT motifs that together with a heat shock element (HSE), leads to the upregulation of genes involved in the heat shock response (Rieping and Schoffl, 1992). The fact that a HSE was not present on the *At-RLK3* promoter region most probably caused the decrease in expression, since it was proposed that the CCAAT motif and the HSE acted cooperatively in the induced expression of genes containing both elements. In the last instance, the influence of anoxia on expression was also tested. Even though only three time points were taken, it is clear that *At-RLK3* expression was only slightly affected by anoxic conditions.

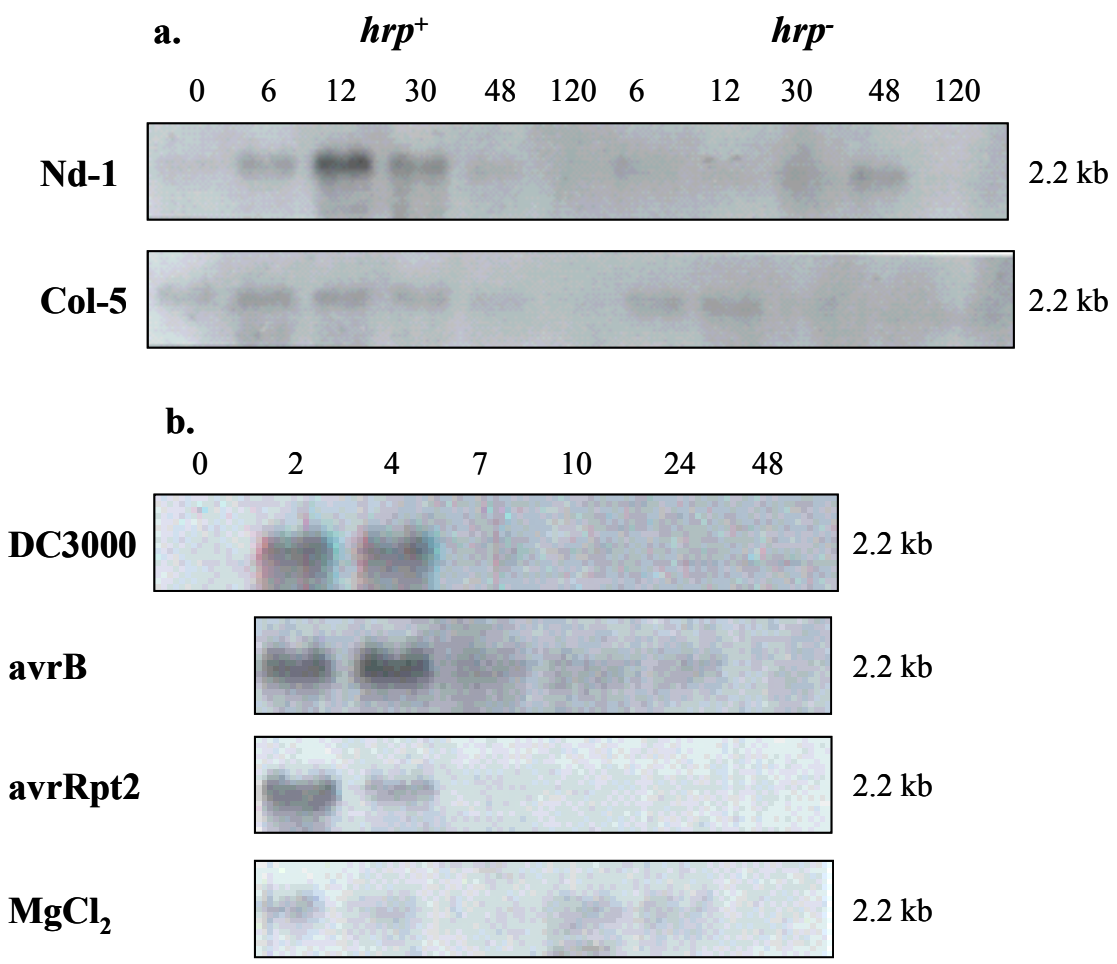


Figure 3.16. Expression of *At-RLK3* mRNA in 10 day old seedlings vacuum infiltrated with different bacterial pathogens. (a) *A. thaliana* Nd^{-1} and Col-5 ecotype seedlings were vacuum infiltrated with wild type *Ralstonia* GMI1000 hrp^+ and mutant hrp^- strains. (b) *A. thaliana* Col-0 ecotype seedlings were vacuum infiltrated with the wild type *Pseudomonas* DC3000 strain expressing or not expressing the *avrB* and *avrRpt2* avirulence gene respectively. As a control, 10 mM $MgCl_2$ was also infiltrated. The time of each harvesting is indicated at the top of each panel. Each panel represents total RNA probed with the labeled *At-RLK3* probe. The sizes of the hybridizing fragments are as indicated.

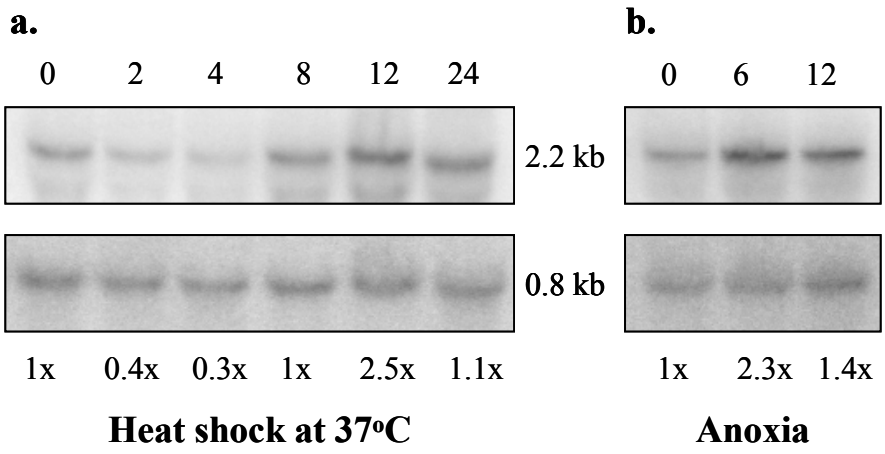


Figure 3.17. Expression of *At-RLK3* mRNA in cell suspension cultures and in 10 day old seedlings during various environmental conditions. *A. thaliana* cell suspension cultures were treated with (a) heat shock at 37°C and (b) seedlings subjected to anoxic conditions. The time of each harvesting is indicated at the top of each panel. The top panel of each treatment represents total RNA probed with the labeled *At-RLK3* probe, while the bottom panel indicates hybridization with the actin control probe. The sizes of the hybridizing fragments are as indicated. Induction levels are indicated at the bottom of each panel.

From the Northern blots, it was clear that several factors influenced the expression of *At-RLK3*. Included in this group was any form of oxidative stress, salicylic acid, dehydration stress (excluding the effect of NaCl) and pathogenic challenges. Off all four factors mentioned, it was only the former whose influence on expression was not supported by analysis of the promoter region (Table 3.1). Two factors, ABA and heat stress, did however not have the expected effect on *At-RLK3* expression. Despite having several *cis*-acting elements present on the promoter region implicating a role for these factors in the expression of the gene, the effect was negligible.

It was therefore decided to re-analyze the first three factors at shorter time intervals to evaluate their effectiveness to induce the expression of *At-RLK3*. Should At-RLK3 act as key receptor protein to detect the presence of the factor, its own expression should increase shortly after treatment. The water control showed no effect on *At-RLK3* expression. The effect of oxidative stress conditions was tested using H_2O_2, menadione and ozone. The latter was shown to lead to a very sharp increase in ROS after exposure to ozone (Mudd, 1997).

When H_2O_2 was directly applied, *At-RLK3* expression increased within 30 min while menadione only induced expression after 1 hour (Fig. 3.18). Ozone exposure led to increased expression within 1 h with a maximal value reached at 2 h. The delay in the increase was most probably due to the plant having to produce H_2O_2 via plasma membrane bound enzymes. Salicylic acid and osmotic stress both led to increased expression within 1 h. Combined, all three treatments led to a very fast increase in expression with H_2O_2 being the fastest. This indicated that At-RLK3 plays a key role in the perception of these different signals. The central component of all these treatments is H_2O_2, making it a possible ligand that is bound by At-RLK3.

Of all the characterized RLKs thus far described, the expression of *SFR2* most closely resembled that of *At-RLK3*, with both defense related and developmental conditions inducing expression of the gene (Pastuglia *et al.*, 1997). Although being part of the S-class of RLKs, SA, pathogens and wounding induced its expression. Analysis of the *SFR2* promoter region revealed a SA-responsive element TCA-1, the elicitor-responsive element ELI box 1, the wound responsive elements PI WUN and WUN, a MeJA-responsive element, a HSE heat shock element, a G-box and TGA auxin-responsive element and a GARE gibberellin-responsive element.

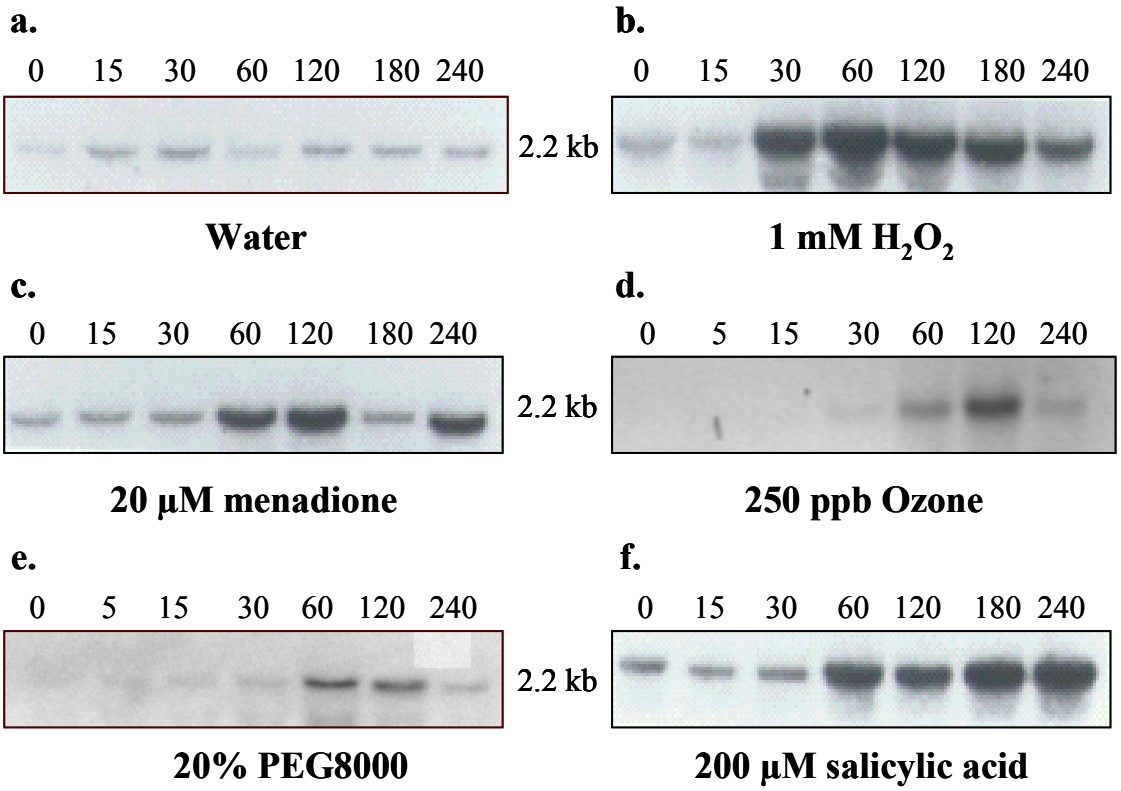


Figure 3.18. Expression of *At-RLK3* mRNA in cell suspension cultures during shorter time intervals. *A. thaliana* cell suspension cultures were treated with (a) water, (b) 1 mM H_2O_2, (c) 20 µM menadione, (d) 250 ppb ozone, (e) 20% (v/v) PEG8000 and (f) 200 µM salicylic acid. The time of each harvesting is indicated in min at the top of each panel. Each panel represents total RNA probed with the labeled *At-RLK3* probe. The sizes of the hybridizing fragments are as indicated.

The only common element between the two promoter regions is different SA-responsive elements, allowing SA induced expression for both genes. The induced expression of *SFR2* by *Ralstonia* and *E. coli* is therefore most probably via increased SA levels and the SA-responsive element (Pastuglia *et al.*, 1997). The expression patterns of the two genes are therefore very similar, but the ligand bound by the two different receptors is most likely different.

Another gene whose expression pattern resembles that of *At-RLK3*, is *RPK1* whose expression is primarily regulated by water stress (Hong *et al.*, 1997). In addition, ABA, salt and cold stress, all components of osmotic stress, also regulate *RPK1* expression. Even though the *RPK1* promoter region of the gene was not described, it must have several, if not all of the dehydration responsive regions, present on the *At-RLK3* promoter region.

Several plant RLKs showed multiple hybridizing mRNA transcripts when Northern blots were done using the full length genes as probe (Tobias *et al.*, 1992; Schaller and Bleecker, 1993; Wang *et al.*, 1996; Suzuki *et al.*, 1997; Kim *et al.*, 2000b). In the case of *ARK1*, it was found that the second mRNA species encoded only the extracellular domain of the receptor protein (Tobias *et al.*, 1992). The authors ascribed this to the presence of an in-frame stop codon present in the first intron of the gene which leads to alternative splicing of the transcript. Similar scenarios were implicated for *BcRK1* (Suzuki *et al.*, 1997) and *CHRK1* (Kim *et al.*, 2000b).

In order to determine whether a similar situation prevails in the case of *At-RLK3*, the nucleotide sequences of the first introns of a number of published RLKs were aligned (Fig. 3.19) using ClustalW (http://www.ebi.ac.uk/clustalw/). As can be seen, the first five nucleotides of the RLKs are identical with all five sharing a stop codon (TAG) in an identical position. This clearly indicated that all five genes should be expressed as multiple copies because of differential transcription.

All the Northern blots thus far presented (Fig. 3.9 – 3.18) were done using the portion of the *At-RLK3* gene encoding the kinase domain as probe. Should the gene be expressed as multiple copies with the second fragment consisting of only the extracellular domain nucleotide sequence, the kinase probe will not recognize a second mRNA fragment encoding the extracellular domain.

A control Northern blot was therefore done using as probe the gene portion encoding the extracellular domain of At-RLK3. Similar to the previous Northern blots, a single hybridizing fragment was found (results not shown). Therefore, in contrast to other published RLKs, *At-RLK3* is expressed as a single mRNA species despite having a stop codon present within the first intron. The reason for the absence of multiple transcripts could be that no poly-adenylation sequence with a conserved sequence of AATAAA was found within the first intron of *At-RLK3*. Thus, even though multiple transcripts could be found, the shorter transcript would be unstable due to the absence of a poly-A tail and will subsequently be broken down.

3.5 Summary

At-RLK3 is proposed to act as a putative receptor protein that spans the plasma membrane. It falls within the cysteine-rich group of RLKs since it has two C-X8-C-X2-C motifs that are implicated to be involved in the SA-responsive induction of gene expression. The gene is present as a single copy that is expressed as a single 2.2 kb transcript. Northern blot analysis revealed that At-RLK3 is most probably involved in the plant defense reactions during pathogen attack. However, a secondary role during osmotic stress was also implicated. Since the protein is predicted to function as a serine/threonine protein kinase, At-RLK3 is most probably able to initiate a signal transduction cascade in order to adapt to the different changing environments.

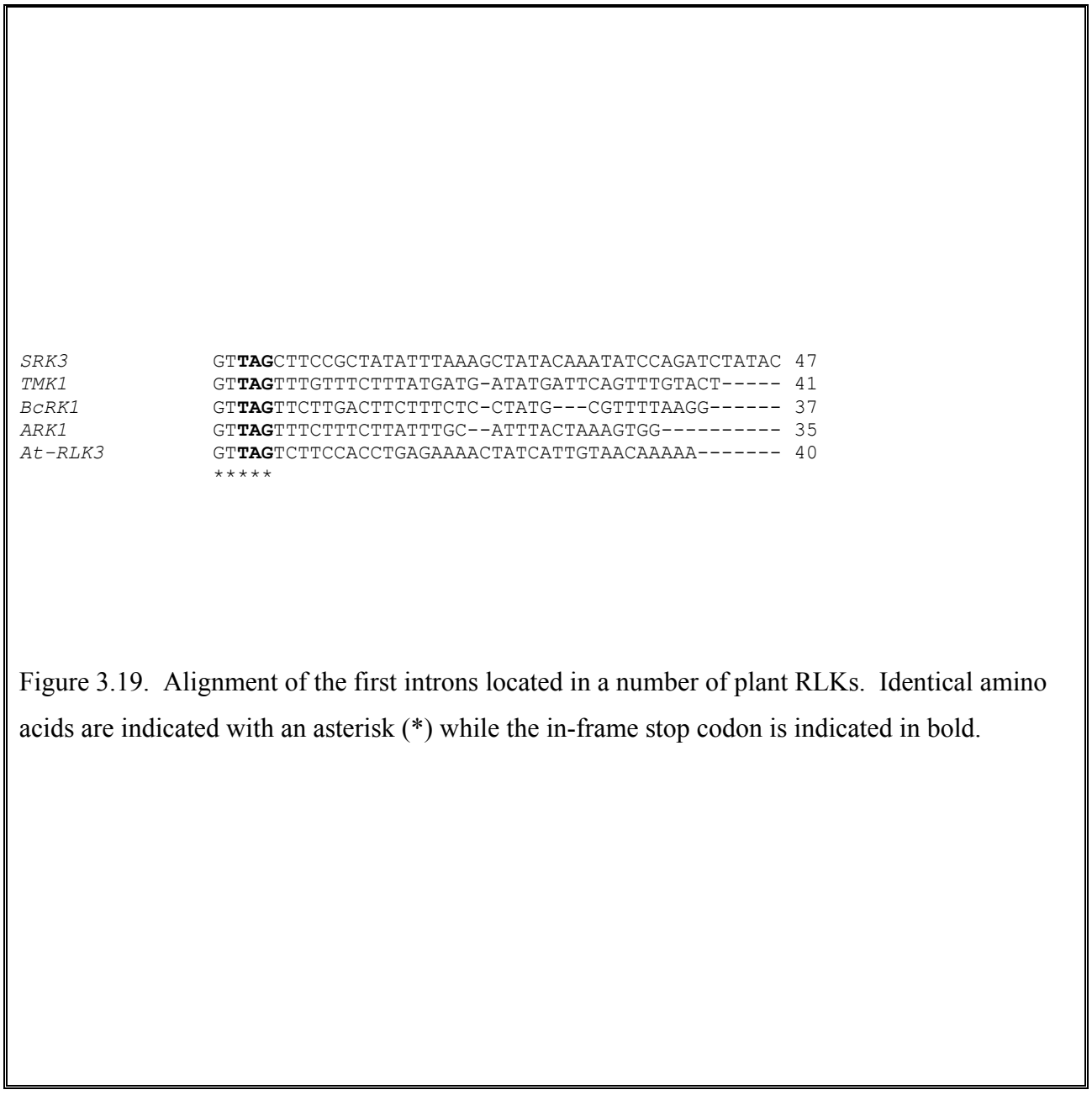


Figure 3.19. Alignment of the first introns located in a number of plant RLKs. Identical amino acids are indicated with an asterisk (*) while the in-frame stop codon is indicated in bold.

Chapter 4

Biochemical characterization of At-RLK3

Biochemical characterization of At-RLK3

4.1 Introduction

The transfer of signals from the outside of the cell to the inside requires receptor proteins that have special characteristics. Signals generated on the outside of the cell are in the form of ligands, molecules that are produced as a result of the change. To adapt, the plant cell must have the ability to recognize the changing environment by binding this ligand. Once bound, the activated protein must be able to transfer the signal to downstream proteins, thus allowing the cells to adapt. This specialized signal transduction event is performed by receptor proteins such as RLKs that have a number of unique characteristics that make them ideal receptors.

All RLKs thus far identified have a central hydrophobic region that is typical of a type 1a integral membrane protein, thus positioning the receptor right at the outer surface of the cell (Weinstein *et al*., 1982). This hydrophobic region is immediately followed by a number of basic amino acids, ensuring that the protein is efficiently incorporated into the plasma membrane. Linked to this is a 20 amino acid N-terminal signal peptide that is characteristic of proteins that are translocated to the ER for secretion to the plasma membrane (von Heijne, 1990). Even though this region is implicated in ER transport, several RLKs lack the signal peptide (Schmidt *et al*., 1997; Li and Wurtzel, 1998; Silva and Goring, 2002). Singer (1990), however, did propose that Type 1b integral membrane proteins lacking the characteristic signal peptide could still be directed to the plasma membrane via the usual ER translocator protein machinery.

Plasma membrane or microsomal fraction localization was shown for a number of RLKs (Chang *et al.*, 1992; Mu *et al.*, 1994; He *et al.*, 1996; Stein *et al.*, 1996; Ahmed *et al.*, 1997; Feuillet *et al.*, 1998; Muschietti *et al.*, 1998; Jinn *et al.*, 2000; Kim *et al.*, 2000b; Friedrichsen *et al.*, 2000; Nam and Li, 2002). Unique within this group is Wak1, an RLK that is both bound to the plasma membrane and associated with the cell wall (He *et al*., 1996). The structure of the protein therefore ensures that the RLK is located at the prime spot for efficient signal detection.

Binding of the ligand is presumed to be through either homo- or hetero-dimerization of the RLK (Wang *et al.*, 1998; Torii, 2000). This dimerization reaction then leads to

the phosphorylation of serine and threonine residues within the kinase domain of the RLK. This phosphorylation can be through either autophosphorylation (Wang *et al.*, 2001) or transphosphorylation as was proposed for instance for BRI1 (Li, 2003). CrRLK1 predominantly autophosphorylates, indicating that dimerization might in this case not be important (Schulze-Muth *et al.*, 1996). It is this phosphorylation event that characterizes activation of the RLK that eventually leads to initiation of the signal transduction cascade.

Plant RLKs all fall within the larger group of plant protein kinases that has 11 conserved sub-domains (Hanks and Quinn, 1991). Whereas mammalian RPKs are tyrosine specific, all plant RLKs exhibit serine/threonine specificity (Table 2.1). This difference is due to the amino acid differences of sub-domain VIb (DLKPEN vs. DLAARN) and VIII (G[T/S]XX[Y/F]IAPE vs. FPIKWMAPE) between plant and mammalian protein kinases. The single exception was shown to be PRK1 that showed dual serine and tyrosine kinase activity, which the authors attributed to a possible unique function for the protein in pollen (Mu *et al.*, 1994). It was however also suggested that the changed specificity could have been due to the high mutation rate in *E. coli* where the gene was expressed.

The kinase activity of several of the identified RLKs was proven (Table 2.1). The ratio of phosphorylation between serine and threonine residues differed among different RLKs, indicating the possibility that a single RLK can function in more than one signaling event by being differentially phosphorylated (Bassett *et al.*, 2000). A number of RLKs showed significantly higher kinase activity in the presence of Mn^{2+} as cofactor when compared to Mg^{2+} (Schaller and Bleecker, 1993; Horn and Walker, 1994; Schulze-Muth *et al.*, 1996; Takahashi *et al.*, 1998; Nishiguchi *et al.*, 2002).

4.2 Aim

After characterizing the expression of the *At-RLK3* gene in Chapter 3, it was imperative to study the protein itself to determine its basic characteristics. This chapter therefore concentrates on the biochemical characterization of At-RLK3 and its expression.

4.3 Materials and methods

4.3.1 Materials

4.3.1.1 Biological material

All DNA manipulations of *At-RLK3* were done using *E. coli* HB101 (*supE*44*hsdS*20($r^-_\beta m^-_\beta$) *recA*13*ara*-14*proA*2*lacY*1*galK*2*rpsL*20*xyl*-5 -1) as the host cell. Expression of the full-length *At-RLK3* cDNA, as well as the two subdomains, was done in yeast (*Saccharomyces cerevisiae* W303a [*MAT*a *ade2-1 leu2-3*,*112 ura3-1 his3-11*, *15 trp1-1 can1-100 ssd1-1*]) and bacteria (*E. coli* BL21(DE3) [F'*amp*T*hsdS*$_\beta$($r_\beta m_\beta$)*galdcm*(DE3)]). For the expression in yeast, the pYX plasmid vector system (Ingenius) was used, while the expression in *E. coli* was done using the pET-21d plasmid vector system (Novagen).

4.3.1.2 Other materials

All PCR amplification reactions were done using the Expand High-Fidelity PCR system (Roche). All DNA fragments were purified from agarose gels using the Geneclean system (Bio101). All modifying enzymes used were obtained from Roche. For the Southern blot analysis, Hybond N^+ nylon membrane (Amersham) was used. DNA probes were prepared using the *Redi*prime^{TM}II system (Amersham). The Wizard Plus DNA purification system was obtained from Promega. The recombinant polypeptide was extracted from *E. coli* using the B-Per protein extraction kit (Pierce). The recombinant polypeptide was purified using Ni-NTA Agarose (Qiagen). Dialysis was performed using the Snakeskin dialysis tubing from Pierce. Protein kinase C was obtained from Invitrogen. The Hi-Trap affinity column, goat anti-rabbit horseradish conjugated antibody and ECL detection system was obtained from Amersham-Pharmacia Biotech. All other chemicals used were of highest purity.

4.3.2 Methods

4.3.2.1 Expression of *At-RLK3* in yeast cells

For the expression of *At-RLK3* in yeast, the cDNA fragment was amplified directly from TL3 containing the truncated *At-RLK3* gene using Bovis 10 and 14 as primers (Appendix 1) (3.3.2.1.6.1). The two primers contained *Nco*1 and *Sal*1 restriction sites respectively to allow for the directional cloning of the amplified fragments into pYX111, 112 and 212. For each reaction, 1 ng TL3 plasmid was used as template DNA. To ensure that the amplified fragments contained as few mutations as possible, 2 U Expand High-Fidelity Taq DNA polymerase with proof-reading ability was used for the amplification. The cycling parameters were as described with the exception that an annealing temperature of 60^{o}C was used.

The success of the reaction was verified by separating a small aliquot of each reaction on a 1% (w/v) agarose gel (3.3.2.1.3). The rest of the reaction was separated on a 1% (w/v) preparative agarose gel and the amplification products purified from the gel using Geneclean according to the manufacturers' instructions.

After purification, 100 ng of the amplified DNA was digested with 10 U *Nco*1 and *Sal*1 in buffer H (3.3.2.1.4). One hundred nanograms of the respective yeast expression vectors, pYX111, 112 and 212, were digested similarly. To prevent self-ligation, the digested vectors were dephosphorylated with 1 U alkaline phosphatase in the presence of 50 mM Tris-HCl pH 8.5, 0.1 mM EDTA for 1 h at 37°C (Sambrook *et al*., 1989). The digested cDNA fragments and plasmids were separated on an agarose gel (3.3.2.1.3) and subsequently purified from the gel. For the ligation reaction, 50 ng of both the purified cDNA fragment and the respective plasmid were ligated using 1 U T4 DNA ligase in the presence of 66 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1 mM DTE and 1 mM ATP. The ligation was performed overnight at 4°C (Sambrook *et al.*, 1989).

E. coli HB101 was transformed with the ligation reactions using a method described by Inoue *et al.* (1990). Competent cells were prepared as follows. A few *E. coli* colonies were inoculated in SOB medium (2% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.05% (w/v) NaCl, 2.5 mM KCl and 10 mM $MgCl_2$) and grown at 18^{o}C to an

A_{600} of 0.6. The cells were harvested by centrifugation at 3000 *g* for 10 min at 4°C. The pellet was resuspended for 10 min in ice-cold transformation buffer (10 mM 1,4-piperazine diethanesulfonic acid [Pipes] pH 6.7, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl), re-centrifuged and then resuspended in transformation buffer. To this was added 7% (v/v) dimethyl sulfoxide (DMSO) after which the cells were quick-frozen in liquid nitrogen and stored at –80°C. For each transformation reaction, a 200 µl aliquot was thawed on ice, the DNA added and incubated on ice for 30 min. After a 30 sec 42°C heat shock, 1 ml SOB medium was added and the cells allowed to recover at 37°C for 1 h. The cells were collected through a brief centrifugation step, resuspended in a small amount of SOB and plated on LB plates (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 1.5% (w/v) agar) containing 50 $\mu g.ml^{-1}$ ampicillin.

Transformed colonies were screened for recombinant plasmids using PCR by adding a small amount of cells to a prepared PCR reaction (3.3.2.1.6.1). The products of the PCR reactions were separated on an agarose gel (3.3.2.1.3). Colonies containing recombinant plasmids were grown in LB-medium containing 50 $\mu g.ml^{-1}$ ampicillin after which plasmid DNA was prepared from the cells using the Wizard miniprep DNA purification system according to the manufacturer's instructions.

To confirm the identity of the inserts, 200 ng isolated plasmid DNA was digested with 10 U *Nco*1 and *Sal*1 in buffer H (3.3.2.1.4) and separated on an agarose gel (3.3.2.1.3). The digested plasmid DNA was transferred to a nylon membrane as described (3.3.2.1.5). The DNA fragment encoding the kinase domain of At-RLK3 was amplified from TL3 (3.3.2.1.6.1) using Bovis 11 and 13 (Appendix 1) as primers. Probe labeling (3.3.2.1.6.2) and hybridization (3.3.2.1.7) were performed as described.

Wild type *S. cerevisiae* W303 was transformed with recombinant pYX111, 112 and 212 plasmids containing the truncated *At-RLK3* gene (Agatep *et al*., 1998). As controls, non-recombinant plasmids were also transformed into the yeast cells. Transformed colonies were selected due to their ability to grow on SC-uracil plates (0.67% (w/v) yeast nitrogen base lacking amino acids, 2% (w/v) glucose, 1.5% (w/v)

agar pH 5.8, SC drop-out mix lacking uracil (http://www.umanitoba.ca/faculties/medicine/biochem/gietz/media.html)).

Yeast colonies containing the recombinant and empty pYX vectors respectively were inoculated into 10 ml SC-ura and grown for 3 days at 30^{o}C. After three days, 1 ml cells were transferred to an eppendorf tube and centrifuged at 12 000 *g* in a microcentrifuge. The pellets were resuspended in 100 µl sterile water and counted using a haemocytometer. Equal amounts of cells were transferred to eppendorf tubes, the cells pelleted and resuspended into protein loading buffer (65 mM Tris-HCl pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol, 50 mM dithiotreitol [DTT], 0.01% (w/v) BPB, 0.05 % (w/v) octylphenol decaethylene glycol ether [Triton X-100], 2 mM phenylmethylsulfonyl fluoride [PMSF]) and boiled for 5 min. In a separate experiment, the cells were first broken with glass beads on a Vortex mixer before the loading buffer was added.

Equal volumes of the protein were separated on a SDS poly-acrylamide gel electrophoresis (SDS-PAGE) gel according to Laemmli (1970). The gel system had a 12% (w/v) separating gel (12% (w/v) acrylamide {30 acrylamide : 0.8 bis-acrylamide ratio}, 0.35 M Tris-HCl pH 8.8, 0.1% (w/v) SDS, 0.04% (w/v) ammonium peroxydisulfate [APS], 0.66 µl N, N, N', N'-tetramethylethylendiamine [Temed].ml^{-1} gel) and 6% (w/v) stacking gel (6% (w/v) acrylamide {30% acrylamide : 0.8% bis-acrylamide ratio}, 0.125 M Tris-HCl pH 6.8, 0.1% (w/v) SDS, 0.04% (w/v) APS, 0.66 µl Temed.ml^{-1} gel). The polypeptides were separated on the gel using the following buffer: 0.1 M Tris-HCl pH 8.3, 0.15 M glycine, 0.1% (w/v) SDS. After separation, the gels were stained with warm 0.2% (w/v) Coomassie Blue R250, 0.1% (w/v) Coomassie Blue G250, 7% (v/v) acetic acid, 50% (v/v) methanol for 15 min (Fish and Jagendorf, 1982). The gels were destained overnight in 43% (v/v) methanol, 7% (v/v) acetic acid and the resulting polypeptides photographed.

In an attempt to isolate larger proteins from the yeast cells, equal numbers of yeast cells were broken with glass beads in an extraction buffer (50 mM Tris-HCl pH 8.0, 0.05 % (w/v) Triton X-100, 2 mM PMSF) by vortexing the tubes vigorously. An equal volume of the supernatant was separated on a SDS-PAGE gel.

4.3.2.2 Expression of *At-RLK3* in *E. coli*

4.3.2.2.1 Cloning of *At-RLK3* domains into pET21d

For the expression of *At-RLK3* in *E. coli*, the pET21d plasmid expression vector was used. By including an in-frame *Nco*1 and an *Eco*R1 palindrome as part of the up- and downstream primers respectively, the amplified fragments was cloned directionally into the plasmid vector. The ATG that forms part of the *Nco*1 palindrome acted as the start codon of the peptide, whilst on the 3' end, the plasmid provided an in-frame stop codon. Included on the expressed polypeptide were six histidine residues that formed part of the carboxyl part of the polypeptide that allowed the purification of the polypeptide using Nickel-chelated agarose columns.

Two different constructs were generated in pET21d. The first contained the full-length cDNA fragment amplified using Bovis 10 and 11, while the second contained the cDNA sequences encoding the kinase domain that was amplified using Bovis 13 and 11 (Appendix 1). The PCR conditions used were as described (3.3.2.1.6.1). To verify the success of the amplification, a small aliquot of each reaction was separated on a 1% (w/v) agarose gel (3.3.2.1.3). The remainder of the reactions was separated on a 1% (w/v) preparative agarose gel and the amplified fragments purified (4.3.2.1).

For the construction of the recombinant plasmids, 100 ng of each of the amplified fragments and 200 ng pET21d plasmid DNA respectively were digested with 10 U *Nco*1 and *Eco*R1 (3.3.2.1.4). The digested plasmid was then dephosphorylated (4.3.2.1). Fifty nanogram of each plasmid and amplified fragment combination was ligated overnight and competent *E. coli* HB101 cells transformed with it (4.3.2.1). Transformed *E. coli* colonies were selected on LB-medium containing 50 $\mu g.ml^{-1}$ ampicillin. Recombinant plasmids were identified using both PCR with the respective primers (3.3.2.1.6.1) and digestion of prepared plasmid DNA (4.3.2.1). One colony for each recombinant plasmid was inoculated into 250 ml LB medium containing 50 $\mu g.ml^{-1}$ ampicillin. The cultures were grown overnight at 37°C and the plasmid DNA extracted using the Midi-prep isolation kit from Qiagen. The inserts were sequenced to determine the correctness of the nucleotide sequence of the inserts. Competent *E. coli* BL21 (DE3) cells were transformed with the recombinant plasmid and transformants selected on LB medium containing 50 $\mu g.ml^{-1}$ ampicillin (4.3.2.1).

4.3.2.2.2 Expression and purification of *At-RLK3* from *E. coli*

To verify the expression of the inserts, *E. coli* BL21 (DE3) cultures containing the different recombinant and non-recombinant constructs, were inoculated separately into 5 ml LB medium containing 50 µg.ml^{-1} ampicillin. The cultures were grown overnight at 37°C and then diluted 1 in 25 into fresh medium. The cells were grown until an A_{600} of 0.6 was reached when expression was induced with the addition of 1 mM isopropylthio-ß-D-galactoside (IPTG). After 3 h, 1 ml of the respective cultures was transferred to eppendorf tubes, centrifuged at top speed, the pellet resuspended in 25 µl SDS loading buffer, boiled for 5 min, where after 20 µl of each sample was separated on a 12% (w/v) acrylamide gel and the gel stained with Coomassie (4.3.2.1).

Localization of the recombinant protein in *E. coli* was determined using the B-Per protein extraction kit. Bacterial cells were recovered from 1.5 ml of the induced culture by centrifuging the cells at 12 000 *g*. The pellet was resuspended in the B-per solution, vortexed for 1 min, centrifuged and the supernatant containing the soluble protein transferred to a new tube. The pellet was resuspended in a 1 to 10 dilution of B-per solution containing 0.2 mg.ml^{-1} lysozyme. The tube was vortexed for 1 min, centrifuged and the pellet resuspended in the diluted B-per solution. This was repeated another two times before the inclusion body pellet was resuspended in 20 mM Tris-HCl pH 8, 1 mM EDTA, 2 mM PMSF. Equal volumes of both the soluble and inclusion body protein were mixed with protein loading buffer and separated on a SDS-PAGE gel (4.3.2.1). Inclusion bodies containing the recombinant protein were purified from a 250 ml induced culture using the B-per solution as described. To enhance the isolation of the inclusion bodies, the lysozyme incubation step was increased to 5 min. Finally, the inclusion bodies were frozen and stored at –20°C until used.

The recombinant protein was simultaneously purified and refolded on a 5 ml Ni-chelated agarose column according to Rogl *et al*. (1998). Twenty-five milligram recombinant protein was centrifuged at 12000 *g* for 10 min at 4°C, washed once with 40 ml 100 mM Tris-HCl pH 8.0 and finally resuspended in 5 ml of the same buffer.

The inclusion bodies were solubilised in 3.2 M urea, 0.6% (w/v) sodium *N*-lauroyl sarcosinate, 90 mM NaCl, 20 mM Tris-HCl pH 8, 4.5% (v/v) glycerol. A 3 ml nickel-chelated agarose column was prepared by transferring the gel solution into a 5 ml plastic syringe. After allowing the agarose to settle, the column was equilibrated with 20 mM Tris-HCl pH 8, 0.2 M NaCl, 10% (w/v) glycerol, 0.1% (w/v) sodium *N*-lauroyl sarcosinate for several h at room temperature.

The solubilized inclusion bodies were passed twice through the column and the column was washed with 100 ml of the same buffer where 0.1% (v/v) Triton X-100 replaced the sarcosinate. The column was washed with the same buffer containing 10 mM imidazole to remove weakly bound protein contaminants. Finally, the refolded recombinant protein was eluted from the column in one step using the same buffer containing 0.3 M imidazole. Individual fractions of 0.5 ml each were collected and 5 µl of each fraction analyzed on SDS-PAGE gels (4.3.2.1). The fractions containing the peptide were pooled and dialyzed overnight against 2.85 mM Tris-HCl pH 8.0, 28 mM NaCl, 1.43% (w/v) glycerol, 0.014% (v/v) Triton X-100 in Snakeskin dialysis tubing to remove the imidazole. The protein was concentrated approximately seven-fold with solid PEG35000 to end up with a final buffer composition of roughly 20 mM Tris-HCl pH 8.0, 200 mM NaCl, 10% (w/v) glycerol, 0.1% (v/v) Triton X-100. The concentration of the purified polypeptide was determined as described by Bradford (1976) using 0.5 $mg.ml^{-1}$ gamma globulin as a standard. The concentration was expressed as $mg.ml^{-1}$ protein. After the concentration was determined, the polypeptide was stored at -20^{o}C.

4.3.2.3 *In vitro* characterization of At-RLK3

4.3.2.3.1 Autophosphorylation activity of At-RLK3

The ability of the purified recombinant kinase to transfer the gamma phosphate of ATP onto itself was determined (Mu *et al.*, 1994). Two µg of the purified polypeptide was incubated in the presence of 30 mM Tris-HCl pH 7.4, 20 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 10 mM $MgCl_2$, 2 mM $MnCl_2$, 10 µM ATP, 50 µCi [γ-^{32}P]-ATP at 30^{o}C for 30 min. As a control, 2 µg total *E. coli* proteins were treated similarly. After labeling, the reaction was stopped by adding 1% (v/v) octylphenolpoly (ethyleneglycolether) (Nonidet P40). The labeled

proteins were precipitated by adding 9 volumes acetone and overnight incubation at –20°C. The protein was recovered by centrifugation at 12 000 *g* at 4°C in a microcentrifuge, washed once with ice-cold 80% (v/v) acetone, dried, dissolved in SDS loading buffer and resolved on a 12% (w/v) SDS-PAGE gel (4.3.2.1). The polypeptides were transferred to a PVDF membrane using a Biorad Mini Trans-blot Electrophoretic Transfer system according to the manufacturer's instructions. The transfer buffer consisted of 25 mM Tris-HCl pH 8.3, 192 mM glycine, 20% (v/v) methanol. After transfer, the membrane was washed four times for 30 min each with 5% (w/v) trichloroacetic acid (TCA), 1% (w/v) sodium pyrophosphate to remove all unincorporated radio-active nucleotides. The membrane was then exposed to an X-ray film.

At-RLK3 was immunoprecipitated from 100 µg total *A. thaliana* protein using different quantities of the antibodies raised against the purified kinase domain and the extracellular domain respectively (4.3.2.4). The protein and antibodies were incubated overnight at 4^{o}C in the presence of 0.4 mg.ml^{-1} BSA, 0.5 mM PMSF and 0.15 M NaCl. The antibody-At-RLK3 complex was precipitated from the solution using 50 µl Protein-A sepharose for 60 min at 4^{o}C and then centrifuged at 10 000 *g* for 5 min. The pellet was washed three times with 20 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.1 M NaCl, 1% (v/v) Triton X-100, once with the same buffer containing 1 M NaCl and once with the kinase buffer (20 mM HEPES pH 7.5, 15 mM $MgCl_2$, 5 mM ethylene glycolbis (aminoethylether)tetraacetic acid [EGTA], 1 mM DTT). The At-RLK3 protein was labeled radio-active in the same kinase buffer containing 2 µCi [γ-^{32}P]-ATP, 0.5 mg.ml^{-1} myelin basic protein (MBP), 0.1 mM ATP for 30 min at room temperature. After centrifugation at 3000 *g* for 2 min, the protein-antibody-Protein-A sepharose complex was resuspended in loading buffer to a final concentration of 65 mM Tris-HCl pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol, 50 mM DTT, 0.01% (w/v) BPB, 2 mM PMSF, boiled for 5 min, centrifuged and the supernatant separated on a 12% (v/v) acrylamide gel (4.3.2.1). The gel was washed with 5% (w/v) TCA, 1% (w/v) sodium pyrophosphate. This washing was continued until no isotope could be detected in the washing solution using a Geiger counter. The gel was then dried and exposed to a phospho-imaging screen.

To determine which co-factor At-RLK3 preferentially use, 10 µg of the purified kinase polypeptide was labeled with 20 µCi [γ-^{32}P]-ATP in the presence of either 10 mM $MgCl_2$ or 10 mM $MnCl_2$ in a kinase buffer (50 mM Tris-HCl pH 7.1, 1 mM DTT, 0.1% (v/v) Triton X-100, 20 µM ATP) as described (Schaller and Bleecker, 1993). After incubation for 40 min at 30^{o}C, the labeled polypeptides were treated as described above.

4.3.2.3.2 Amino acid phosphorylation specificity

Two microgram of the purified polypeptide was labeled in the presence of 70 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 5 µCi [γ-^{32}P]-ATP and 0.15 U Protein Kinase C at 30°C for 2 h. The labeled polypeptide was precipitated, washed once with 80% (v/v) acetone and dried (4.3.2.3.1). Amino acid analysis was done using a modified method as described by Boyle *et al.* (1991). The dried polypeptide was dissolved in 6 N HCl and placed at 110°C for 1 h. The polypeptide was lyophilized overnight under vacuum in a Speed-Vac and the dried pellet dissolved in 5 µl water. As a control, 2 µl of a 2 mg.ml^{-1} unlabelled phosphoserine, -threonine and –tyrosine mixture was added to the labeled polypeptide. The hydrolyzed amino acids were separated on thin-layer cellulose plates in one dimension according to Schulze-Muth *et al*. (1996). The separating buffer was 37.5% (v/v) butanol, 25% (v/v) pyridine, 7.5% (v/v) acetic acid. The standards were visualized by spraying the plates with 0.2% (w/v) ninhydrin in ethanol. The standards were marked and the plate exposed to an X-ray film.

4.3.2.4 Preparation of antibodies

Two antibodies were prepared. The first antibody was raised against the purified kinase domain of At-RLK3 when 100 µg of the purified polypeptide (4.3.2.2.2) was dissolved in 500 µl 20 mM Tris-HCl pH 8.0, 200 mM NaCl, 10% (v/v) glycerol. An equal volume Freunds complete adjuvant was added to the polypeptide, the solution thoroughly mixed and injected subcutaneously into a New Zealand White rabbit. Three rabbits were immunized after 5 ml blood was collected as the pre-immune serum controls. Two booster injections of 100 µg of the purified polypeptide resuspended in Freunds incomplete adjuvant were administered with 4 week intervals.

Ten days after the final booster injection, the total blood volume of the rabbits was collected through exsanguination.

The second antibody was raised against a unique amino acid sequence located on the extracellular domain of At-RLK3. Using a software program, the extracellular region of At-RLK3 was searched for a peptide sequence that showed both high antigenic and surface probability, as well as good hydrophilicity characteristics. Two such regions were identified, namely AKSTPSSSDNHY (amino acids 173 – 184) and PAGDQDNPTNND (amino acids 272 – 283) (Fig. 3.2). The amino acid sequence of both regions was aligned with those included in the Swissprot and Genbank gene databases. The first peptide sequence showed homology with three amino acid sequences present in another protein from *A. thaliana*, while the second did not share any significant homology with any other plant proteins. The latter was therefore used for peptide synthesis. To ensure that the peptide remained immuno-reactive, it was linked to the keyhole limpet hemocyanin (KLH) carrier protein. Three rabbits were immunized with the peptide as described, with the exception that 1 mg of the peptide was used for each immunization.

After the blood was collected, it was allowed to clot overnight at 4^{o}C, the clot removed and the serum cleared by centrifugation at 10000 *g* for 10 min. Immunoglobulins (IgG) were collected from the serum using ammonium sulfate precipitation (Harlow and Lane, 1988). Contaminating serum protein was first precipitated using 30% (w/v) ammonium sulfate by incubating it overnight at 4^{o}C. After centrifuging the solution at 3000 *g* for 30 min, total IgG was precipitated from the cleared serum using 50% (w/v) ammonium sulphate at 4^{o}C overnight. After centrifugation, the precipitated immunoglobulins were dissolved in PBS (50 mM potassium phosphate pH 7.2, 150 mM NaCl), dialyzed overnight against three changes of PBS at 4^{o}C and the concentration determined (4.3.2.2.2).

The titer of the antibodies from the three different rabbits of each immunization was determined using enzyme-linked immunosorbent assay (ELISA) (Harlow and Lane, 1988). The wells of a microtiter dish were covered overnight at 4^{o}C with 50 µg of the purified kinase domain dissolved in PBS or the synthesized peptide (unlinked and linked to KLH) in 50 mM Na_2CO_3 pH 9.6. After three washes with PBST (PBS +

0.05% (v/v) Tween 20), the wells were blocked with PBST containing 3% (w/v) BSA for 1 h at room temperature. The wells were washed twice with PBST and then covered with serial dilutions of the different antisera in PBST and left at room temperature for 1 h. The unbound antibodies were removed by washing the wells twice with PBST. The secondary goat anti-rabbit antibody with a conjugated alkaline phosphatase diluted 1 to 9500 in PBST was added to each well and the plates incubated at room temperature for 1 h. After three washes with PBST, 2 mg p-nitrophenyl phosphate in 50 mM Na_2CO_3, 1 mM $MgCl_2$ pH 9.8 was added to each well and the reaction was allowed to proceed for 60 min at room temperature. The reaction was stopped by adding 0.4 M NaOH and the A_{405} measured.

After ELISA, the antiserum of the rabbit that showed the best immunological response against the purified extracellular domain peptide, was used for further purification. The antibodies were purified using a Hi-Trap affinity column (Porankiewicz-Asplund *et al*., 2000) when 12.5 µmole peptide dissolved in 0.2 M $NaHCO_3$, 0.5 M NaCl pH 8.3 was bound to the column according to the manufacturers instructions. The column was equilibrated with 10 mM sodium phosphate buffer pH 7.0, 0.15 M NaCl. After diluting the serum 1:1 with PBS, the serum was circulated overnight through the column at 4^{o}C to allow the antibodies to bind to the peptide. The column was then washed with the same buffer until the A_{280} value of the collected fractions was zero. The antibodies were eluted from the column using 0.1 M glycine pH 2.5 and the appropriate fractions collected and transferred to dialysis tubing. The antibodies were concentrated by covering the tube with solid PEG35000 until the desired volume was obtained. The antibodies were then finally dialyzed overnight at 4^{o}C with PBS and the concentration determined (4.3.2.2.2).

4.3.2.5 Western blot analysis of *A. thaliana* protein

To analyze At-RLK3 levels in treated *A. thaliana* cells, 25 µg total protein was separated on a 10% (w/v) separating acrylamide gel with a 6% (w/v) stacking gel (4.3.2.1). After separation, the polypeptides were transferred to a PVDF membrane (4.3.2.3.1). The membrane was stained in 2 $mg.ml^{-1}$ Ponceau S, 3% (w/v) TCA, where after the protein standards were marked with a pencil. After destaining the membrane with water, it was quenched in TBST containing 8% (w/v) fat-free milk

powder for 1 h at room temperature. The primary antibody was appropriately diluted in TBST containing 4% (w/v) fat-free milk powder and the membrane incubated in it for 1 h at room temperature. After washing the membrane three times for 10 min with TBST, the secondary goat anti-rabbit horseradish conjugated secondary antibody diluted 1:2000 in TBST with 4% (w/v) fat-free milk powder was added and the membranes incubated at room temperature for 1 h. The membranes were finally washed twice for 5 min and twice for 15 min in TBST at room temperature where after the detection of the cross reacting polypeptides were detected using the ECL detection kit according to the manufacturer's instructions.

4.3.2.6 Plasma membrane isolation and immuno-detection of At-RLK3

Plant plasma membrane isolation was done using the dextran-PEG two-phase system as described by Larsson (1985) with modifications.

All manipulations were carried out at 4^{o}C or on ice, while all solutions were kept at 4^{o}C. *A. thaliana* plant tissue was harvested and homogenized with a homogenizer 3 times for 30 sec each in two volumes extraction buffer (10 mM Tris-HCl pH 7.8, 0.25 M sucrose, 5 mM EDTA, 1 mM PMSF). Cell debris was removed from the solution by centrifugation at 10 000 *g* for 10 min. The cleared supernatant was centrifuged at 50 000 *g* for 30 min, the supernatant containing total soluble protein removed and the microsomal pellet resuspended in 500 µl resuspension buffer (5 mM potassium phosphate buffer pH 7.8, 0.33 M sucrose, 3 mM KCl). Of this suspension, 0.5 g was added to a 3.5 g dextran-PEG3350 phase mixture to give a 4 g phase system with a final composition of 6.2% (w/w) Dextran T500, 6.2% (w/w) PEG3350, 0.33 M sucrose, 3 mM KCl, 5 mM potassium phosphate buffer pH 7.8. After mixing the system thoroughly, the tubes were centrifuged at 1 500 *g* for 3 min to facilitate phase separation. The upper phase was recovered, added to a new phase system and repurified. The final upper phase was then diluted four-fold in 5 mM potassium phosphate buffer pH 7.8, 0.33 M sucrose, 3 mM KCl and the plasma membranes recovered with centrifugation at 100 000 *g* for 30 min. The pellet was finally dissolved in 10 mM Tris-HCl pH 7.8, 1 mM EDTA, 0.33 M sucrose, 1 mM PMSF. The protein concentration was then determined (4.3.2.2.2).

The K^+-stimulated, Mg^{2+} dependent ATPase activity was determined (Hodges and Leonard, 1974, Faraday and Spanswick, 1992). A standard curve of nmol phosphate against A_{820} was made (http://www.gentest.com/products/transporters/atpase_assay). The preparation of the Tris salt of ATP using Dowex 50W was done as described (Hodges and Leonard, 1974).

ATPase activity was determined when 1 µg of the microsomal fraction, the purified internal membrane fraction and purified plasma membrane fractions was respectively diluted in 500 µl reaction buffer (20 mM Tris-MES pH 6, 50 mM KCl, 3 mM $MgSO_4$) containing 1 mM sodium azide, 0.1 mM sodium molybdate, 0.33 M sucrose, 0.01% (v/v) Triton X-100, 3 mM Tris-ATP. To a second reaction, 0.1 mM vanadate was added. The reactions were incubated at 38°C for 30 min, where after 1 ml stop reagent (2% (v/v) H_2SO_4, 5% (w/v) SDS, 0.7% (w/v) sodium molybdate) was added and mixed. To this, 50 µl of a 10% (w/v) ascorbic acid reduction solution was added, the tubes incubated at room temperature for 30 min and the A_{820} values determined.

Western blot analysis was done as described (4.3.2.5) using 5 µg protein from each purification step for separation on the acrylamide gel. The dilutions of the purified anti-peptide antibody used were as indicated. For the chase experiment, the diluted antibody was incubated for 30 min at room temperature with 1 µg of the synthesized peptide in TBST containing 4% (w/v) fat-free milk powder and then used directly for the Western blot.

4.3.2.7 In-gel kinase protein assays

In-gel kinase assays were performed as described by Romeis *et al.* (1999). Total cellular protein was isolated from ground *A. thaliana* plant tissue by resuspending it in two volumes ice-cold extraction buffer (25 mM Tris-HCl pH 7.5, 15 mM EGTA, 15 mM $MgCl_2$, 2 mM DTT, 0.1 mM Na_3VO_4, 5 mM NaF, 60 mM β-glycerophosphate, 1 mM PMSF, 0.1% (v/v) Nonidet P40 and 4 $\mu g.ml^{-1}$ of antipain, chymostatin, leupeptin, pepstatin and aprotinin respectively) and incubating the reactions on ice for 1 h. After centrifugation at 20 000 *g* for 15 min at 4°C, the cleared supernatant was recovered and the protein concentration determined (4.3.2.2.2).

Of each treated plant sample, 25 µg total protein was precipitated with acetone (4.3.2.3.1), dissolved in 65 mM Tris-HCl pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol, 50 mM DTT, 0.01% (w/v) BPB and separated on a 10% (w/v) acrylamide gel containing 0.25 $mg.ml^{-1}$ MBP in the separating gel as substrate. After separation, the gel was washed three times for 30 min each with copious amounts of wash buffer (50 mM Tris-HCl pH 8.0, 20 % (v/v) isopropanol). The gel was then incubated in three 30 min changes of 50 mM Tris-HCl pH 8.0, 5 mM β-mercaptoethanol. The proteins were completely denatured in 50 mM Tris-HCl pH 8.0, 5 mM β-mercaptoethanol, 6 M guanidinium chloride for 1 h at room temperature. The proteins were renatured overnight at 4°C by washing the gel with 5 changes of renaturation buffer (50 mM Tris-HCl pH 7.5, 5 mM β-mercaptoethanol, 0.04 % (v/v) Tween-20). The gel was equilibrated with two 30 min washes in 50 mM Tris-HCl pH 7.5, 2 mm DTT, 2 mM EGTA, and 15 mM $MgCl_2$. The proteins were labeled in the same buffer containing 50 µCi [γ-^{32}P]-ATP for 1 h at 37°C. The gels were finally washed with several changes of 5% (w/v) TCA, 1% (w/v) sodium pyrophosphate until no radio-activity was present in the wash buffer. The gel was finally dried and exposed to a phospho-imaging screen.

To confirm the activation of At-RLK3 upon H_2O_2 treatment, 10 day old *A. thaliana* seedlings (3.3.2.1.1) were treated with water and 1 mM H_2O_2 respectively (3.3.2.2.2). Total protein was extracted by incubating the frozen tissue in two volumes extraction buffer (25 mM Tris-HCl pH 7.5, 1 mM EGTA, 1 mM EDTA, 5 mM DTT, 0.1 mM Na_3VO_4, 5 mM NaF, 50 mM β-glycerophosphate, 1 mM PMSF, 0.1% (v/v) Nonidet P40 and 4 $\mu g.ml^{-1}$ of antipain, chymostatin, leupeptin, pepstatin and aprotinin respectively) for 30 min on ice. After centrifugation at 20 000 *g* for 15 min at 4°C, the cleared supernatant was recovered and the protein concentration determined (4.3.2.2.2).

At-RLK3 was immunoprecipitated from 100 µg total protein (4.3.2.3.1) using 5 µg of the purified anti-peptide antibody (4.3.2.4) for 2 h at 4°C. After adding 50 µl protein A sepharose, the reaction was placed at 4°C for another hour. After centrifugation at 3000 *g* for 5 min at 4°C, the pellet was washed as described (4.3.2.3.1). It was finally resuspended in 40 µl kinase buffer (20 mM HEPES pH 7.5, 15 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT, 150 mM NaCl) containing 1 $mg.ml^{-1}$ MBP, 25 µM ATP and 1

μCi [γ-^{32}P]-ATP. The reaction was incubated at 37^{o}C for 30 min, centrifuged at 12 000 *g* for 5 min and 30 μl of the supernatant transferred to a new tube. After adding 45μl of a 10% (v/v) TCA solution, 25 μl of each reaction was spotted on P81 Whatman phospho-cellulose paper. The paper was washed four times for 15 min each with 0.5% (v/v) H_3PO_4, dried and each sample counted using the Cerenkov channel of a scintillation counter. The activation of At-RLK3 was determined by subtracting the control value from the value of each time interval and then expressing this value relative to that of time zero.

4.4 Results and discussion

4.4.1 *At-RLK3* expression in *S. cerevisiae*

For the characterization of At-RLK3, it was imperative to obtain pure protein. It was therefore decided to express *At-RLK3* in either a bacterial or yeast expression system. *S. cerevisiae* was initially chosen as host cell, since it is a eukaryotic organism that should manage effective expression of the plant gene. The pYX vector series was chosen, since the three vectors employ different combinations of two different origins of replication and two different promoter regions that allow varying levels of constitutive expression of the cloned gene.

Indicated in figure 4.1a (i) are the three recombinant plasmid molecules used for expression of the truncated *At-RLK3* gene in yeast. The inserts were cloned into these plasmids using an *Nco*1 restriction site that has an internal ATG which acted as the initiation codon for expression. On the 3' end of the cDNA insert, the *Sal*1 restriction site allowed the fragment to be inserted into the plasmids in such a way that an in frame hemagglutinin tag was attached to the carboxyl part of the polypeptide. The identity of the inserts were confirmed with a Southern blot using the truncated cDNA fragment as probe (Fig. 4.1b [i]). To test the expression of the gene in yeast, yeast cultures containing the three recombinant and three empty plasmids respectively, were grown. Protein recovered from these cell cultures were separated using SDS-PAGE (Fig. 4.2).

In a first attempt, the yeast cells were broken just by boiling it in the loading buffer. This buffer contained both PMSF to inhibit any possible proteases, as well as Triton X-100 to solubilize all membrane-bound proteins. As shown, the expression profiles of the yeast cells containing the recombinant plasmids were identical to that of the cells containing the empty plasmids (Fig. 4.2a). It was also clear that the maximum size of the released polypeptides was in the order of 66 kDa with very few larger polypeptides being released. This posed a potential problem in that At-RLK3 was around 70 kDa in size. Therefore, the cells were next broken using glass beads. This extraction method yielded much larger polypeptides (Fig. 4.2b). Again, no unique polypeptide was present in the cells containing the recombinant plasmids.

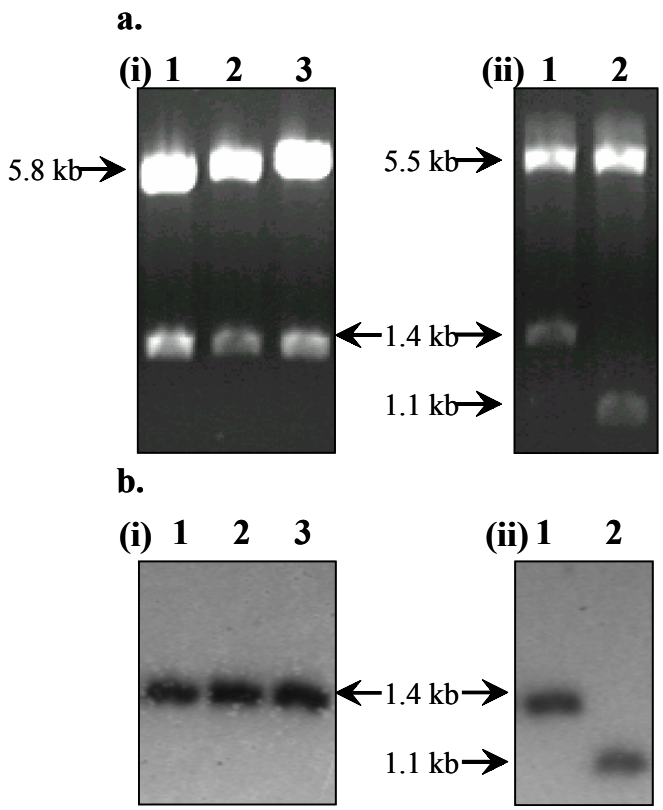


Figure 4.1. Recombinant plasmid vectors used for the expression of *At-RLK3* in yeast and *E. coli*. (a) Agarose gel electrophoresis of the full length 1.4 kb *At-RLK3* cDNA fragment cloned into (i) pYX111 (Lane 1), pYX112 (Lane 2) and pYX212 (Lane 3) for expression in yeast and (ii) the 1.4 kb cDNA (Lane 1) and 1.1 kb fragment encoding the kinase domain (Lane 2) cloned into the pET21d(+) vector for expression in *E. coli*. In (b), the transferred recombinant plasmids were probed with the labeled 1.4 kb *At-RLK3* cDNA fragment and exposed to an x-ray film.

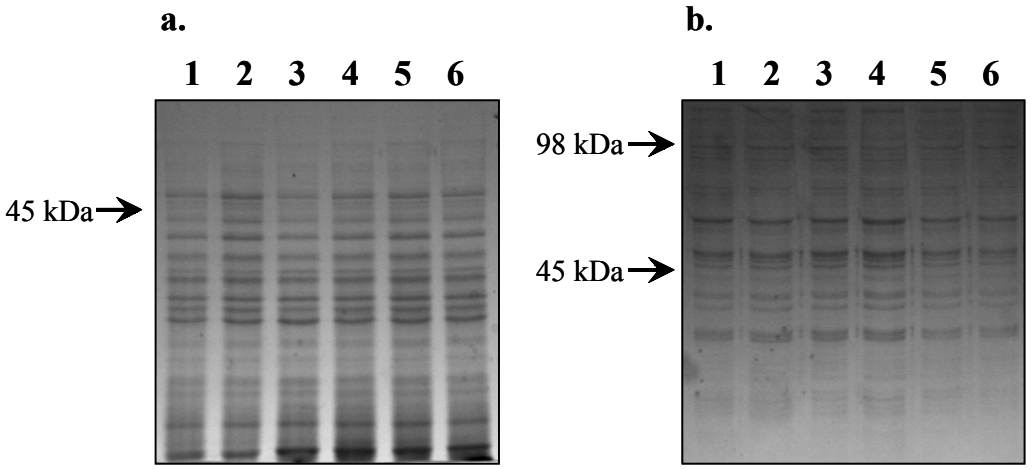


Figure 4.2. Expression of the truncated *At-RLK3* gene in *S. cerevisiae*. Lanes 1, 3 and 5 contains total protein from yeast cells containing the empty pYX111, 112 and 212 plasmids respectively, while lanes 2, 4 and 6 contains protein from yeast cells harboring the respective recombinant plasmids. (a) Yeast cells were broken open by boiling the cells in loading buffer while in (b) the cells were broken with glass beads in loading buffer.

It was therefore concluded that no expression of *At-RLK3* occurred within the yeast cells. It was only later found that a major portion of the 5'-end of the gene was missing from the isolated cDNA fragment. It is therefore most probable that expression of the gene was not successful since the natural start codon of the gene was absent. Another possibility was that its expression was inhibited in yeast, since At-RLK3 might have been detrimental to the survival of the yeast cells. It was then decided to employ a different strategy for the expression of *At-RLK3*.

4.4.2 *At-RLK3* expression in *E. coli*

For the expression of *At-RLK3* in *E. coli*, both the truncated cDNA fragment and the portion encoding the kinase domain were cloned into the pET vector (Fig. 4.1a [ii]). The identity of the inserts were again confirmed using Southern blot analysis (Fig. 4.1b [ii]). As was the case with the expression in yeast, the recombinant plasmid containing the full length cDNA fragment again failed to produce a recombinant polypeptide in the bacterial cells (results not shown).

However, in the case of the recombinant plasmid harboring the cDNA sequence encoding the kinase domain, a unique 38 kDa polypeptide was formed after treating the cells with IPTG (Fig. 4.3a). This polypeptide was absent in the cells containing the empty pET21 plasmid, as well as the cells not treated with IPTG. The polypeptide was the correct size as predicted by cDNA analysis. By separating the insoluble protein from the soluble proteins, it was established that the recombinant polypeptide was synthesized in large quantities that was precipitated as inclusion bodies within the cells (Fig. 4.3b). Using the B-Per extraction solution, the inclusion bodies were purified to a high degree of homogeneity (Fig. 4.4a). The recombinant polypeptide was completely denatured using urea and then purified and refolded to an active state on a Ni-chelated agarose column. The eluted fractions from the column containing the recombinant polypeptide were collected (Fig. 4.4b), dialysed against a buffer to remove excess imidazole and then concentrated.

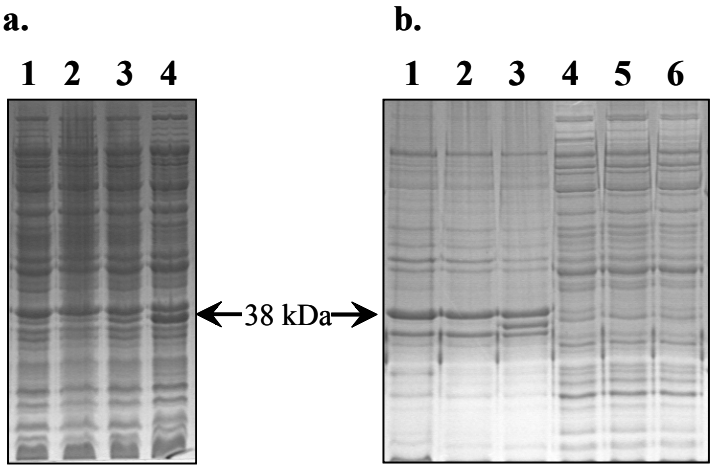


Figure 4.3. Synthesis of the kinase domain of At-RLK3 in *E. coli*. In (a), lanes 1 and 2 represents *E. coli* cells with the empty pET21 plasmid and lanes 3 and 4 the recombinant plasmid encoding the kinase domain of At-RLK3. The cells in lanes 2 and 4 were treated with 1 mM IPTG while that of lanes 1 and 3 were not. In (b) bacterial cells with the empty pET21 plasmid (lanes 1 and 4) and cells with the recombinant plasmid (lanes 2, 3, 5 and 6) were fractionated into the insoluble (lanes 1 to 3) and the soluble fractions (lanes 4 to 6). In addition, some were treated with 1 mM IPTG (lanes 1, 3, 4 and 6) and others not (lanes 2 and 5).

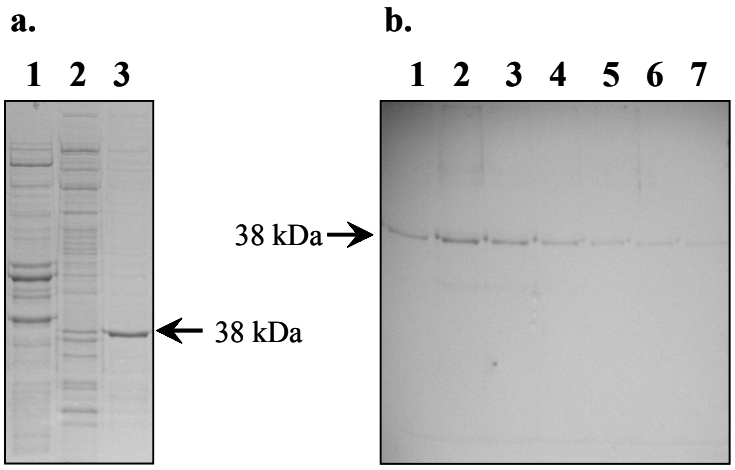


Figure 4.4. Purification and refolding of the kinase domain of At-RLK3. In (a), the total *E. coli* extract was fractionated into the soluble fraction (lane 1), the insoluble fraction (lane 2) with lane 3 containing the inclusion bodies purified from insoluble fraction. In (b), eluted fractions from the Ni-column containing the purified recombinant polypeptide (lanes 1 to 4) were pooled and dialysed.

4.4.3 *In vitro* characterization of At-RLK3

Amino acid sequence analysis of At-RLK3 showed that the protein had all the consensus subdomains characteristic of a functional protein kinase in *A. thaliana* (Fig. 3.4). Furthermore, subdomain VIb of the protein specified that phosphorylation should be serine/threonine specific. The purified kinase domain was therefore characterized *in vitro*.

When the kinase activity of the purified polypeptide was determined, total *E. coli* protein was used in a control reaction (Fig. 4.5a). In the presence of radio-labeled ATP, the total *E. coli* protein produced a single radio-active polypeptide. This indicated that the kinase conditions were sufficient to allow phosphorylation of the included proteins. The 38 kDa purified At-RLK3 kinase domain was also labeled, indicating that it is an active kinase that is able to undergo autophosphorylation. The level of phosphorylation was however very low. This activity was in accordance with several other RLKs for which autophosphorylation activity was shown (Table 2.1).

To confirm that the native enzyme was a functional protein kinase in *A. thaliana*, At-RLK3 was immuno-precipitated from a total protein extract and its autophosphorylation activity determined (Fig. 4.5b). A single 70 kDa labeled polypeptide was immunoprecipitated using both the kinase domain and purified extracellular domain specific antibodies. The size of the radio-labeled polypeptide corresponded very well to that predicted using its cDNA sequence. At-RLK3 was thus able to autophosphorylate itself, but these levels were again extremely low and could only be detected after prolonged exposure to a phospho-imaging screen. In addition, since MBP was included as substrate protein, At-RLK3 is thus also able to transphosphorylate other proteins.

Both Mn^{2+} and Mg^{2+} could act as cofactors for enzymes in the cell. The preference of At-RLK3 was tested when the purified polypeptide was incubated in the presence of a kinase buffer having either Mn^{2+} or Mg^{2+}. Of the two, Mg^{2+} was a better cofactor for At-RLK3, since the amount of labeling was several fold higher than that of the Mn^{2+} labeled polypeptide (Fig. 4.6a). This is in contrast to several other RLKs that showed higher activity in the presence of Mn^{2+} (Schaller and Bleecker, 1993; Horn and

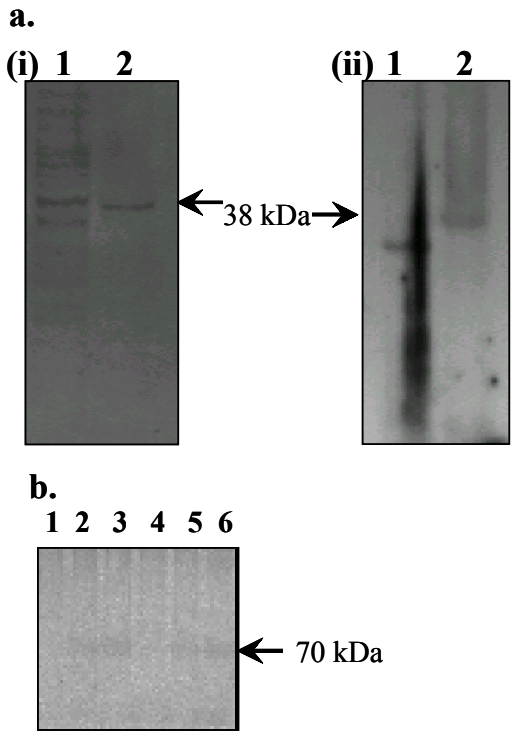


Figure 4.5. The *in vitro* phosphorylation ability of At-RLK3. In (a) 2 μg total *E. coli* protein (lane 1) and 2 μg of the purified kinase domain (lane 2) was labeled radioactive, separated on a SDS-PAGE gel, transferred to a nitrocellulose membrane (i) and then exposed to an x-ray film (ii). In (b), At-RLK3 was immunoprecipitated from 100 μg total *A. thaliana* protein using 10 (lanes 1 and 4), 50 (lanes 2 and 5) and 100 μg (lanes 3 and 6) kinase domain specific (lanes 1 to 3) and extracellular domain specific (lanes 4 to 6) antibodies respectively. The sizes of the polypeptides are indicated in kDa.

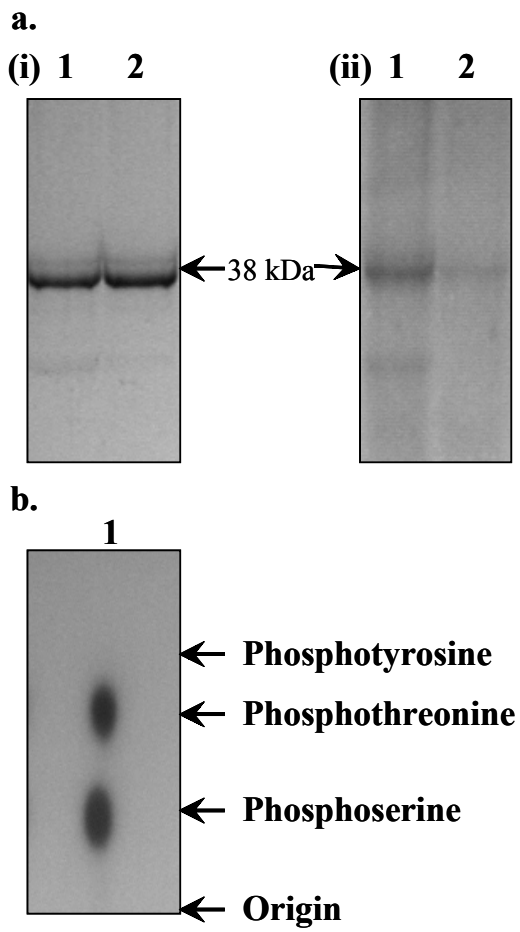


Figure 4.6. The cofactor usage and amino acid specificity of At-RLK3 phosphorylation. In (a) 10 μg of the purified At-RLK3 polypeptide labeled in the presence of buffers containing 10 mM $MgCl_2$ (lane 1) or 10 mM $MnCl_2$ (lane 2), was separated on an SDS-PAGE gel (i) which was then exposed to a x-ray film (ii). In (b), radio-labeled At-RLK3 was hydrolyzed and separated on a thin-layer chromatography plate. Unlabeled phosphorylated standards are marked as indicated.

Walker, 1994; Schulze-Muth *et al.*, 1996; Takahashi *et al.*, 1998; Nishiguchi *et al.*, 2002).

Since the At-RLK3 autophosphorylation activity was very low, all attempts to determine which amino acids within the kinase domain were labeled, were initially unsuccessful. As an alternative, the purified kinase domain was phosphorylated by protein kinase C using gamma labeled ATP. The labeled polypeptide was hydrolyzed and the individual amino acids separated on a thin-layer cellulose chromatography plate (Fig. 4.6b). At-RLK3 phosphorylation only occurred on serine and threonine with no labeling present on tyrosine. This is in accordance to all plant RLKs thus far characterized (Table 2.1). The only exception to this was PRK1 that showed dual serine and tyrosine phosphorylation (Mu *et al.*, 1994).

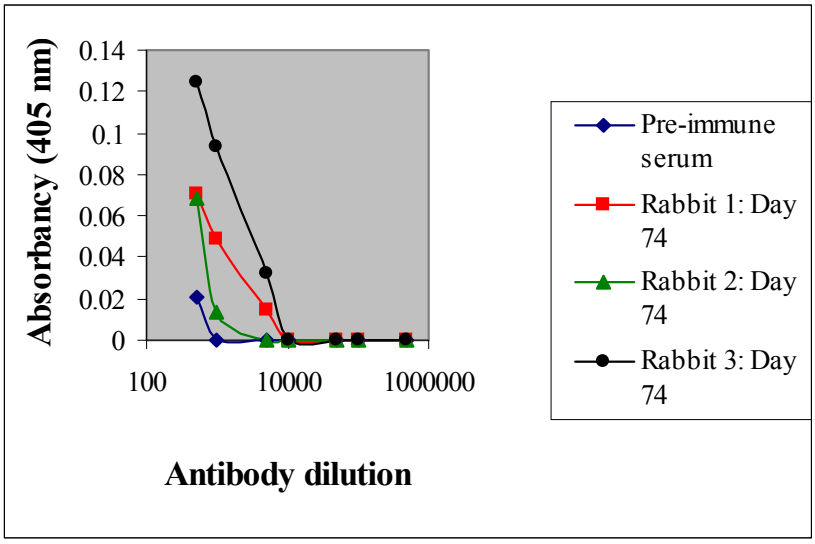
Regarding its kinase activity, At-RLK3 therefore represents a normal plant RLK that is able to undergo autophosphorylation and trans-phosphorylation by other enzymes. In addition, it can also trans-phosphorylate other substrates, indicating that is a versatile enzyme that can play a crucial role in signaling events.

4.4.4 At-RLK3 expression and localization

Two different antibodies were prepared for At-RLK3. The first antibody was raised against the kinase domain purified from *E. coli* cells. It is however known that the kinase domains of the plant protein kinases share great homology amongst each other. Therefore a second antibody was prepared against a unique peptide sequence located on the extracellular domain of At-RLK3. This antibody was expected to have a higher specificity against the native protein in *A. thaliana*.

The titer of the generated kinase domain antibodies was very low in all three rabbits (Fig. 4.7a). The best titer was found for the third rabbit and was therefore used for Western blot analysis. When the ELISA for the anti-peptide antibody was done, it was found that the majority of antibodies in all three rabbits were directed against the KLH carrier protein (Table 4.1). This led to an anti-peptide antibody titer that was only slightly higher than that of the pre-immune serum (Fig. 4.7b). These contaminating anti-KLH antibodies could lead to non-specific cross-reacting peptides

a.



b.

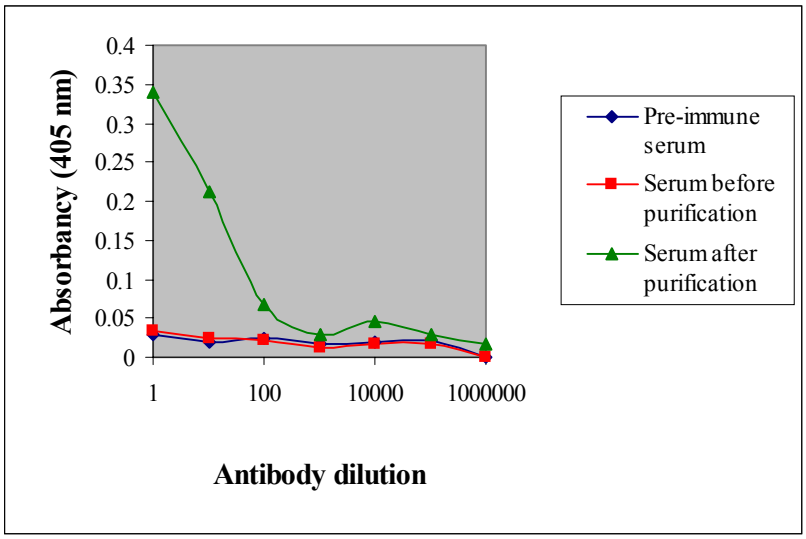


Figure 4.7. Titer determination of the antibodies raised against (a) the purified kinase domain and (b) the unique peptide located on the extracellular domain of At-RLK3. A single value was determined for each dilution.

Table 4.1. ELISA determination of antibody titer raised against the extracellular domain peptide. The indicated values represent the absorbency values at a wavelength of 405 nm.

	Rabbit nr 1	**Rabbit nr 1**	**Rabbit nr 2**	**Rabbit nr 2**	**Rabbit nr 3**	**Rabbit nr 3**
Antigen Antibody	**Peptide**	**Peptide + KLH**	**Peptide**	**Peptide + KLH**	**Peptide**	**Peptide + KLH**
0 µg	0.057	0.029	0.034	0.036	0.068	0.058
1 µg	0.489	0.903	0.442	0.896	0.26	1.00
0.1 µg	0.441	0.835	0.331	0.915	0.212	1.038
0.01 µg	0.359	0.746	0.109	0.648	0.151	0.878
1 ng	0.285	0.591	0.032	0.367	0.106	0.758
0.1 ng	0.05	0.268	0.00	0.063	0.00	0.331
0.01 ng	0.007	0.123	0.00	0.005	0.00	0.194

during a Western blot. It was therefore decided to purify the antibodies directed against the extracellular peptide from the serum of the first rabbit using a Hi-Trap column to which the synthesized peptide was bound. The resulting purified antibodies gave an improved titer compared to both the pre-immune and the unpurified serum (Fig. 4.7b).

Northern blot analysis previously showed that *At-RLK3* expression was induced when cell suspension cultures were subjected to oxidative and osmotic stress, SA and infection with various pathogens. It was therefore decided to test whether this induced expression was also found on protein level (Fig. 4.8). In all cases, the kinase domain antibody recognized a single 70 kDa polypeptide that corresponded well with the immunoprecipitated polypeptide.

As was the case with the Northern blots, any form of oxidative stress led to increased At-RLK3 levels (Fig. 4.8a – c). H_2O_2 and menadione treatments led to maximum protein levels within 30 min while the maximum protein levels for the ozone treatment were only found at 60 min. Salicylic acid treatment also led to higher At-RLK3 levels, but the increase was gradual over a long time interval with the maximum level only reached after 2 h. In all cases, the increases were transient with values decreasing later on. While some variation in At-RLK3 levels after treatment with water was found, the levels were never higher than that at time 0 indicating constitutive expression. The fact that the protein levels increased very shortly after the different treatments, indicates that the newly synthesized protein must play a crucial role in the plant when it is subjected to these different stresses.

4.4.5 Localization and activation of At-RLK3

Since the cDNA sequence of *At-RLK3* predicted that it will be a transmembrane protein, an attempt was made to localize it within the plasma membrane of the cell. Seedlings were fractionated into the soluble protein, internal membrane and plasma membrane fractions. K^+ stimulated, Mg^{2+} dependent ATPase activity was determined as a marker enzyme to follow the purification steps (Table 4.2). As the membranes were separated from the soluble protein fraction, the specific ATPase activity increased. The separation of the plasma membrane from the internal membranes were

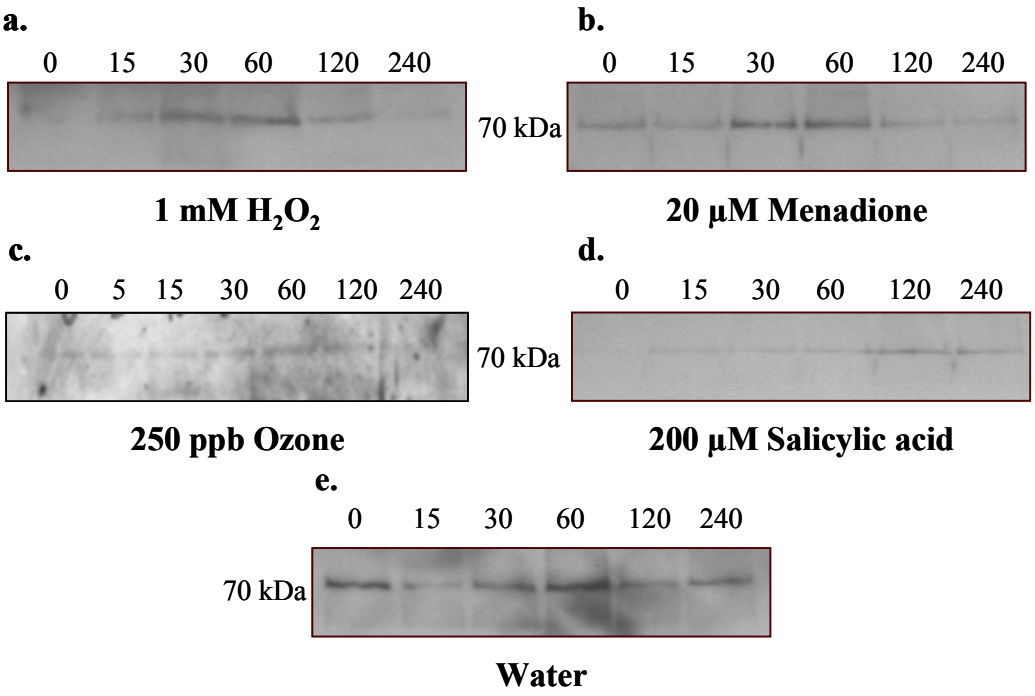


Figure 4.8. Western blot analysis of At-RLK3 from treated *A. thaliana* cells. Cell suspension cultures were treated with (a) 1 mM H_2O_2, (b) 20 µM Menadione, (c) 250 ppb ozone, (d) 200 µM salicylic acid and (e) water. The time of each harvesting is indicated in min at the top of each panel. Each panel represents total cellular protein probed with the antibody raised against the kinase domain of At-RLK3. The size of the cross reacting polypeptide is as indicated.

Table 4.2. Marker enzyme analysis of phase-partitioned membranes from *A. thaliana* seedlings.

TP – total cellular protein, CP – soluble cytosolic protein, MF – microsomal fraction, IM – internal membrane fraction, PM – plasma membrane fraction.

	Specific activity nmol phosphate.mg^{-1} protein.min^{-1}					**Enrichment**
Marker enzyme	**TP**	**CP**	**MF**	**IM**	**PM**	**PM / IM**
K^+, Mg^{2+} ATPase	0.089	0.072	0.211	0.230	0.248	1.08
K^+, Mg^{2+} ATPase + orthovanadate	0.074	0.05	0.198	0.239	0.219	
% inhibition	18%	30%	6%	-4%	12%	

however not totally effective, as some ATPase activity remained within the internal membrane fraction with the enrichment fraction only being 1.08.

Orthovanadate, a known inhibitor of plasma membrane ATPases (Larsson, 1985), was used to determine the inhibition profile of the respective fractions. Even though both the plasma membrane and internal membrane fractions showed comparable ATPase activity, a 12% inhibition of activity was found in the plasma membrane fraction, while the internal membrane fraction showed a 4% increase in activity. The ATPase activity residing in the internal membrane fraction was therefore not of the K^+ stimulated, Mg^{2+} dependant ATPase type, which therefore indicated that the different fractions were quite pure.

Using the antibody raised against the unique amino acid sequence located on the extracellular domain of At-RLK3, the presence of the protein in the different fractions were determined (Fig. 4.9a). Two polypeptides present within the plasma membrane fraction cross-reacted with the antibody, while the other fractions showed no cross-reacting polypeptides. The size of the larger polypeptide was 70 kDa, which again correlated well with the polypeptides detected in both the Western blots and the immunoprecipitated protein (Fig. 4.8). In contrast, when the blot was probed with the pre-immune serum, no cross-reacting polypeptide was found (results not shown).

To determine whether this 70 kDa polypeptide was indeed At-RLK3, a chase experiment was performed. For the chase, the antibody was incubated in the presence of 1 μg of the synthesized peptide before doing the Western blot. The rationale behind this was that should the antibodies bind to the peptide *in vitro*, they would be unable to bind to At-RLK3 present on the membrane. This was the case, since the antibodies used in the chase experiment failed to cross-react with both the two plasma membrane polypeptides (Fig. 4.9b). These results therefore confirmed the plasma membrane localization of *At-RLK3*, similar to several other RLKs (Chang *et al.*, 1992; He *et al.*, 1996; Stein *et al.*, 1996; Ahmed *et al.*, 1997; Feuillet *et al.*, 1998; Jinn *et al.*, 2000; Kim *et al.*, 2000b; Friedrichsen *et al.*, 2000; Nam and Li, 2002).

The second cross-reacting polypeptide that also disappeared when the chase experiment was done, most probably represent a break-down product of At-RLK3.

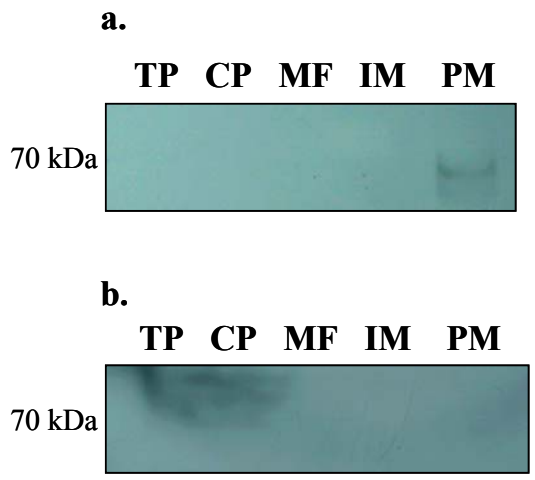


Figure 4.9. Localization of At-RLK3 in *A. thaliana*. In (a) 5 µg protein of each purified fraction was probed with the purified antibody against the extracellular domain peptide sequence. In the chase experiment (b), the antibody was first incubated in the presence of 1 µg peptide and then used directly for the Western blot. TP – total cellular protein, CP – cytosolic protein, MF – microsomal fraction, IM – internal membranes, PM – plasma membranes.

The reason for this is three-fold. The first is that a single mRNA species was detected during the Northern blot analysis (Fig. 3.9 – 3.18) despite the presence of the in-frame stop codon (Fig. 3.19). Secondly, no RLP was found to exist within the *Arabidopsis* genome when the extracellular domain was aligned with all gene sequences included in the genome database. Finally, when the peptide used for the generation of the antibodies was aligned against the *Arabidopsis* genome database, no homology with any other *Arabidopsis* protein was found (4.3.2.4).

4.4.6 The activation of At-RLK3

To assess the generation of a signal transduction event in the form of a phosphorylated protein, total protein from treated *A. thaliana* cell suspension cultures was subjected to in-gel kinase activity determination (Fig. 4.10). The resulting auto-radiographs are shown. The expression pattern of *At-RLK3* was reconfirmed using Northern blots. The *At-RLK3* expression pattern in the water treated cells remained constitutive (Fig. 4.10a). This was also reflected in the in-gel kinase assay where the phosphorylation status of the proteins remained unchanged (Fig. 4.10a).

Oxidative stress in the form of both H_2O_2 and menadione led to increased expression of *At-RLK3* shortly after treatment (Fig. 4.10b, c), but compared to previous results (Fig. 3.18), the induced expression levels was much lower. However, in contrast to the water treatment, several polypeptides were activated by phosphorylation. Menadione treatment led to the activation of three protein kinases 75, 69 and 60 kDa in size respectively (Fig. 4.10c). All three polypeptides were phosphorylated within 5 min after treatment, with the 75 kDa polypeptide reaching its maximum level 2 h after treatment. Maximal activity for the 69 and 60 kDa polypeptides was reached 1 hour after treatment. Even though it was weak, a 69 kDa polypeptide was inducibly phosphorylated within 5 min after H_2O_2 treatment reaching its maximum level 30 min after treatment (Fig. 4.10b).

Osmotic stress led to the induced expression of *At-RLK3* within 1 h after treatment (Fig. 4.10d). This induction was accompanied by the activation of a 69 kDa protein 15 min after the onset of treatment, reaching its maximum level 2 h after treatment.

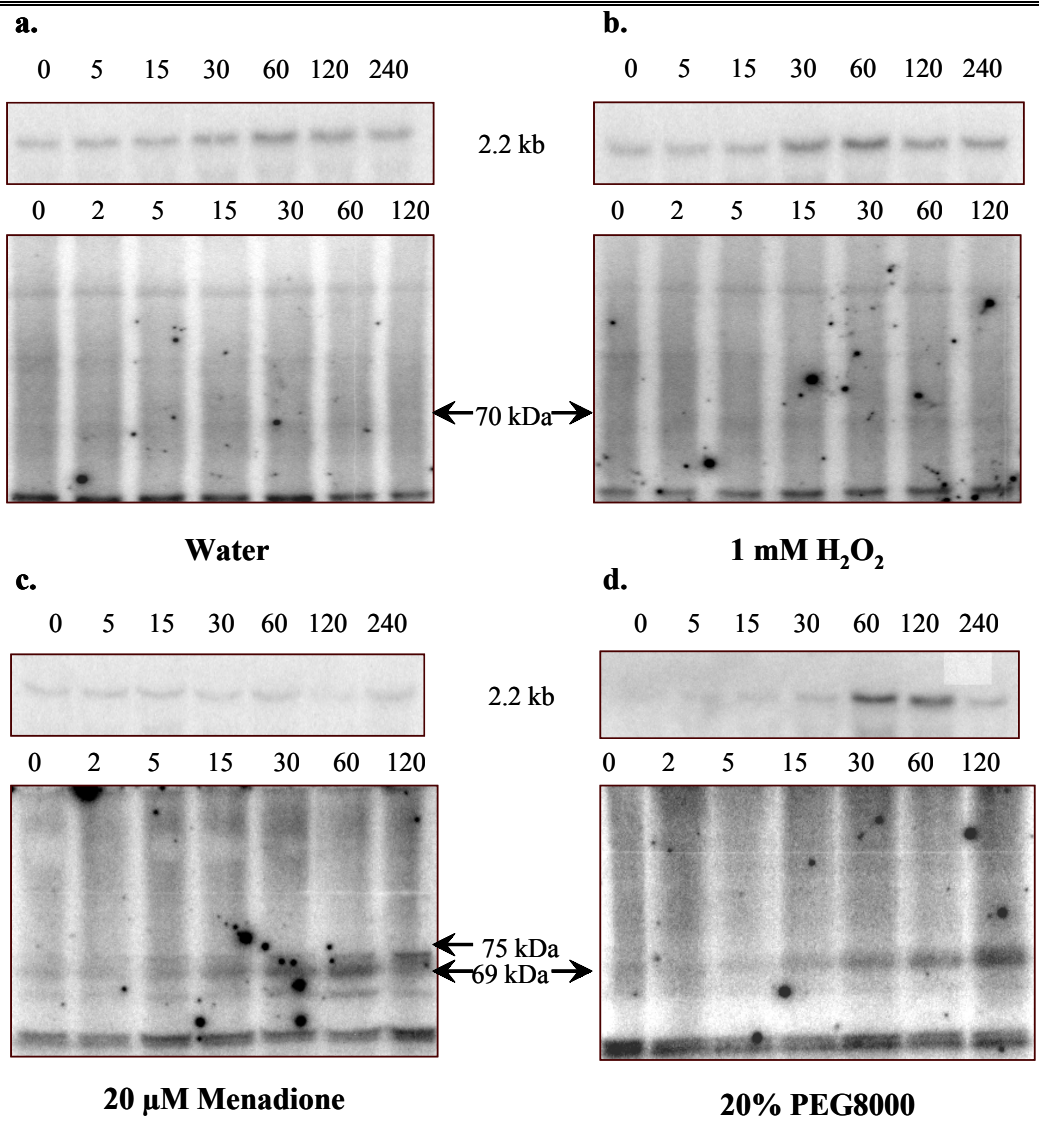


Figure 4.10. In-gel protein kinase assays of treated *A. thaliana* cells. Cell suspension cultures were treated with (a) water, (b) 1 mM H_2O_2, (c) 20 µM Menadione and (d) 20% (w/v) PEG8000. The top panel of each result is a Northern blot with the size of the hybridizing mRNA indicated in kb, while the bottom panel is the in-gel kinase assay with sizes of the activated polypeptides indicated in kDa.

A. thaliana cells were also treated with SA, but no activation of any polypeptides in the 70 kDa size range was found (results not shown).

Since H_2O_2, menadione and osmotic stress all led to increased *At-RLK3* transcription as well as the activation of a 69 kDa polypeptide by phosphorylation, it was important to determine whether this activated polypeptide is indeed At-RLK3 with a predicted size of 70 kDa. To test this, plants were treated with H_2O_2 and water and At-RLK3 was immunoprecipitated from the protein mixture. A kinase reaction was performed where MBP was used as substrate, after which the labeled MBP was determined by means of scintillation counting. This was repeated only once.

Water treatment of plants led to no phosphorylated MBP protein with the levels staying close to that at time 0 (Fig. 4.11). This indicated that At-RLK3 activity was not influenced by the water treatment. H_2O_2 treatment on the other hand led to increased labeled MBP levels 5 min after treatment reaching a maximum at 15 min. This implies that shortly after treatment with H_2O_2, At-RLK3 is activated through phosphorylation either through auto- or transphosphorylation as was previously established.

Mention must be made of the low levels of *At-RLK3* activation. It is important to realize that the in-gel kinase assays were performed on protein extracts from cell suspension cultures. In these suspensions, all cells are immediately exposed to H_2O_2 when treated and therefore respond as a coordinated unit leading to the activation of all At-RLK3 protein molecules present in these cells. This results in high levels of labeled MBP. When intact plants are however treated with H_2O_2, only the uppermost level of leaf cells are initially exposed to the H_2O_2. Therefore, the activated At-RLK3 proteins present in the exposed cells represent only a fraction of all immunoprecipitated At-RLK3 protein. It is therefore safe to accept the low At-RLK3 activation levels as being correct.

Having said this, one can assume that the activated 69 kDa polypeptide in H_2O_2, menadione and osmotic stress treated cell suspension cultures is indeed At-RLK3. The activation of At-RLK3 in the menadione and osmotic stress treatments are most probably also through the presence of H_2O_2. It was previously shown that osmotic

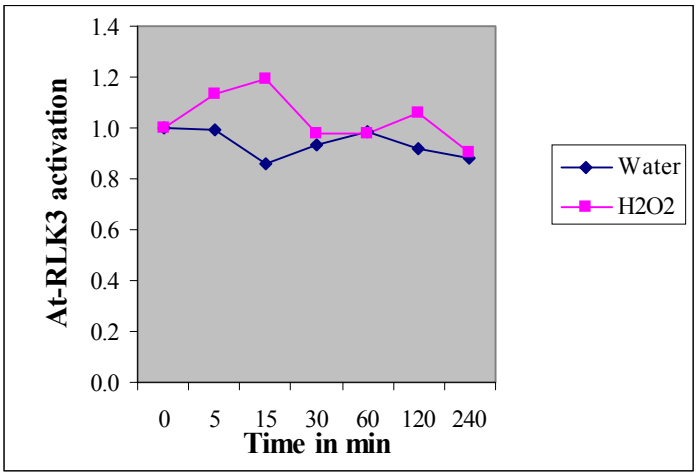


Figure 4.11. Activation of immunoprecipitated At-RLK3 after treating plants with water or H_2O_2.

stress leads to oxidative stress conditions, most probably through H_2O_2 accumulation (Smirnoff, 1993). Menadione treatment on the other hand also leads to elevated H_2O_2 and O_2^- levels (Borges *et al.*, 2003).

The key component for At-RLK3 activation therefore seems to be H_2O_2. This statement is strengthened by the fact that various other treatments, all generating oxidative stress conditions at some stage, induce *At-RLK3* expression. This was true for proline and hypo-osmotic stress treatments (Fig. 3.9), SA (Fig. 3.14) and GA treatment (Fig. 3.11) and infiltration with both compatible and incompatible pathogens (Fig. 3.17).

The activation of At-RLK3 and induced expression of *At-RLK3* by osmotic stress is most probably due to the osmotic component of the stress and not the ionic component, since salt stress did not lead to induced expression of the gene (Fig. 3.9). It would be interesting to further characterize *RPK1* from *Arabidopsis* that was shown to be induced upon the onset of various osmotic stress conditions (Hong *et al.*, 1997). Since the activation of the protein was not described, it would be interesting to see whether oxidative stress also plays a role in the regulation of the gene. Once that was established, the interaction of At-RLK3 and RPK1 needs to be established, since it is possible that the combined action of these two enzymes could be involved in the osmotic response of *Arabidopsis*.

Finally, it is of interest that even though SA induced *At-RLK3* expression, At-RLK3 was not activated upon treatment (results not shown). This was despite the fact that SA probably mediates the oxidative burst leading to cell death during the plant defense reaction (Shirasu *et al.*, 1997) by inhibiting H_2O_2 scavenging enzymes (Conrath *et al.*, 1995; Durner and Klessig, 1995). Du and Klessig (1997), however, postulated that H_2O_2 functions upstream of SA. Therefore, application of SA will lead to the induced expression of *At-RLK3* through the SA responsive elements present on the promoter region (Table 3.1) as was suggested (Ohtake *et al.*, 2000; Du and Chen, 2000). The H_2O_2 levels will however not be affected and thus no activation of At-RLK3 will occur.

4.5 Summary

At-RLK3 is an active serine/threonine specific protein kinase in *A. thaliana*. The protein is localized in the plasma membrane, an ideal location for a receptor protein responsible for detecting changes in the extra-cellular environment. The importance of At-RLK3 was confirmed when Western blot analysis indicated that protein levels increased similarly to mRNA levels shortly after the application of the different chemicals. The main element responsible for At-RLK3 activation appears to be H_2O_2. For this reason, the enzyme is thought to play a crucial role in detecting any changing environment that results in elevated H_2O_2 levels. These results are further supported by the expression profiles of *At-RLK3* as discussed in chapter 3.

Chapter 5

Defining a function for At-RLK3 in *A. thaliana*

Defining a function for At-RLK3 in *A. thaliana*

5.1 Introduction

Plants are continuously subjected to both biotic and abiotic stresses and need to adjust accordingly. One key component needed for the adjustment to both is ROS that is implicated to play an important role in signaling leading to the differential expression of genes (Bartosz, 1997).

There are three ways in which ROS could modulate gene expression (Apel and Hirt, 2004). The presence of ROS could activate sensor proteins that in turn could activate signaling cascades leading to altered gene expression. ROS could also be responsible for the modification of existing signaling components thereby altering their activity and function. Finally, it is also possible that ROS could affect gene expression by directly modifying transcription factor activity.

In yeast, a two component signaling system in the form of a histidine kinase together with a transcription factor functions as a redox sensor (Singh, 2000). When exposed to oxidative stress conditions, the kinase autophosphorylates on a histidine residue within the protein. This phosphate group is then transferred to the transcription factor, leading to its activation and the subsequent activation of gene expression.

While plants contain several two-component histidine kinases, the involvement of these proteins in the recognition of ROS still has to be proven (Hwang *et al.*, 2002). Doubt was however expressed whether a receptor specific for H_2O_2 exists (Neill *et al.*, 2002). Currently, a lot of research is directed towards the identification of a redox sensor.

5.2 Aim

The aim of this chapter is to define a putative function for At-RLK3 by studying transgenic plants overexpressing an antisense copy of the gene. By subjecting the transgenic plant to various challenging conditions, it was hoped that a distinctive phenotype unique to the transgenic plant could be established.

5.3 Materials and methods

5.3.1 Materials

5.3.1.1 Biological material

The Columbia ecotype of *A. thaliana* was used for the generation of transgenic plants. The *Agrobacterium tumefaciens* C58C1 strain carrying the pGV2260 binary plasmid vector was used to transfer the chimeric constructs into the plant cells. All DNA manipulations of *At-RLK3* were done using *E. coli* HB101 (*supE*44*hsdS*20($r^-_\beta m^-_\beta$) *recA*13*ara*-14*proA*2*lacY*1*galK*2*rpsL*20*xyl*-5 -1) as the host cell. For the transformation of *A. thaliana* Columbia, two different binary vector systems were used. The first was pKYLX71:35S, a binary vector that allows constitutive expression of the transferred chimeric gene within the plant. The second binary vector used was pTA7002 (Aoyama and Chua, 1997) which allows inducible expression of the transgene after spraying the plants with dexamethazone (DEX). The pGem3Z cloning vector was obtained from Promega.

5.3.1.2 Other material

The Qiagen Plasmid mini DNA extraction kit was obtained from Qiagen. DNA fragments were purified from both solutions and agarose gels using the Geneclean kit from Bio101. DEX was obtained from Sigma Aldrich. For all RT-PCR reactions, the Titan One Tube RT-PCR system from Roche was used.

5.3.2 Methods

5.3.2.1 Cloning of the full length *At-RLK3* gene

As previously mentioned (3.4.1.1), the originally cloned cDNA encoding *At-RLK3* was a truncated gene with a portion of the 5'-end of the gene missing. For the generation of transgenic plants overexpressing an antisense copy of the gene, it was important to clone the full-length *At-RLK3* gene.

Since the complete *A. thaliana* genome sequence was available, a DNA primer (Bovis 7) was made that annealed to the 5' end of the gene (Appendix 1). Using the Bovis 7 and 11 primers, the full-length gene, as well as the portion of the gene encoding the kinase domain (4.3.2.2.1), was amplified from an *A. thaliana* cDNA library prepared in the pFL61 plasmid vector (Minet *et al.*, 1992) as described (3.3.2.1.6.1). A control PCR reaction was done where the template DNA was omitted from the reaction. After the full length cDNA fragment was purified from the agarose gel using the Geneclean kit according to the manufacturer's instructions, it was digested with *Eco*RI (3.3.2.1.4) and cloned into pGem3Z as described (4.3.2.1). After transferring the plasmid to competent *E. coli* HB101 cells (4.3.2.1), colonies containing recombinant plasmid DNA were selected through α-complementation (Sambrook *et al.*, 1989) by plating the cells on LB agar plates containing 80 $\mu g.ml^{-1}$ 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and 0.5 mM IPTG. Colonies containing the recombinant plasmids were inoculated into 5 ml LB medium containing 50 $\mu g.ml^{-1}$ ampicillin and grown overnight at 37°C. Plasmid DNA was prepared from the cultures using the Qiagen plasmid mini kit according to the manufacturer's instructions.

Of each extract, 200 ng plasmid DNA was digested with *Eco*RI (3.3.2.1.4) and separated on a 1% (w/v) agarose gel (3.3.2.1.3). The digested DNA was transferred to a nylon membrane (3.3.2.1.5) and hybridized (3.3.2.1.7) with the radio-actively labeled truncated *At-RLK3* cDNA fragment (3.3.2.1.6). After selecting the appropriate recombinant plasmids, the inserts were sequenced to confirm the identity thereof.

5.3.2.2 Production of transgenic plants

To produce transgenic plants expressing an antisense copy of *At-RLK3*, two different binary plasmid vectors were used, namely pKYLX71:35S and pTA7002. For each plasmid, two different chimeric constructs were prepared. The first contained the full length gene in the antisense orientation, while the second contained the region encoding the kinase domain in the antisense orientation. Once transformed, transgenic plants containing the chimeric construct from pKYLX71:35S were selected on GM medium containing 50 $\mu g.ml^{-1}$ kanamycin, while those having the construct from pTA7002 were selected on germination (GM) medium (0.5x MS salts pH 5.8, 1% (w/v) sucrose, 5 $mg.l^{-1}$ silver thiosulphate, 0.8% (w/v) plant agar) containing 30 $\mu g.ml^{-1}$ hygromycin.

5.3.2.2.1 Preparation of chimeric constructs

5.3.2.2.1.1 pKYLX71:35S

For the amplification of the full length *At-RLK3* gene and the portion encoding the kinase domain, the Bovis 15 and 16 and Bovis 1 and 2 primer combinations were respectively used (Appendix 1). While Bovis 15 and 1 had *Xba*1 restriction sites, Bovis 16 and 2 had *Sac*1 restriction sites. The different fragments were amplified as described (3.3.2.1.6.1), separated on an agarose gel (3.3.2.1.3), purified from the gel (5.3.2.1) and digested with *Xba*1 and *Sac*1 (3.3.2.1.4). pKYLX71:35S was digested similarly and the insert ligated into the linear plasmid (4.3.2.1). After *E. coli* transformation, colonies containing recombinant plasmid DNA were selected (4.3.2.1). The identity of the inserts were confirmed after a recombinant plasmid was digested with *Xba*1 and *Sac*1 (3.3.2.1.4), separated on an agarose gel (3.3.2.1.3), transferred to nylon membrane (3.3.2.1.5) and hybridized (3.3.2.1.7) with a radio-actively labeled *At-RLK3* probe (3.3.2.1.6).

5.3.2.2.1.2 pTA7002

For the generation of chimeric constructs in pTA7002, a similar strategy was followed as for pKYLX71:35S (5.3.2.2.1.1) with the exception that different primer sets were used for the amplification. To amplify the full length gene, Bovis 3 and 4 were used, while for the fragment encoding the kinase domain, Bovis 5 and 6 were used (Appendix 1). Bovis 3 and 5 had an internal *Xba*1 restriction site, while Bovis 4 and 6

had a *Sal*1 restriction site. After the inserts were digested, they were ligated into the isoschizomeric *Spe*1 and *Xho*1 restriction sites located on the plasmid. To confirm the identity of the insert, a recombinant plasmid was digested with *Eco*RI (3.3.2.1.4) and hybridized as described (5.3.2.2.1.1).

5.3.2.2.2 Transformation of *A. tumefaciens*

A. tumefaciens was transformed with the different recombinant plasmids using electroporation (Main *et al.*, 1995). Competent cells were prepared when *A. tumefaciens* cells were grown overnight in LB medium containing 50 $\mu g.ml^{-1}$ rifampicin and 50 $\mu g.ml^{-1}$ ampicillin at 28^{o}C. Ampicillin was added to the medium to ensure that the bacterium retained the pGV2260 binary plasmid, since it provided the virulence region needed to transfer the chimeric constructs into the plant cells. This overnight culture was used to inoculate LB medium containing 5 mM $MgSO_4$ and was grown at 28^{o}C until an A_{600} of between 0.5 and 1.0 was reached. After chilling the culture on ice for 30 min, the cells were harvested by centrifugation at 3000 *g* for 10 min at 4^{o}C, the cells resuspended in 20 mM $CaCl_2$ and aliquots stored at -80^{o}C.

For transformation, the cells were thawed on ice, 100 ng of the different recombinant plasmids added, mixed and incubated on ice for 1 min. After transferring the cells to the electroporation cuvette, the cells were pulsed at 12.5 $kV.cm^{-1}$, collected, resuspended in LB medium and incubated at 28^{o}C for 2 hr. Those cells transformed with pKYLX71:35S were plated on LB plates containing 12.5 $\mu g.ml^{-1}$ tetracyclin, while those transformed with pTA7002 were plated on LB plates containing 50 $\mu g.ml^{-1}$ kanamycin. The plates were incubated at 28^{o}C for 2 days to allow the transformed colonies to develop.

5.3.2.2.3 Transformation of *A. thaliana*

A. thaliana was transformed with the different chimeric constructs using a modified protocol as described by Clarke *et al*. (1992). *A. thaliana* seed was sterilized (3.3.2.1.1) and germinated on GM medium for 3 days at 23^{o}C in the dark and then grown at a light intensity of 50 $\mu mol.m^{-2}.s^{-1}$ as described (3.3.2.1.1). Roots were excised, placed on callus inducing medium (CIM) (1x Gamborg's B5 salts, 0.05% (w/v) MES pH 5.8, 2% (w/v) glucose, 0.8% (w/v) plant agar) containing 5 $mg.l^{-1}$

silver thiosulphate, 0.5 mg.l^{-1} 2,4-dichlorophenoxyacetic acid [2,4-D], 0.05 mg.l^{-1} kinetin) and incubated for three days at 23^{o}C.

The transformed *A. tumefaciens* cultures were inoculated in LB cultures containing the appropriate antibiotics and grown for 2 days at 28^{o}C. The cultures were diluted to an A_{600} of 0.1 with a dilution buffer (1x Gamborg's B5 salts, 0.05% (w/v) MES pH 5.8, 2% (w/v) glucose). After cutting the root explants into small pieces, the pieces were resuspended in the diluted *A. tumefaciens* cultures for 2 min, the pieces were recovered, the excess *A. tumefaciens* solution removed and the pieces incubated on CIM plates for 3 days. The roots were then washed with sterile water to get rid of the excess bacteria, resuspended in molten shoot overlay medium (SOM) (1x Gamborg's B5 salts, 0.05% (w/v) MES pH 5.8, 2% (w/v) glucose, 0.8% (w/v) low melting point agarose) containing 850 mg.l^{-1} vancomycin, 5 mg.l^{-1} 2-isopentinyladenine (2-IP), 0.15 mg.l^{-1} IAA and the appropriate antibiotics. For tissue transformed with pKYLX71:35S, 50 μg.ml^{-1} kanamycin was added and for those transformed with pTA7002, 30 μg.ml^{-1} hygromycin was added. This tissue was finally dispersed on shoot inducing (SIM) plates (same as SOM, but including 0.8% (w/v) plant agar in place of the agarose) and incubated to allow the formation of transgenic calli and shoots over several weeks.

Shoots with well expanded leaves were transferred to shoot elongation medium (SEM) (0.5x MS salts pH 5.8, 1% (w/v) sucrose, 0.8% (w/v) plant agar) in tissue culture vessels sealed with gas-permeable film and allowed to set seed. The collected seed were vernalized at 4^{o}C for 1 week and then germinated on GM medium containing the different antibiotics. When the seedlings were 5 cm high, they were transplanted into soil (peat moss, compost, potting grit and vermiculite in a 2:2:2:1 ratio), incubated for a week at high humidity and then transferred to the glass house to set seed over two to three months. These T3 seeds were used for all subsequent analyses.

5.3.2.3 Analysis of transgenic plants

5.3.2.3.1 Cultivation of transgenic plants

For both the Southern blot and RT-PCR analysis, seed from the wild type and transgenic lines was sterilized as described (3.3.2.1.1). The wild type seed was germinated on GM medium (5.3.2.2.3), while the transgenic seed was germinated on GM medium containing kanamycin or hygromycin respectively (5.3.2.2.3). For the induced expression of the antisense *At-RLK3* fragments in the pTA7002 chimeric constructs, the plants were sprayed with a solution of 0.03 mM DEX, 0.01% (v/v) Tween 20 until micro-droplets were visible on the surface of the plants. These plants were harvested 3 h later.

5.3.2.3.2 Southern blot analysis of transgenic plants

Genomic DNA was isolated from both wild type and transgenic plants as described (3.3.2.1.2). After digestion with *Eco*RI (3.3.2.1.4), the digested DNA was separated on a 0.8% (w/v) agarose gel (3.3.2.1.3), transferred to a nylon membrane (3.3.2.1.5) and probed with a radio-actively labeled *At-RLK3* probe (3.3.2.1.6).

5.3.2.3.3 Expression analysis of transgenic plants

Total RNA was extracted from the harvested plant tissue as described (3.3.2.2.3). The reverse transcribed polymerase chain reaction (RT-PCR) was used to quantify antisense *At-RLK3* mRNA levels in the transgenic lines using the Titan One Tube RT-PCR system. Two primers (Bovis 30 and 31) were designed to amplify the portion of the gene encoding the kinase domain of At-RLK3 (Appendix 1). Each 10 µl RT-PCR reaction contained 10 ng total RNA, 25 pmole of each primer, 6 mM DTT, 0.25 mM dNTPs, 4 mM Tris-HCl pH 7.5, 25 mM KCl, 0.02 mM EDTA, 0.1% (v/v) Tween 20, 0.1% (v/v) Nonidet P40, 10% (v/v) glycerol and 0.2 µl of the enzyme mix as supplied in the kit. The amplification regime for the reaction was one cycle of 55^{o}C for 30 min and 94^{o}C for 1 min, 30 cycles of 94^{o}C for 10 sec, 55^{o}C for 1 min and 68^{o}C for 4 min and a final step of 68^{o}C for 7 min. The amplified fragments were separated on an agarose gel as described (3.3.2.1.3).

To quantify the expression levels of the antisense mRNA, a control RT-PCR reaction was performed for every transgenic line using the Bovis 26 and 27 primers that

specifically amplify the 18S rRNA fragments (Appendix 1). This gene is also constitutively expressed in plant tissue, similar to the actin gene. The yield of both amplified fragments was determined using a scanning densitometer. The values obtained for the antisense *At-RLK3* fragments were expressed relative to the values of the respective 18S rRNA fragments, thereby allowing the correlation of the phenotype of the transgenic plant with the level of antisense *At-RLK3* gene expression.

In order to define a role for At-RLK3 in *A. thaliana*, 10 day old wild type and C4 transgenic seedlings were both treated with 1 mM H_2O_2 and 200 µM SA respectively (4.3.2.7). After total RNA was extracted from the harvested plant tissue (3.3.2.2.3), the activation of the plant defense reaction was confirmed using RT-PCR of the *PR-2* gene. The gene was amplified using two primers that were gene-specific, namely Bovis 37 and 38 (Appendix 1). The reactions were performed as described with a reverse transcription (RT) temperature of 48^{o}C and a PCR annealing temperature of 51^{o}C. As control, the 18S rRNA gene was also amplified to allow the quantification of the expression of the *PR-2* gene (see above).

5.4 Results and discussion

5.4.1 Cloning of the full-length *At-RLK3* gene

For the generation of transgenic plants expressing an antisense copy of the gene, the full length gene encoding At-RLK3 was directly amplified from a cDNA library using gene specific primers (Fig. 5.1a). As a control, the portion of the gene encoding the kinase domain was also amplified. Both amplification reactions yielded a PCR product with the expected sizes of 2 kb for the full length gene and 1.1 kb for the kinase domain. The control reaction with no template DNA did not produce an amplified fragment.

The putative full length gene was cloned into pGem3Z and recombinant plasmids were selected. After digestion, the inserts of the recombinant plasmids were hybridized with the radio-actively labeled truncated *At-RLK3* gene (Fig. 5.1b). The probe hybridized with the 2 kb inserts, indicating that the cloned inserts did share homology with the *At-RLK3* gene. The identities of the cloned inserts were finally confirmed when the inserts were sequenced and found to encode the full length *At-RLK3* gene.

5.4.2 Production of transgenic plants

Antisense technology was employed to reduce the *in vivo* At-RLK3 levels in *A. thaliana*. This was done by constructing four different chimeric constructs in two different binary plasmid vectors. The pKYLX71:35S vector allows constitutive expression of the transferred gene *in vivo*, while with pTA7002, the expression can be induced by treatment with DEX. Two different constructs were prepared in each vector, the first being the full length *At-RLK3* gene cloned in the antisense orientation, while the second was the portion encoding the kinase domain cloned into the antisense orientation. To confirm the identity of the inserts of the recombinant plasmids, the full length gene was cut from the two recombinant plasmids (Fig. 5.2a). Since the gene was inserted into the *Sac*1 and *Xba*1 restriction sites of pKYLX71:35S, digestion of the recombinant plasmid yielded the 2 kb insert that hybridized with the *At-RLK3* probe (Fig. 5.2b).

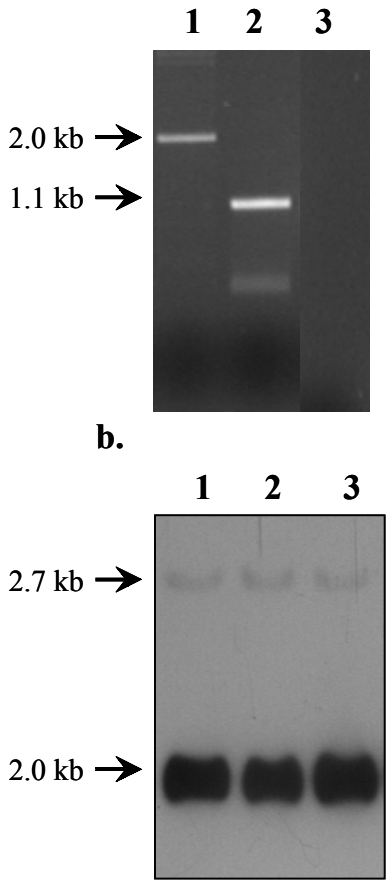


Figure 5.1. Cloning of a full-length *At-RLK3* gene from an *A. thaliana* cDNA library. (a) PCR amplification of the *At-RLK3* gene. Lane 1 represents the amplified full-length gene, lane 2 the gene portion encoding the kinase domain and lane 3 the negative control. (b) Southern blot analysis of recombinant plasmids using the truncated *At-RLK3* gene as probe. Lanes 1 to 3 represents different digested recombinant plasmids.

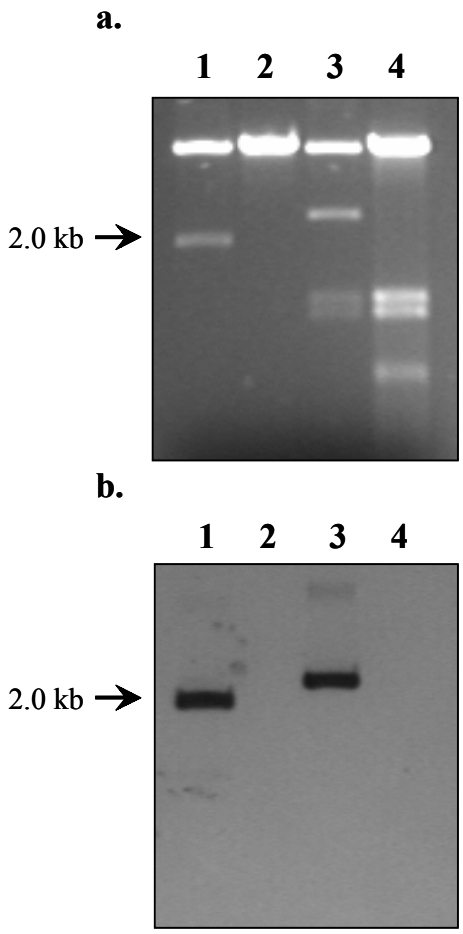


Figure 5.2. Cloning of the full-length *At-RLK3* gene into the two binary plasmid vectors. (a) Agarose gel electrophoresis of digested plasmid DNA. In lane 1 and 2, recombinant and non-recombinant pKYLX71:35S plasmid DNA were digested with *Sac*1 and *Xba*1, while lane 3 and 4 contain recombinant and non-recombinant pTA7002 plasmid DNA digested with *Eco*RI. (b) Autoradiogram of digested plasmid DNA probed with the radio-actively labeled *At-RLK3* probe.

To clone *At-RLK3* into pTA7002, the *Spe*1 and *Xho*1 palindromes had to be used. Since *At-RLK3* had internal *Spe*1 and *Xho*1 palindromes, the primers used to amplify *At-RLK3* did not include these palindromes, but rather those for *Xba*1 and *Sal*1 that are isoschizomers for *Spe*1 and *Xho*1. After ligation into pTA7002, these restriction sites were however lost. Since pTA7002 has *Eco*RI restriction sites on either side of the poly-cloning site, the recombinant and non-recombinant plasmids were digested with *Eco*RI (Fig. 5.2a). When digested from the plasmid, the insert was slightly larger than that of the pKYLX71:35S plasmid, since the *Eco*RI sites were not directly adjacent to the *Spe*1 and *Xho*1 restriction sites. This larger insert also hybridized with the labeled *At-RLK3* probe (Fig. 5.2b), confirming that both recombinant plasmids contained the full length *At-RLK3* gene cloned in the antisense orientation. Similarly, it was confirmed that the portion of the gene encoding the kinase domain was also successfully cloned into the respective plasmids (results not shown).

After infection of the root explants with *A. tumefaciens*, the tissue was incubated on CIM medium that allowed the formation of calli (Fig. 5.3a). Once the calli were well established, they were transferred to SIM medium that stimulated the formation of shoots and leaves (Fig. 5.3b - d). Those calli that showed good leaf and shoot formation were transferred to separate containers where they were allowed to set seed (Fig. 5.3e).

Finally, putative transgenic seed was collected from 11 developed calli. Four lines (C1 – C4) constitutively expressed an antisense copy of the portion of the *At-RLK3* gene encoding the kinase domain and two lines (E1 + E2) the full length antisense *At-RLK3* gene. Another set of transgenic lines was obtained where three lines inducibly expressed the portion of the *At-RLK3* gene encoding the kinase domain after DEX treatment (D1 – D4) and another two the full length antisense *At-RLK3* gene (F1 + F9). The transformed status of the different lines was verified using antibiotic selection. Transformed lines generated using the recombinant pKYLX71:35S plasmids germinated successfully on GM medium (Fig. 5.4a) and GM medium containing kanamycin (Fig. 5.4c), but not on GM medium containing hygromycin (Fig. 5.4b). This was similarly tested for lines produced using the pTA7002 recombinant plasmids (results not shown).

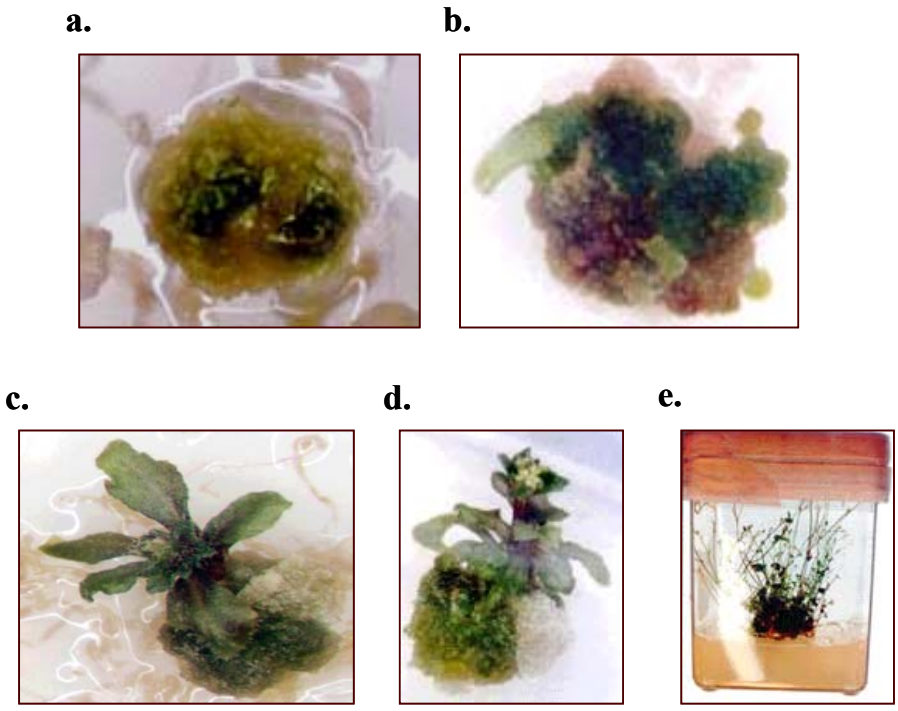


Figure 5.3. The generation of transgenic plants. (a) – (e) represents different stages in the process of generating the plants from transformed callus tissue.

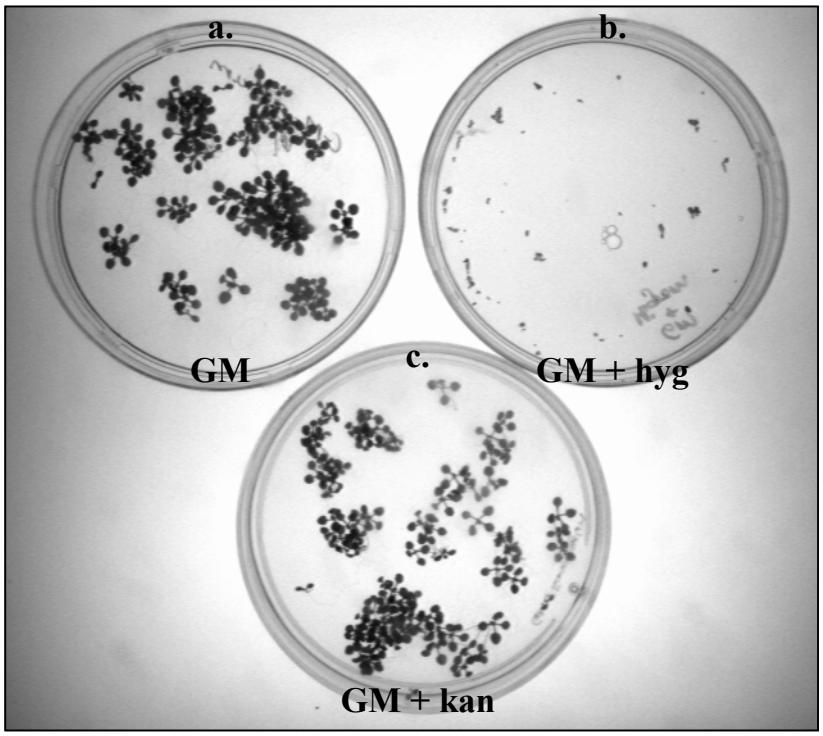


Figure 5.4. Testing of the transformed status of the different transgenic lines obtained. Seed obtained from calli transformed with the different pKYLX71:35S recombinant plasmids was germinated on (a) GM medium, (b) GM medium containing 50 $\mu g.ml^{-1}$ hygromycin and (c) GM medium containing 50 $\mu g.ml^{-1}$ kanamycin respectively.

5.4.3 Genetic analysis of transgenic *A. thaliana* lines

During both the multiplication of seed and the testing of antibiotic resistance of the transgenic seed, it was found that some of the transgenic plants were phenotypically quite distinct from both the wild type plants and the other transgenic lines. Seedlings from the D1, D4 and F9 transgenic lines all showed retarded germination and growth with D4 expressing the most severe phenotype (Fig. 5.5, 5.10). Besides germinating and growing at a very slow rate, the germinated plants were severely stunted with underdeveloped roots, dark green mature leaves and had a very long seed set time. All the other transgenic lines were however phenotypically similar to the wild type plants (Fig. 5.5, 5.9, 5.10).

These results resembled that obtained by Holt *et al.* (2002). The authors found that some transgenic *Arabidopsis* lines expressing an antisense copy of the *AtTIP49a* gene showed aberrant growth, development and seed set. The severity of the phenotype increased as the amount of antisense *AtTIP49a* mRNA present within the transgenic plants increased. Therefore, by reducing the amount of AtTIP49a *in vivo*, the development of the plant was influenced, even though the protein was thought to be involved in a completely different aspect of the plant development.

This prompted a genetic investigation of the transgenic lines generated in this study both on DNA and mRNA levels. First, the incorporation of the transferred chimeric constructs into the host genomes was investigated (Fig. 5.6). In the wild type plant, a single faint cross-hybridizing fragment was visible, similar to that previously found (Fig. 3.8). This fragment represented the endogenous *At-RLK3* gene present in the genome. This fragment was found in all the transgenic lines except for E4, D1 and F1. In several others such as E1, the size of this fragment differed slightly, most probably due to separation artifacts of the genomic DNA on the gel. The absence of any cross-hybridizing fragments in the lanes containing genomic DNA from E4 and F1 was most probably due to low DNA levels in each lane. The number of cross-hybridizing fragments present in the transgenic plants ranged from 2 (C2 and C4) to 5 (D2) (Fig. 5.6).

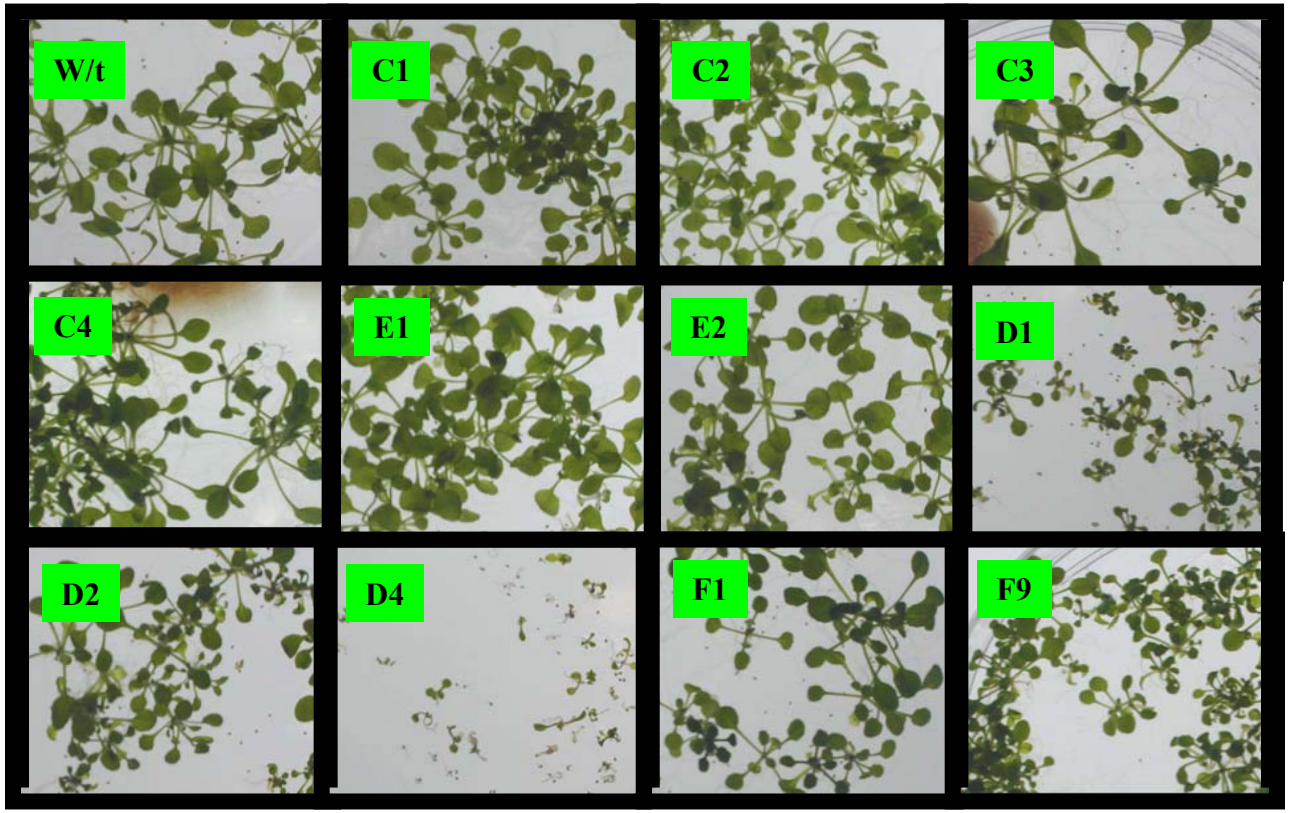


Figure 5.5. Phenotypes obtained for the different transgenic *A. thaliana* lines.

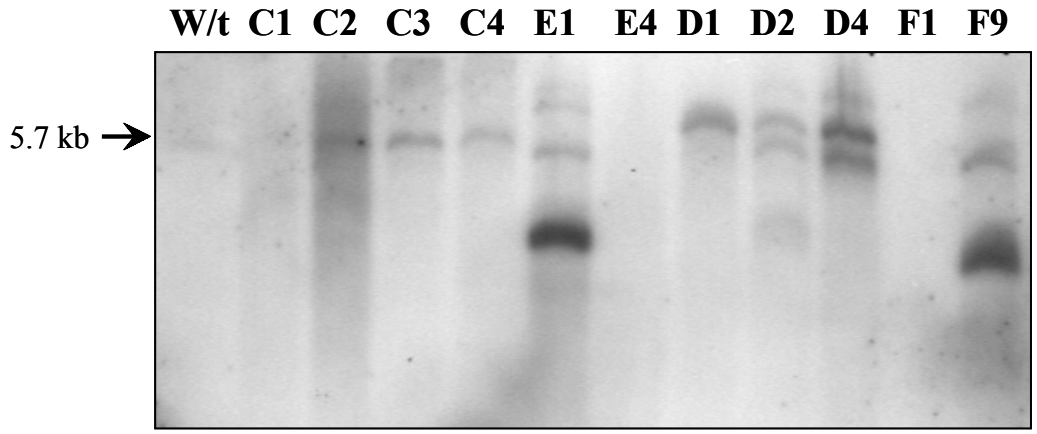


Figure 5.6. Southern blot analysis of wild type and transgenic *A. thaliana* plants. The size of the normal endogenous *At-RLK3* gene is indicated.

The expressed antisense mRNA levels in the transgenic *A. thaliana* lines were next investigated. Instead of the traditional Northern blot analysis using an antisense mRNA probe, an RT-PCR approach was used to quantify the antisense mRNA levels in the different transgenic lines (Fig. 5.7).

During a normal RT-PCR reaction, the first step is the RT of mRNA into first strand cDNA. This step is initiated by a gene-specific primer where after two gene-specific primers are used to exponentially amplify the transcript during the PCR reaction. The reverse transcriptase used is heat sensitive and is denatured during the first PCR step. Normally, both gene-specific primers are included during the preparation of the RT-PCR reaction. An example can be seen in figure 5.8a where the portions of the *At-RLK3* gene encoding the extracellular and kinase domain were successfully amplified using Bovis 3 and 12 and Bovis 30 and 31 respectively when both primers were included from the beginning in the RT-PCR reactions. The sizes of both the amplified fragments corresponded to that predicted (Fig. 5.7).

In wild type plants, the only *At-RLK3* transcript present will be the normal sense mRNA. When a mixture of Bovis 30 and 31 are included in an RT-PCR reaction for *At-RLK3* in wild type plants, a single transcript will be amplified using Bovis 30 as primer for the RT step (Fig. 5.7). Transgenic plants expressing an antisense copy of the *At-RLK3* gene will have a second *At-RLK3* transcript available, namely the antisense mRNA. When the Bovis 30 and 31 mixture is added to the RT-PCR reaction, both sense and antisense mRNAs will be reverse transcribed and amplified. The two amplified fragments will however be indistinguishable from each other since they are the same size.

To distinguish between the amplified sense and antisense cDNAs, a single primer was initially added to each RT-PCR reaction. Once the reverse transcriptase was destroyed during the first denaturation step of the PCR reaction, the second primer was added.

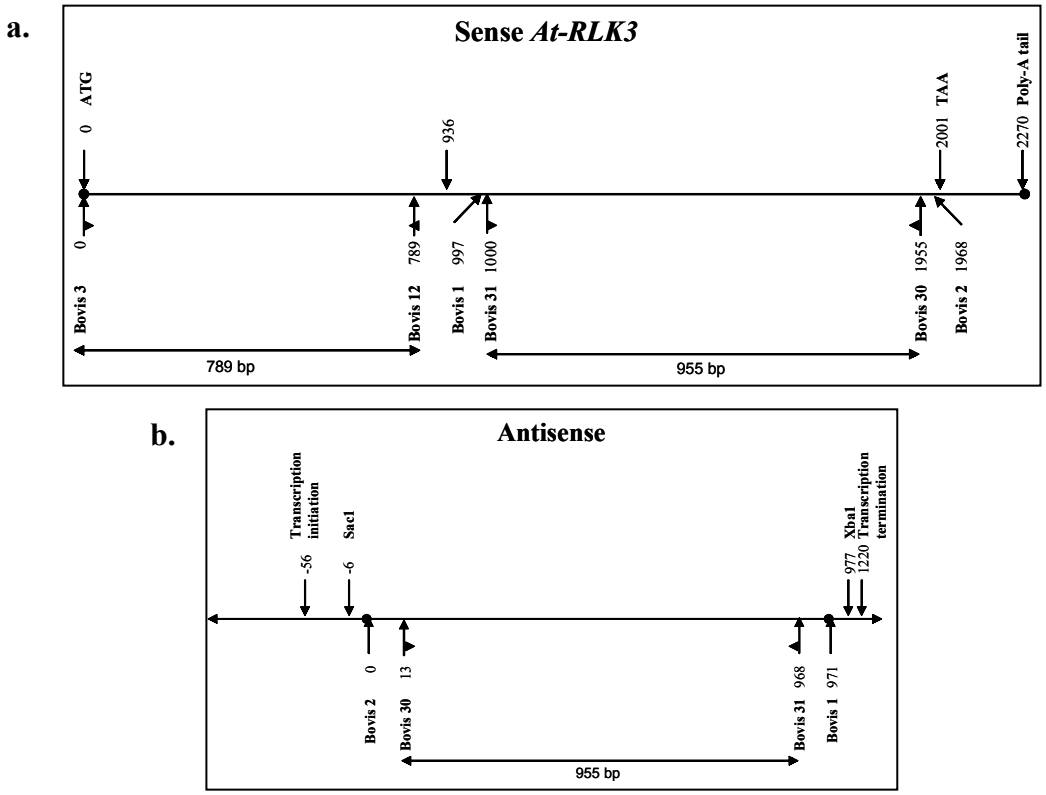


Figure 5.7. RT-PCR strategy for the amplification of antisense *At-RLK3* transcripts. The normal endogenous *At-RLK3* gene is presented in (a) and the portion of the gene encoding the kinase domain in antisense orientation in (b).

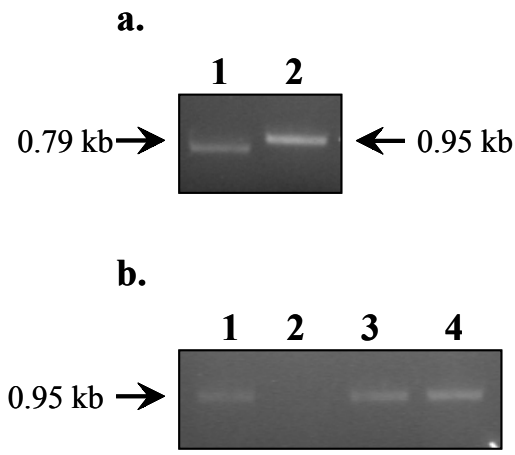


Figure 5.8. Testing of the amplification of antisense transcripts in transgenic plants using RT-PCR. In (a), the portions of the *At-RLK3* gene encoding the extracellular domain (lane 1) and the kinase domain (lane 2) were amplified. In (b), the portion of the gene encoding the kinase domain was amplified from wild type (lanes 1 and 2) and C1 transgenic plants (lanes 3 and 4). In lanes 1 and 3, the Bovis 30 primer was used for the RT-step and in lanes 2 and 4, Bovis 31 was used.

When Bovis 30 and 31 primers were added separately to two different RT reactions using mRNA from wild type plants as template, only Bovis 30 (Fig. 5.7a) managed to initiate the amplification of the *At-RLK3* fragment during the subsequent PCR steps after the addition of Bovis 31 (Fig. 5.8b). The reaction containing Bovis 31 for the RT step did not produce any fragment, since these plants did not express *At-RLK3* in antisense orientation.

When Bovis 30 and 31 were used in similar RT reactions using mRNA from the transgenic C1 plants as template, both reactions produced an amplified fragment (Fig. 5.8b). This meant that the amplified fragment using Bovis 30 as RT primer represent the normal sense mRNA, therefore the fragment found when using Bovis 31 as RT primer, must represent the antisense mRNA being expressed in the transgenic plant (Fig. 5.7b). The fact that it is possible to use this modified RT-PCR technique to discriminate between the sense and antisense mRNAs in the transgenic plants, allowed us to analyze the expression of the antisense *At-RLK3* gene in the different transgenic lines (Fig. 5.9, 5.10).

Using total RNA isolated from the different transgenic lines, the levels of antisense *At-RLK3* mRNA was determined. Transgenic *A. thaliana* lines produced using the pKYLX71:35S plasmid, gave offspring whose phenotypes resembled that of the wild type plants (Fig. 5.9c). This was despite the fact that the antisense mRNA levels differed among the different plants (Fig. 5.9a, b). The highest expression level compared to the 18S rRNA gene was for the C2 and C4 transgenic lines and the lowest for E4.

The transgenic lines produced using the pTA7002 plasmid yielded three lines that had completely different phenotypes compared to the wild type (Fig. 5.10c). The first objective was to correlate the observed phenotypes with the expression level of the antisense gene. This was firstly done on plants not treated with DEX, since the plasmid does allow basal levels of expression of the transferred gene (Aoyama and Chua, 1997).

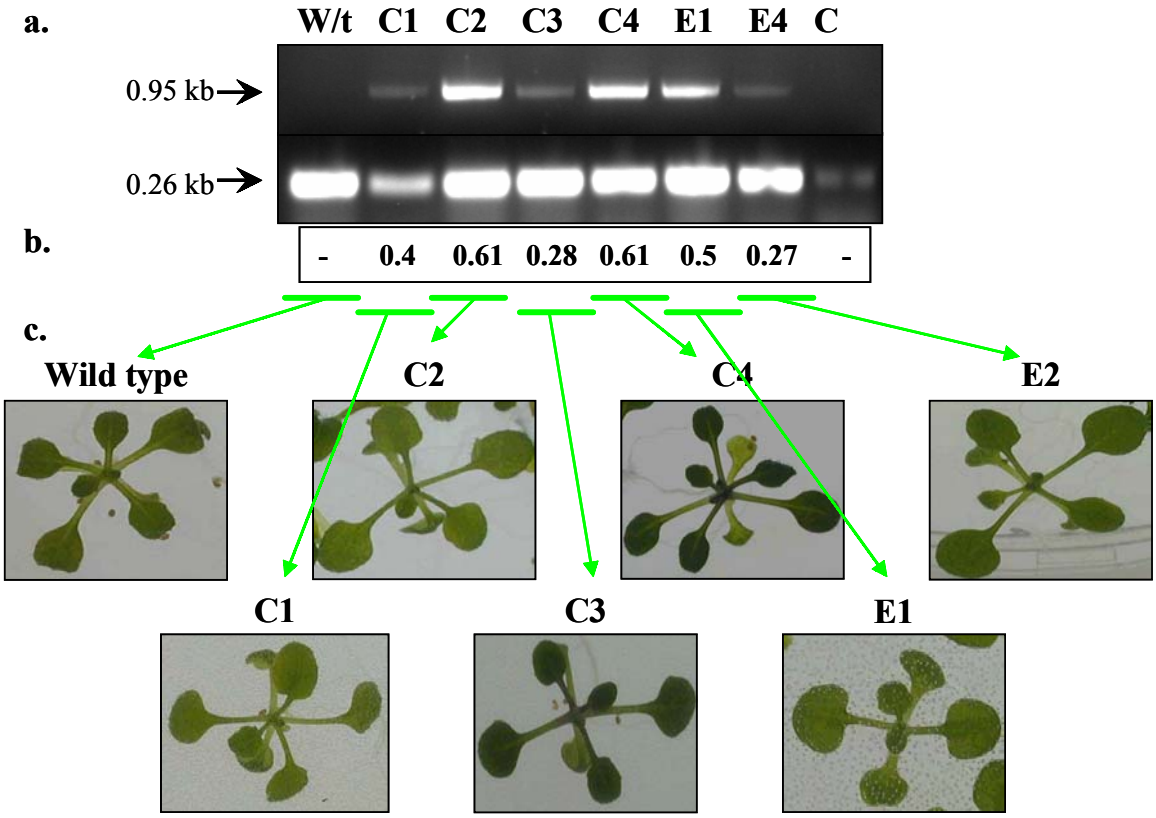


Figure 5.9. Determination of antisense *At-RLK3* mRNA levels in transgenic plants generated by using the pKYLX71:35S binary vector. (a) represents the amplified antisense mRNA encoding the kinase domain of *At-RLK3* in the top panel and the amplified 18S rRNA gene in the bottom panel. In (b), the antisense *At-RLK3* yield was expressed relative to that obtained for the 18S rRNA fragment, while in (c) the phenotypes of the different transgenic plants are indicated.

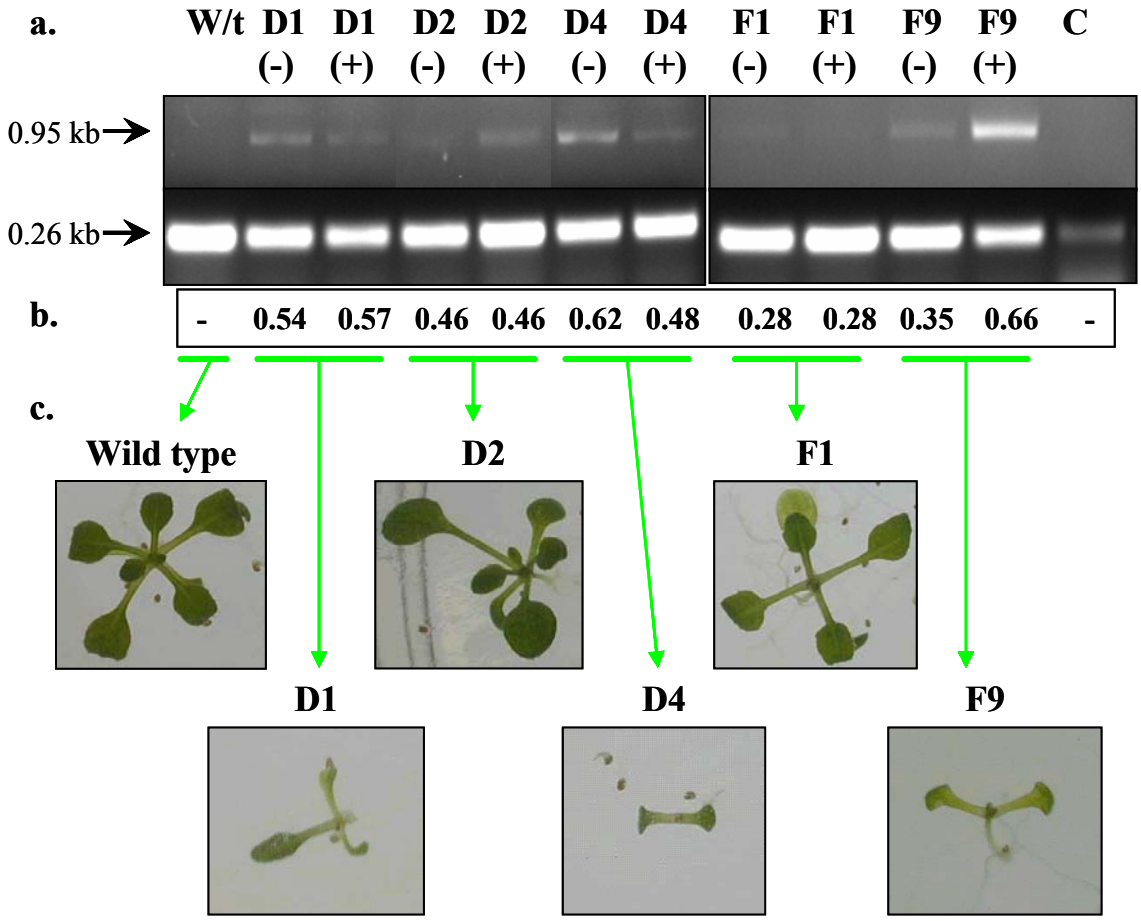


Figure 5.10. Determination of antisense *At-RLK3* mRNA levels in transgenic plants generated by using the pTA7002 binary vector. (a) represents the amplified antisense mRNA encoding the kinase domain of *At-RLK3* in the top panel and the amplified 18S rRNA gene in the bottom panel. In (b), the antisense *At-RLK3* yield was expressed relative to that obtained for the 18S rRNA fragment, while in (c) the phenotypes of the different transgenic plants are indicated.

In the absence of DEX, the transgenes were expressed at various levels within the plants (Fig. 5.10a, b). The highest expression level was found for D4 and the lowest for F1, which was very similar to the expression levels obtained for the transgenic plants that expressed the gene constitutively.

No correlation was found between the phenotype and antisense expression levels of the transgenic plants (Fig. 5.10). F9 had the second lowest expression level of antisense mRNA, yet still produced the abnormal phenotype, while D4, that had a higher antisense mRNA expression level than F9, exhibited a normal phenotype. In addition, C2 and D4 gave the highest expression levels of antisense *At-RLK3* mRNA, yet C2 exhibited a normal phenotype and D4 the abnormal phenotype (Fig. 5.9, 5.10). It is therefore safe to assume that the altered phenotypes of the transgenic plants produced using the pTA7002 plasmid were not due to the high antisense mRNA levels present in the plant, but most probably due to another factor.

Interestingly, in another unrelated project, *At-RLK3* was over-expressed in *A. thaliana* using the pTA7002 plasmid (S Lategan, unpublished results). The transgenic lines obtained also exhibited these aberrant growth characteristics as was found in this study. A possibility is that the pTA7002 plasmid could play a role, since no altered phenotypes were observed in the plants generated using pKYLX71:35S (Fig. 5.9). It is thus possible that the transferred pTA7002 DNA fragment shares homology with a region located within a gene that is crucial for plant development. The insertion of the transferred DNA segment would therefore interrupt the coding region of this gene which would lead to an inactive enzyme and consequently an altered phenotype. This could easily be resolved by sequencing the regions flanking the inserted fragment and determining whether a coding sequence was indeed interrupted.

For the continuance of the project, it was decided not to use any of the transgenic plants generated with pTA7002 for further analysis because of the altered phenotypes. A second reason for the decision was the fact that only F9 showed induced expression of the transgene after treatment with DEX (Fig. 5.10a, b), but since it exhibited the altered

phenotype, it was deemed unsuitable for further analysis. It is however possible that the other lines could also have shown inducible expression, but only later-on. This was not tested.

It was thus decided to use C4 for all subsequent analyses. This transgenic line expressed an antisense copy of the portion of the *At-RLK3* gene encoding the kinase domain, displayed the highest expression levels of antisense mRNA and still exhibited a normal phenotype.

5.4.4 Defining a function for At-RLK3

From the start, the aim was to define a function for At-RLK3 in *Arabidopsis*. After a detailed expression analysis (Chapter 3), it was found that the expression of *At-RLK3* is upregulated by various conditions including pathogen invasion, osmotic stress, ozone stress and salicylic acid. Since oxidative stress in the form of H_2O_2 forms a critical component of all the above-mentioned stress conditions, the effect of H_2O_2 treatment on *At-RLK3* expression was tested. The application of H_2O_2 to a cell suspension culture led to a rapid and dramatic induction of *At-RLK3* gene expression (Fig. 3.15). Furthermore, At-RLK3 is also actively phosphorylated by oxidative and osmotic stress conditions (Fig. 4.10, 4.11). All these results therefore implicated a role for At-RLK3 in the early reaction of the plant towards the presence of H_2O_2. This involvement could be either as a receptor protein since At-RLK3 is plasma membrane bound (Fig. 4.9) or as part of a larger signal perception and transduction complex.

To test this hypothesis, wild type and C4 transgenic plants were treated with H_2O_2 (Fig. 5.11) and SA (Fig. 5.12) respectively. The ability of the plant to react to these compounds was evaluated by determining the activation of the plant defense response, since both compounds are known to activate the plant defense reaction (Chen *et al.*, 1995; Tanaka *et al.*, 2003). This defense activation was determined by RT-PCR of the *PR-2* gene, a marker for the defense reaction (Uknes *et al.*, 1993).

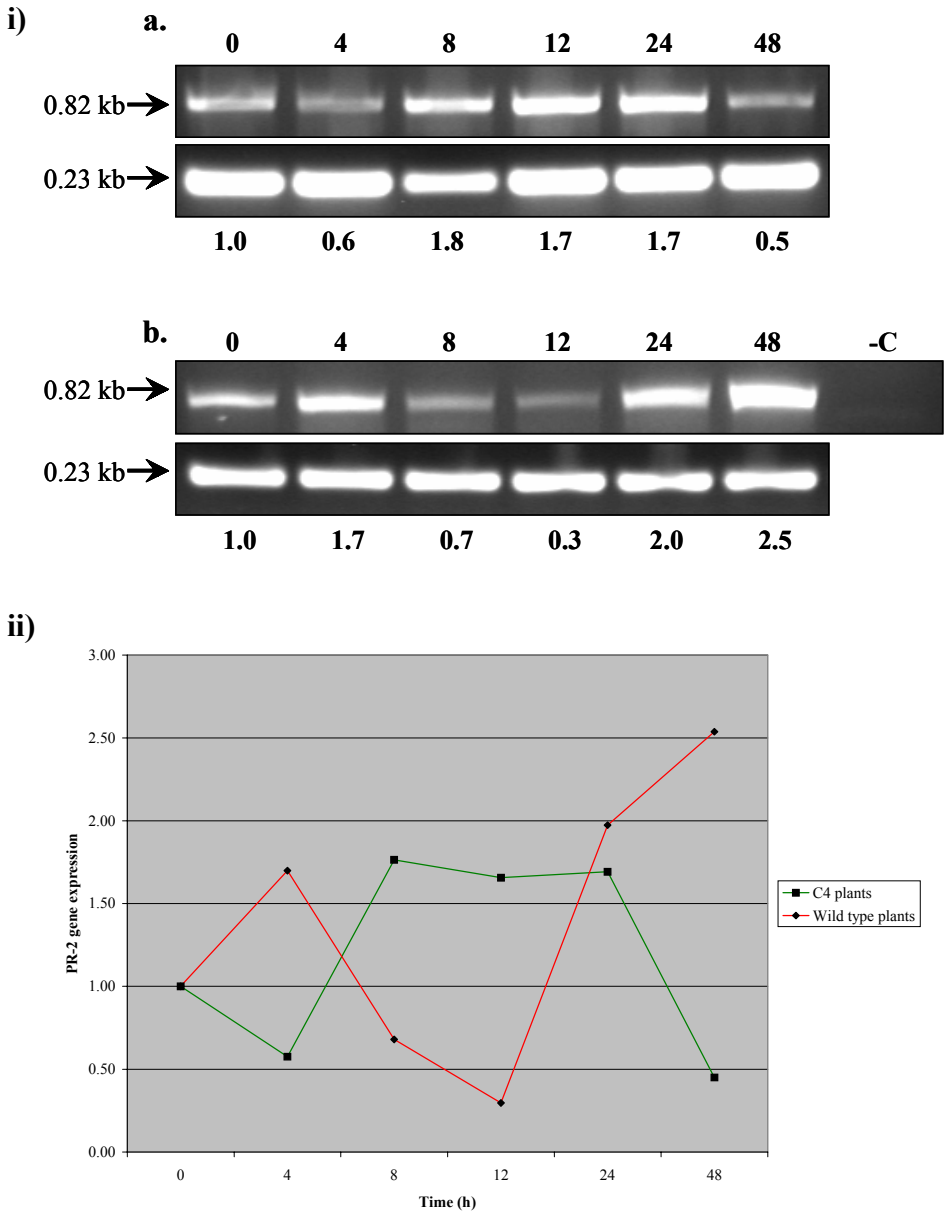


Figure 5.11. *PR-2* gene expression in C4 transgenic and wild type plants when treated with 1 mM H_2O_2. i) RT-PCR analysis of *PR-2* gene expression in (a) C4 transgenic plants and (b) wild type plants treated with 1 mM H_2O_2. The amplified fragment in the top panel represent *PR-2* and the bottom panel the amplified 18S rRNA. (ii) A graphical representation of the PR-2 gene expression after treatment.

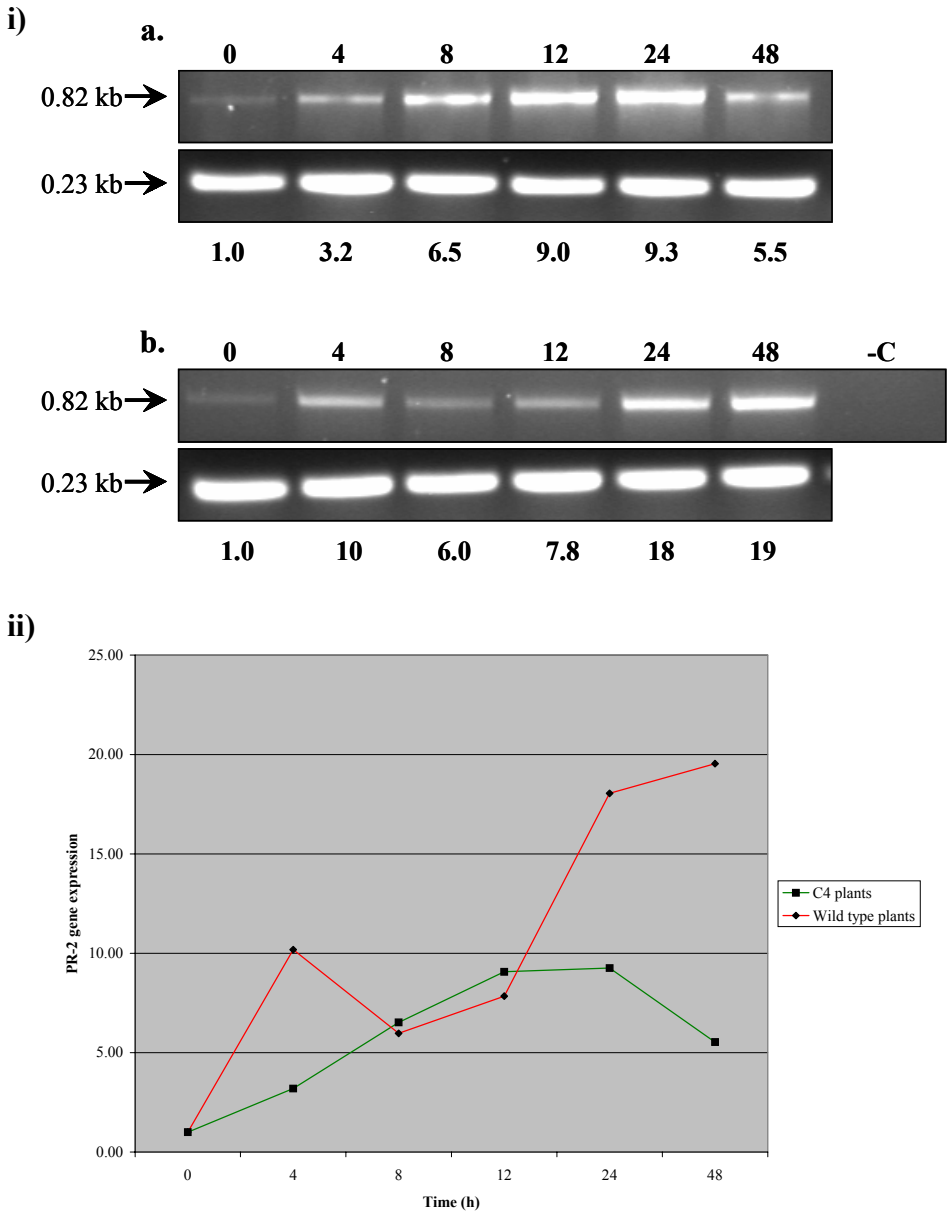


Figure 5.12. *PR-2* gene expression in C4 transgenic and wild type plants when treated with 200 µM SA. i) RT-PCR analysis of *PR-2* gene expression in (a) C4 transgenic plants and (b) wild type plants treated with 200 µM SA. The amplified fragment in the top panel represent *PR-2* and the bottom panel the amplified 18S rRNA. (ii) A graphical representation of the PR-2 gene expression after treatment.

When treated with 1 mM H_2O_2, the wild type plant reacted within 4 h by almost doubling the expression of *PR-2* (Fig. 5.11). In contrast, in the C4 plants the expression of *PR-2* was reduced by almost half that at time 0 resulting in an almost 3 fold difference of *PR-2* expression between the two plants. The expression then recovered in the C4 plants reaching a maximum expression level between 12 and 24 h after treatment where after it declined to its lowest level at 48 h. Following the initial doubling of *PR-2* gene expression in the wild type plants, the expression level decreased up to 12 h, followed by a pronounced induction of expression reaching a maximum at 48 h.

Thus, whereas *PR-2* expression is on the increase 48 h after treatment in the wild type plants, the C4 plants displayed a decrease. This indicates that an effectively induced defense system was present in the wild type plants, while in the transgenic plants, the defense reaction was terminated. A similar situation was found when both plants were treated with 200 μM SA (Fig. 5.12). Even though both reacted with induced *PR-2* gene expression 4 h after treatment, the levels in the wild type plant were about 3 times higher than that of the C4 transgenic plants, indicating a much better initial response to the presence of SA. Again, as was the case with H_2O_2, the transgenic plants reacted with a gradual increase in *PR-2* gene expression, but at 48 h, the expression levels started to decrease. The wild type plants on the other hand showed a temporary decrease in expression, but starting at 8 h, the expression increased almost 4 fold to the highest levels at 48 h, again indicating the establishment of an effective defense response.

It is thus clear that by removing at least a portion of the At-RLK3 protein from the plant cell using the antisense mRNA, the activation of the plant defense reaction is severely affected, but not totally abolished. It is however important to mention that in a future experiment, the reduced At-RLK3 levels in the C4 transgenic plants must be confirmed using Western blots, since antisense mRNA levels on their own is not an accurate reflection of the protein levels within the cells. At-RLK3 could therefore fulfill a primary role in the signal detection and transduction process. The relevance of these results will be discussed in Chapter 6 where a function for At-RLK3 will be proposed.

5.5 Summary

When At-RLK3 is absent from *Arabidopsis*, the establishment of an effective defense system is affected. When treated with H_2O_2 and SA respectively, C4 plants displayed a transient activation of the defense response that seems to be terminated 48 h after treatment, while in the wild type plant this defense response is further strengthened at the corresponding stage. The early reaction of the plants towards the presence of the two compounds also differs. Since H_2O_2 forms part of the response of plants towards SA treatment, it clearly indicates that H_2O_2 and At-RLK3 is involved in a very close relationship within *A. thaliana* to fulfill a crucial role.

Chapter 6

Discussion

Discussion

6.1 Introduction

Being sessile, plants over millennia have developed a complex and finely tuned defense response allowing them to adjust to all types of adverse conditions. These include adaptations to pathogen attacks and environmental and developmental changes. It is only during the last four or five years that researchers are starting to understand the complexity of the adaptations that plants undergo in order to survive.

Part of the complexity of the defense reaction is that several seemingly unrelated events occurring in plants might share common elements (Hancock *et al.*, 2002). This is especially true when one considers the extensive cross talk that occurs during different signaling events where ROS and SA play a role (Bowler and Fluhr, 2000). Any attempt to assign a definite function for At-RLK3 in the defense reaction of *A. thaliana* must therefore be done with great caution. It is also true that a lot more research must be done before a precise function can be given. This discussion would therefore attempt to give a putative function for the protein based on the results obtained during this study, as well as the latest literature.

6.2 ROS and SA interactive signaling

During 2003 and the first half of 2004, several excellent articles appeared where it was shown that both SA and ROS are key components of an integrated defense reaction of plants subjected to various stresses, including pathogens and abiotic stresses.

When plants are exposed to pathogens, plasmamembrane bound NADPH-oxidases, cell-wall bound peroxidases and apoplastic amine oxidases produce ROS in the form of O_2^- and H_2O_2 (Allen and Fluhr, 1997; Desikan *et al.*, 1998; Hammond-Kosack and Jones, 2000). O_2^- cannot enter the cell through diffusion, but is converted to H_2O_2 by superoxide dismutase. Besides being used for cell wall strengthening by peroxidases, H_2O_2 can also enter the cell via diffusion, perhaps facilitated by peroxiporines (Henzler and Steudle, 2000) to activate many plant defenses including PCD (Desikan *et al.*, 1998; Dangl and Jones, 2001). H_2O_2 is also responsible for the activation of PAL that is involved in SA biosynthesis (Desikan *et al.*, 1998). During different

abiotic stresses, the chloroplast together with the mitochondrion, act as sources of ROS production (Foyer and Noctor, 2003; Tiwari *et al.*, 2002). These conditions include temperature extremes, ozone, dehydration and drought (Prasad *et al.*, 1994; Guan *et al.*, 2000).

In addition to the active synthesis of ROS, the inactivation of several ROS scavengers by SA and NO also leads to elevated ROS levels (Klessig *et al.*, 2000). There is thus a clear involvement of SA in the accumulation of ROS, as well as the other way round, indicating a very close relationship between these molecules within the plant defense response.

ROS signaling can be active on three different levels (Apel and Hirt, 2004). On the first, a sensor that detects a rise in ROS levels can be directly activated by ROS that subsequently change gene expression levels. Secondly, ROS can change the activity of different components of the signal transduction pathway by directly oxidizing the proteins. Finally, the direct regulators of transcription, namely transcription factors, can also be targeted and modified to change their activation status.

As yet, no sensor for ROS has been confirmed in plants. In both prokaryotic organisms and yeast, two component signaling systems consisting of a histidine kinase and a response regulator in the form of a transcription factor, acts as redox sensors (Singh, 2000). Upon detecting external ROS, the transmembrane kinase autophosphorylates on an internal histidine residue, then transfers the phosphate group to an aspartate residue within the response regulator thereby activating the transcriptional activity of the protein, leading to induced gene expression. Even though the *Arabidopsis* genome project revealed the presence of various two-component histidine kinases, none has at this stage been proven to act as a redox sensor (Hwang *et al.*, 2002).

In fungi and plants, the two-component sensors form part of a larger protein complex involving MAPK signaling components (Gustin *et al.*, 1998). In plants it was shown that exogenously applied H_2O_2 activates several MAPKs (Desikan *et al.*, 1999, 2001; Kovtun *et al.*, 2000; Jonak *et al.*, 2002). Two of these are activated directly via a MAP kinase kinase kinase (MAPKKK) called ANP1 (Kovtun *et al.*, 2000). It was

thus suggested that various stresses induce the production of ROS which in turn can then activate various MAPK signaling cascades (Apel and Hirt, 2004). In addition to MAPKs, H_2O_2 was also shown to modulate the activity of mammalian protein phosphatases by oxidizing thiol groups within the proteins. This modulation is done by oxidizing a cysteine within the active site of the enzyme (van Montfort *et al.*, 2003).

SA, JA and ethylene accumulate in response to pathogen infection or herbivore damage leading to the induced expression of both distinct and overlapping signaling and defense genes (Glazebrook *et al.*, 2003). This led to a hypothesis that plants are able to differentially activate defense responses based on the particular organism that was detected (Pieterse and van Loon, 2004).

In tobacco, a lipase called SA binding protein 2 (SABP2) acts as a binding protein for SA that activates the enzyme upon binding (Kumar and Klessig, 2003). Cells that undergo SAR accumulate SA, resulting in the upregulation of a group of *PR* genes (van Loon and van Strien, 1999). This activation is via the action of NPR1 (Kinkema *et al.*, 2000) that binds TGA transcription factors (Zhang *et al.*, 2003). Other transcription factors that can act as targets for redox signaling, is the WRKY family, since they contain a redox sensitive zinc finger DNA binding domain that could be the direct target of H_2O_2 (Eulgem *et al.*, 2000). The target *cis*-acting elements, bound by H_2O_2 induced transcription factors on the promoter regions of genes, are still unclear. However, several potential H_2O_2 responsive *cis*-acting elements were proposed (Desikan *et al.*, 2001).

It is thus clear from the above that cellular signaling induced by both SA and H_2O_2 is very complex and overlap significantly. With this report, a direct role for At-RLK3 in these signaling events will be stated, most probably as the redox sensor that detects extracellular H_2O_2 levels. Whether the enzyme works alone or as part of a larger enzyme complex will be discussed.

6.3 At-RLK3 is a typical plant receptor-like protein kinase

At-RLK3 codes for a typical RLK protein. The gene exists as a single copy within the *A. thaliana* genome (Fig. 3.8) similar to the majority of other RLKs. It is located on chromosome 4 and forms part of a large gene cluster where all the encoded protein products share a conserved motif and are SA-responsive (Ohtake *et al.*, 2000). It was proposed that all RLKs involved in plant defense are clustered together on a particular chromosomal region (Komjanc *et al.*, 1999), suggesting a role for At-RLK3 in plant defense.

The gene has seven exons and six introns with the first exon coding for the whole of the extracellular domain of the protein (Fig. 3.1). This is very similar to several other RLKs (Stein *et al.,* 1991; Tobias *et al.*, 1992; Dwyer *et al.*, 1994; Wang *et al.*, 1996; Suzuki *et al*., 1997). An in-frame stop codon is present within the first intron (Fig. 3.19), suggesting that multiple transcripts could be formed from this gene. However, in contrast to other genes containing the in-frame stop codon (Stein *et al.*, 1991; Tobias *et al*., 1992; Chang *et al.*, 1992; Dwyer *et al.*, 1994; Delorme *et al.*, 1995; Suzuki *et al.*, 1997) which produce multiple transcripts (Stein *et al.*, 1991; Giranton *et al*., 1995; Tobias and Nasrallah, 1996; Wang *et al.*, 1996), *At-RLK3* was expressed as a single 2242 bp transcript.

At-RLK3 has the typical three-dimentional structure of a plant RLK with four characteristic domains being present (Fig. 3.2). The first two are both stretches of hydrophobic amino acids containing several other characteristic basic amino acids located at critical regions of the polypeptide. The first is a typical signal peptide located at the amino terminal region of the protein, predicting that the protein will be exported to the plasma membrane via the ER. The second hydrophobic stretch of amino acids is a typical transmembrane domain located in the center of the protein (Weinstein *et al*., 1982). Both these regions predict that At-RLK3 is plasma membrane bound. This was confirmed when a Western blot showed that the protein co-purified with the plasma membrane fraction of *A. thaliana* plant cells (Fig. 4.9). This was similar to several other RLKs that were shown to be located at the plasma membrane (Chang *et al.*, 1992; He *et al.*, 1996; Stein *et al.*, 1996; Ahmed *et al.*, 1997;

Feuillet *et al.*, 1998; Jinn *et al.*, 2000; Kim *et al.*, 2000b; Friedrichsen *et al.*, 2000, Nam and Li, 2002).

The intracellular region of At-RLK3 is a typical protein kinase domain with all 11 conserved subdomains as described by Hanks and Quinn, (1991) being present (Fig. 3.3). This region shared very high homology with several other RLKs indicating a possible evolutionary relationship between them (Fig. 3.3, 3.4). Kinase activity was proven for both the purified recombinant At-RLK3 kinase domain (Fig. 4.5a) and a 70 kDa polypeptide that was immunoprecipitated using At-RLK3 specific antibodies (Fig. 4.5b). Subdomains VIb and VIII both have the consensus amino acid sequences characteristic of a serine/threonine specific protein kinase (Fig. 3.3) which was proven using thin-layer plate chromatography (Fig. 4.6b). Except for PRK1 showing dual tyrosine and serine phosphorylation specificity (Mu *et al.*, 1994), all other RLKs thus far described, exhibit serine/threonine specificity (Table 2.1) again indicating that At-RLK3 is a typical plant RLK. One of the few differences between At-RLK3 and other RLKs, was the fact that the enzyme preferably used Mg^{2+} as cofactor (Fig. 4.6a), whereas all the other tested RLKs prefer Mn^{2+} (Schaller and Bleecker, 1993; Horn and Walker, 1994; Schulze-Muth *et al.*, 1996; Takahashi *et al.*, 1998; Nishiguchi *et al.*, 2002).

The extracellular region of At-RLK3 revealed the presence of two DUFs, regions that have a conserved amino acid sequence which at this stage still do not have a defined function (Fig. 3.5). These same regions were also found in several other RLKs (Takahashi *et al.*, 1998; Lange *et al.*, 1999; Ohtake *et al.*, 2000; Du and Chen, 2000). Located within the two DUF regions were two conserved cysteine-rich regions with a consensus C-X8-C-X2-C amino acid sequence (Fig. 3.2) as described by Takahashi *et al.*, (1998). The cysteines present within these regions are thought to be involved in the formation of disulphide bridges (Ohtake *et al.*, 2000). In addition, the C-X2-C part of the motif can also act as a potential binding site for transition metal ions (Dykema *et al.*, 1999). RLKs containing this sequence were shown to be SA responsive (Ohtake *et al.*, 2000). This led the authors to suggest that genes containing this motif are involved in SA-mediated plant defense responses.

Once the physical characteristics of *At-RLK3* and its encoded polypeptide were confirmed, it was important to determine which aspects of plant life involved At-RLK3. This was done by doing a detailed analysis of *At-RLK3* gene expression, as well as At-RLK3 activation.

6.4 At-RLK3 and H_2O_2: two partners dancing together

The intimate relationship between H_2O_2 and At-RLK3 became apparent during the expression analysis of *At-RLK3*.

Upon treatment with H_2O_2, a dramatic induction of gene expression was found within 30 minutes after adding H_2O_2 to the cell suspension culture (Fig. 3.15a, 3.18b). This was matched both in speed and induction level when the cells were treated with menadione (Fig. 3.15b, 3.18c), a reagent that leads to the production of ROS within cells (Timoshenko *et al.*, 1999). Since H_2O_2 forms part of the elevated ROS levels after menadione treatment, it is safe to assume that the induction of *At-RLK3* expression by menadione is due to H_2O_2. This clearly indicated a direct role for At-RLK3 in the response of *A. thaliana* during oxidative stress conditions.

This hypothesis was further strengthened when it was found that *At-RLK3* expression was also induced under a variety of other stress conditions. Changes in the osmotic potential of the cellular environment led to the induced expression of *At-RLK3*, be it hyper-osmotic (Fig. 3.9b, 3.18e) or hypo-osmotic conditions (Fig. 3.9d). The latter result must however be taken with caution, since this particular treatment could also have led to changes in other factors, such as the amount of carbon available. Even when proline, a plant osmolyte involved in the regulation of the osmotic status of plant cells (Savouré *et al.*, 1997) was added, the expression of *At-RLK3* was induced. Any change in the osmotic potential of cells leads to alteration of the redox state of the chloroplasts (Foyer and Noctor, 2003), leading to elevated intracellular ROS levels. This includes H_2O_2, thereby again emphasising the role of At-RLK3 due to the presence of H_2O_2.

Interestingly, osmotic stress in the form of NaCl did not dramatically induce the expression of *At-RLK3* (Fig. 3.9c, 3.10a). Salt stress imposes both an ionic and an

osmotic stress (Pasternak, 1987) with one of the targets within the plant being photosynthesis (Leung *et al.,* 1994). This results in elevated intracellular ROS levels (Price and Hendry, 1991). A possible reason for this lack of induced expression might be that the salt concentration used was toxic for the cells. It seemed as if the cells were eventually killed since even the actin levels decreased during the treatment (Fig. 3.9c). It is however also possible that during salt stress, the combination of ROS produced is different compared to that produced during osmotic stress, since the nature of the stress caused by different factors, differ (Laloi *et al.*, 2004). It is therefore possible that because of the ionic component of salt stress, the ROS produced contain very little H_2O_2, leading to a very low level of *At-RLK3* induced expression.

Another distinctive anomaly was that ABA did not induce *At-RLK3* expression (Fig. 3.11e). This was despite the fact that ABA and osmotic stress are tightly linked with ABA accumulating in response to water stress (Jensen *et al*., 1996). In addition, in guard cells, ABA stimulated H_2O_2 production induces the closure of stomata (Pei *et al.*, 2000). It was however proposed that Ost1, a protein kinase, functions between ABA perception and ROS signaling in the guard cells, thereby possibly making At-RLK3 redundant (Mustilli *et al.*, 2002). The interaction between ABA and H_2O_2 therefore seems to be tissue specific and may depend on many factors, making this signaling event very difficult to interpret (Neill *et al.*, 2002).

This was highlighted when it was shown that while GA treatment led to increased H_2O_2 levels by inactivating antioxidant enzymes such as catalase, ABA had the opposite effect (Fath *et al.*, 2001). This pattern was reflected in the expression levels of *At-RLK3* when treated with ABA and GA respectively (Fig. 3.11c, e). GA treatment led to the increased expression of *At-RLK3* whereas ABA did not, most probably due to the absence of increased H_2O_2 levels. Treatments with both IAA and BAP also did not lead to a dramatic increase in *At-RLK3* expression (Fig. 3.11a, d), whereas ACC, a precursor for ethylene, only led to a substantial increase in expression 4 h after treatment (Fig. 3.11b). This could be better explained if one considers the expression pattern of JA (Fig. 3.14b), since both ethylene and JA function in the same signaling pathway in response to herbivorous insects and wounding (Ton *et al.*, 2002).

When one considers the role of the two signaling molecules, SA and JA, only SA induced *At-RLK3* expression (Fig. 3.14a, 3.18f). SA mediates the oxidative burst that leads to cell death during the HR after pathogen infection (Shirazu *et al.*, 1997). In addition, SA also plays a major role in modulating the response of plants to various abiotic stresses (Senaratna *et al.*, 2000). During salt and osmotic stress, SA is directly involved in a feedback amplification cycle in concert with ROS (Jabs, 1999; Borsani *et al.*, 2001). SA was also implicated to activate SAR (Uknes *et al.* 1993) and HR (Mauch-Mani and Slusarenko, 1996) by mediating the oxidative burst leading to cell death (Shirasu *et al.*, 1997). SA application was therefore expected to induce the expression of *At-RLK3*, since H_2O_2 forms part of this oxidative burst. Similarly it was shown that exposure to ozone leads to increase levels of ROS with H_2O_2 being one of them (Langebartels *et al.*, 2000). It was therefore not strange that exposure of seedlings to ozone led to increased *At-RLK3* expression (Fig. 3.18d), since SA is also implicated in the response of plants to ozone (Yalpani *et al.*, 1994).

The fact that JA did not induce the expression of *At-RLK3* (Fig. 3.14b) could stem from the fact that SA has an antagonistic effect on JA signaling (Pieterse *et al.*, 2001). This is mediated through cytosolic NPR1 to ensure that during SA mediated SAR, JA signaling is repressed to ensure efficient SA-dependent resistance (Spoel *et al.*, 2003).

Oxygen deprivation of plants leads to the activation of NADPH oxidase via the so-called Rop signaling (Baxter-Burrell *et al.*, 2002). The elevated H_2O_2 levels eventually deactivate Rop, indicating again a role for H_2O_2 in the adaptation of the plant towards anoxia. As was found, hypoxia led to an induction of *At-RLK3* expression, albeit not very substantial (Fig. 3.17b).

The oxidative burst plays a very important role during the response of plants towards pathogen infection. During pathogen infection, enhanced enzymatic reactions lead to the active production of ROS (Hammond-Kosack and Jones, 2000). In addition, SA and NO are responsible for the inactivation of various antioxidant proteins, leading to even higher ROS levels and PCD (Klessig *et al.*, 2000).

In the case of an incompatible interaction whereby disease resistance will occur, the oxidative burst is biphasic (Baker and Orlandi, 1995). The initial and very rapid accumulation of H_2O_2 is followed by a second and prolonged stage of H_2O_2 production. In contrast, compatible interactions only exhibit the first transient accumulation of H_2O_2. This difference could therefore explain the expression pattern of *At-RLK3* in different plant-pathogen interactions (Fig. 3.16). In the incompatible interaction between avirulent *Ralstonia* and resistant *A. thaliana* Nd-1, the expression of *At-RLK3* is induced, while in the compatible interaction, no such induction profile was found (Fig. 3.16a). This activation of expression occurred 12 h after infiltration and most probably represents the second prolonged oxidative burst which is absent in the compatible interactions. It is thus possible that the first short oxidative burst was missed due to the long time intervals between sampling. During infection with *Pseudomonas*, increased expression of *At-RLK3* was found in all three interactions, be it compatible or incompatible (Fig. 3.16b). The reason for this discrepancy might be located in the nature of the respective pathogens (Deslandes *et al.*, 1998). These results clearly indicate that the induced expression of *At-RLK3* during these different interactions was due to the elevated H_2O_2 levels.

In contrast to the induced expression of *At-RLK3*, three different treatments led to decreased *At-RLK3* expression. When both methionine and valine were added to the cell suspension cultures, the expression decreased (Fig. 3.12b, c). This was also found during the heat shock response when the cell suspensions were subjected to elevated temperatures (Fig. 3.17a), clearly indicating that At-RLK3 plays no role in cellular activities associated with these conditions.

The expression pattern of *At-RLK3* therefore indicated that all conditions involving elevated ROS levels induce the expression of *At-RLK3*. Since H_2O_2 forms part of ROS, and since H_2O_2 application on its own leads to induced *At-RLK3* expression, it is safe to say that H_2O_2 is the primary agent involved with At-RLK3. Thus far, no other RLK exhibited such a complex expression pattern (Table 2.1).

The expression of several RLKs is induced upon SA treatment (Table 2.1). In addition to SA, pathogenic organisms and wounding also induce the expression of *SFR2*, *LRPKm1* and *Wak1* (Pastuglia *et al.*, 1997; Komjanc *et al.*, 1999; He *et al.*,

1998). This indicates that a factor common to all three stimuli could regulate the expression of these genes. In contrast to *At-RLK3*, the expression of *RPK1* was induced upon ABA treatment, dehydration and salt stress, but repressed upon GA treatment (Hong *et al.*, 1997). It is thus possible that while At-RLK3 acts as a receptor protein under ABA-independent osmotic stress signaling, RPK1 could play a role during the ABA-dependent signaling of osmotic stress, specifically during salt stress. Even though the expression of some RLKs was shown to be induced upon H_2O_2 treatment (Desikan *et al.*, 2001), none have been studied in detail thus far.

The expression profiles of *At-RLK3* corresponded very well with the presence of several conserved *cis*-acting elements present in the promoter region of the gene (Table 3.1). The *At-RLK3* promoter has a number of sites that confers dehydration regulated expression of the gene. Included are both MYC and MYB recognition sites, which could regulate the osmotic responsive expression of *At-RLK3*. Also present, were three elements linked to ABA-responsiveness even though *At-RLK3* expression was not induced upon ABA treatment (Fig. 3.11e). This again emphasizes the complex regulation of osmotic adaptation in plants.

Finally, two potential SA responsive elements were also found (Table 3.1). Again, this was in accordance with the expression pattern of *At-RLK3*, since expression of the gene was induced upon SA treatment. Another important site was the *as-1* site (TGACG motif). This motif was shown to be activated by SA through different oxidative species, excluding H_2O_2 that did not play a role in the activation (Garretón *et al.*, 2002). Even though genes containing the *as-1* motif were shown to be induced by H_2O_2, the motif was thought not to be involved (Dai and An, 1995). The activation of gene expression by H_2O_2 must therefore be through another as yet unknown element. The presence of this motif on the promoter region however strongly indicated that *At-RLK3* expression could be regulated by the NPR1/TGA interaction that will be described later on.

The induced expression of *At-RLK3* was also evident on protein level (Fig. 4.8). H_2O_2, menadione and ozone treatments led to a transient increase in protein levels. In the case of H_2O_2, the levels increased shortly after treatment only to decrease 60 min later. A similar increase was also found in plants treated with SA, but only at a much

later stage. This clearly indicates the very swift response of the plant when treated with H_2O_2, but a much slower response after SA treatment. Since the induced expression pattern of *At-RLK3* matched the increase in polypeptide levels, the level of At-RLK3 regulation is on transcriptional level.

In addition to being inducibly expressed by H_2O_2, At-RLK3 was also activated when intact plants were sprayed with H_2O_2 (Fig. 4.11). This activation was within 5 minutes after spraying, indicating a very rapid response of the enzyme towards the sprayed H_2O_2. H_2O_2, menadione and osmotic stress led to the activation of several protein kinases (Fig. 4.10), including a 69 kDa polypeptide. Since At-RLK3 is 70 kDa in size and activated by H_2O_2, it is thus safe to assume that this polypeptide represents At-RLK3. Significantly, SA treatment of a cell suspension culture did not lead to the activation of this 69 kDa protein (results not shown), indicating that At-RLK3 is not activated shortly after SA treatment.

When all these results were collectively analysed, it led to the hypothesis that At-RLK3 could act as a redox sensor protein responsible for the detection of exogenous H_2O_2. This hypothesis will be discussed next by analysing the results obtained when wild type and transgenic plants were treated with H_2O_2 and SA respectively.

6.5 Could At-RLK3 act as a redox sensor in *A. thaliana*?

When treated with H_2O_2 and SA, the expression of *PR-2* is induced as part of the onset of SAR (Lamb and Dixon, 1997). This induced expression was exploited to test the role of At-RLK3 in wild type and transgenic plants expressing an antisense copy of *At-RLK3* when treated with H_2O_2 and SA respectively.

Wild type plants showed a biphasic induction of *PR-2* gene expression when treated with both H_2O_2 (Fig. 5.11) and SA (Fig. 5.12). After an initial activation of expression 4 h after treatment, a transient decrease was found. This induced expression was indicative of two effective and functional signaling events taking place within the cells allowing the activation of *PR-2* expression. In the case of H_2O_2, the induced expression was most probably through an active redox sensor protein leading to a 1.7 fold induction of expression. Since SA is bound by the lipase SABP2

receptor (Kumar and Klessig, 2003), a different signaling pathway was most probably used leading to a 10 fold induction of expression.

After the first induction of *PR-2* gene expression, a stronger induction was found 48 h after treatment (Fig. 5.11, 5.12). This activation was most probably due to several proposed feedback loops. The treatment of cells with H_2O_2 leads to the activation of plasma membrane bound NADPH oxidases through ROP proteins (Baxter-Burrell *et al.*, 2002), as well as the activation of cell wall bound peroxidases (Bolwell *et al.*, 2002). Both would lead to an increase in extracellular H_2O_2 levels. SA, on the other hand, was shown to be involved in both positive and negative feedback loops whereby the defense responses are controlled and amplified (Shah, 2003). As part of the feedback regulation, ROS is produced through a partially understood mechanism (Després *et al.*, 2003). Therefore, in both cases the latter increase in *PR-2* gene expression could be mediated by H_2O_2 that acts as a second messenger.

The close relationship between H_2O_2 and At-RLK3 in signaling became evident when the results obtained when C4 transgenic plants were treated with H_2O_2 (Fig. 5.11) and SA (Fig. 5.12) respectively, were considered. When treated with SA, the transgenic plants showed an increase in *PR-2* gene expression 4 h after treatment, even though the levels were only about a third of that of the wild type plants. In contrast, while H_2O_2 treatment led to increased *PR-2* expression in the wild type plants 4 h after treatment, the expression in the transgenic plants decreased by almost half that of the wild type plants. This result is significant if one considers the premise that SA is bound by the SABP2 receptor, no receptor for H_2O_2 has been described. One could therefore assume that since *PR-2* gene expression is not induced in transgenic plants lacking At-RLK3, but in the wild type plants having At-RLK3, that At-RLK3 must play a crucial role in the immediate perception of H_2O_2. This argument was strengthened when one considers the later stages of *PR-2* gene expression. Whereas an amplified *PR-2* gene expression pattern was found in the wild type plants treated with both H_2O_2 and SA, a dramatic decrease in *PR-2* gene expression was evident in both sets of transgenic plants, indicating that an amplified defense reaction was not initiated. These observations led to a hypothetical function of At-RLK3 as a redox sensor (Fig. 6.1, 6.2). In this hypothesis, At-RLK3 is predicted to act as the primary redox sensor

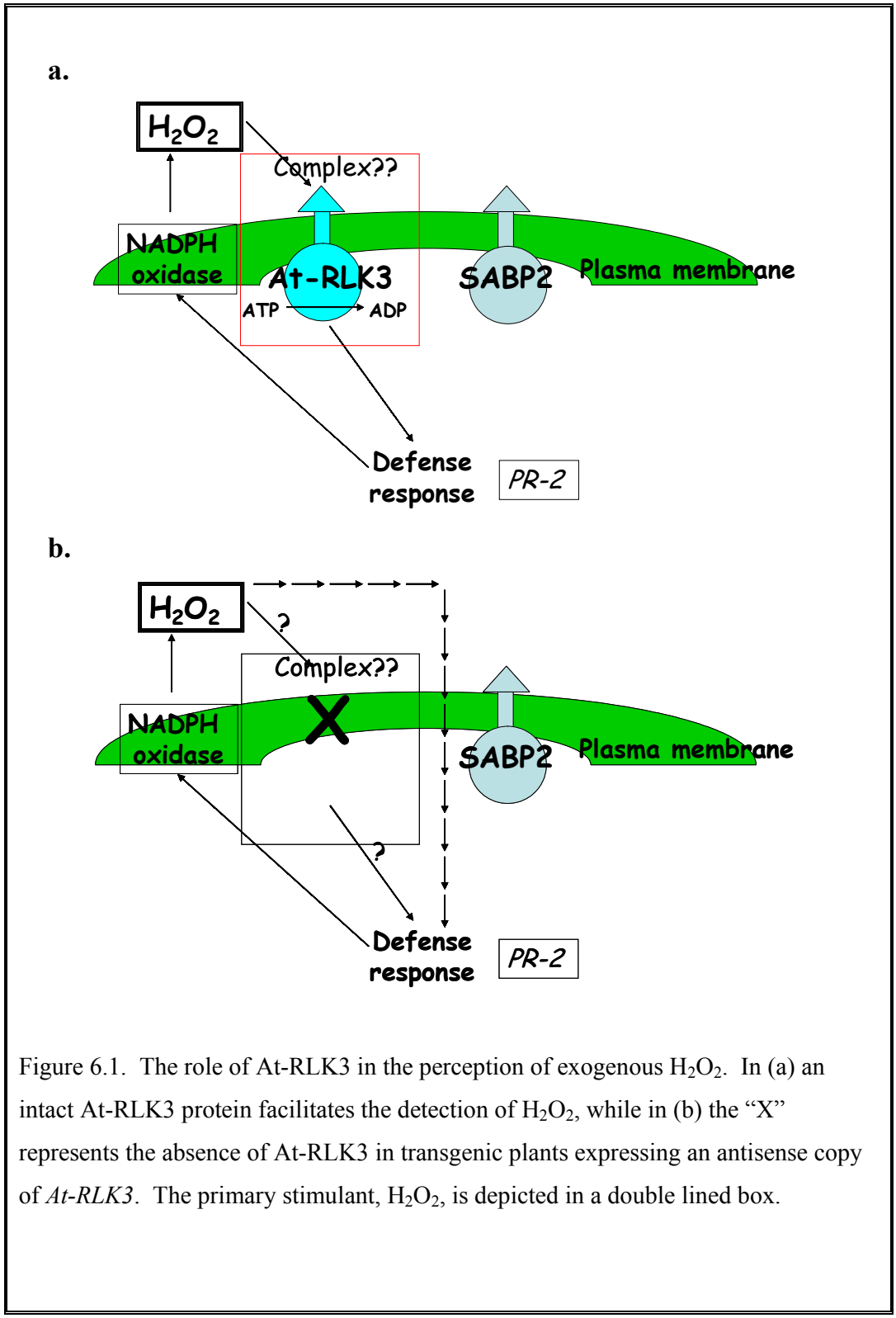


Figure 6.1. The role of At-RLK3 in the perception of exogenous H_2O_2. In (a) an intact At-RLK3 protein facilitates the detection of H_2O_2, while in (b) the "X" represents the absence of At-RLK3 in transgenic plants expressing an antisense copy of *At-RLK3*. The primary stimulant, H_2O_2, is depicted in a double lined box.

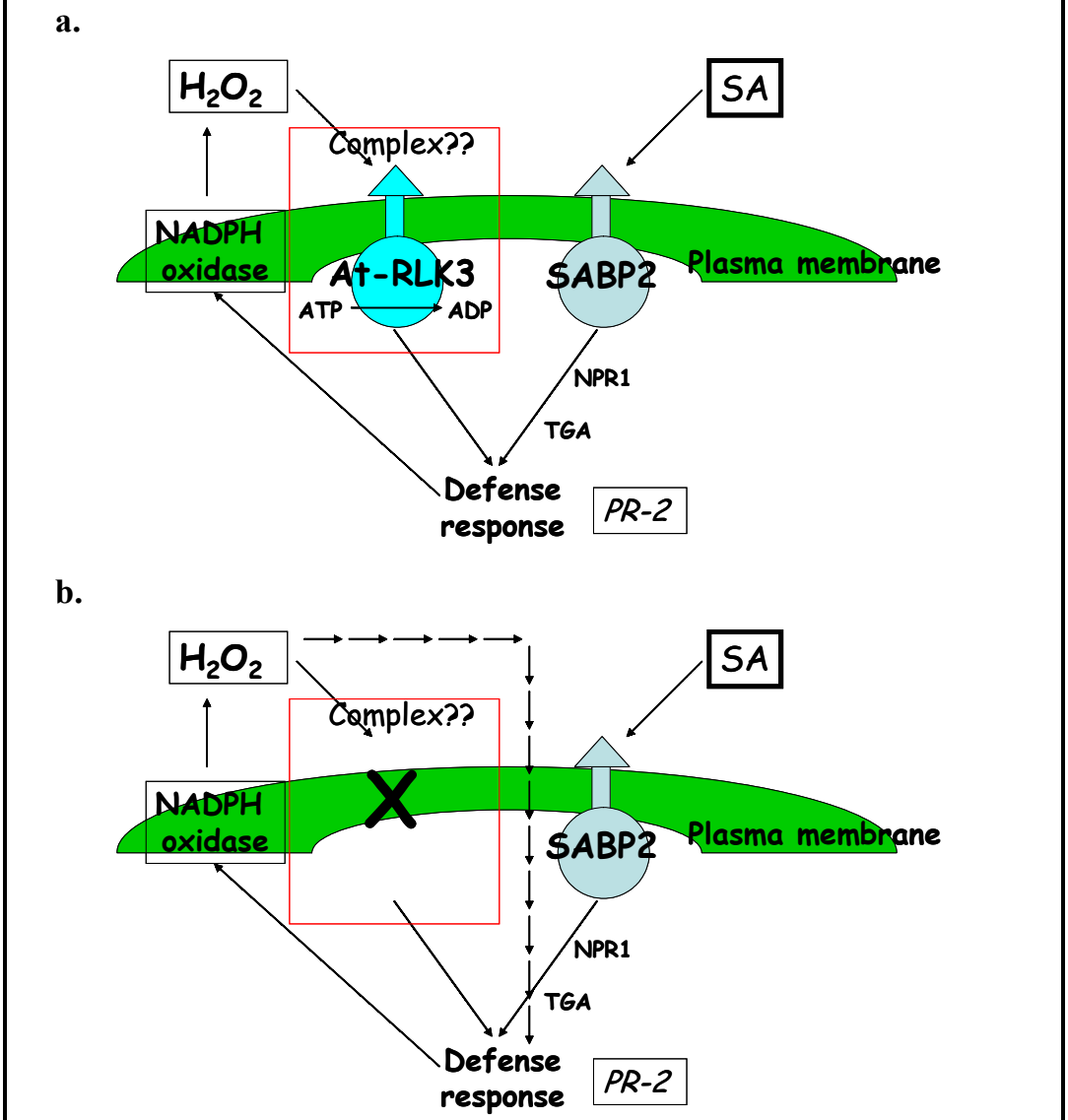


Figure 6.2. The role of At-RLK3 in the perception of exogenous SA. In (a) an intact At-RLK3 protein facilitates the detection of SA, while in (b) the "X" represents the absence of At-RLK3 in transgenic plants expressing an antisense copy of *At-RLK3*. The primary stimulant, SA, is depicted in a double lined box.

protein in *A. thaliana*. When H_2O_2 is present on the outside of the cell, it is detected by At-RLK3 (Fig. 6.1a). Upon detection, At-RLK3 is activated through autophosphorylation (Fig. 4.11) on serine or threonine residues (Fig. 4.6b). Since *At-RLK3* is also able to trans-phosphorylate other proteins (Fig. 4.10), this message can then be transduced to the nucleus, most probably via a MAPK cascade as was described by Samuel *et al.* (2000). This signaling event will then lead to the activation of the plant defense system, including the enhanced expression of *PR-2* (Fig. 5.11) and *At-RLK3* (Fig. 3.15a). Part of the induced defense response is the activation of plasma membrane bound NADPH oxidases (Baxter-Burrell *et al.*, 2002) and cell wall associated peroxidases (Bolwell *et al.*, 2002). This leads to higher H_2O_2 levels that is recognized by the elevated At-RLK3 protein level (Fig. 4.8a), eventually leading to the amplified defense response as was found 48 h after treatment (Fig. 5.11).

In the absence of At-RLK3 (Fig. 6.1b), *PR-2* gene expression was not activated within 4 h after treatment (Fig. 5.11). There was however some activation of *PR-2* gene expression from 4 h up to 24 h, whereafter the expression decreased. This could be attributed to the fact that H_2O_2 can move freely over the plasma membrane by means of diffusion, perhaps facilitated by peroxiporines (Henzler and Steudle, 2000). Once inside the cell, it would be able to activate many plant defense reactions (Desikan *et al.*, 1998; Dangl and Jones, 2001), perhaps by causing redox changes within the cells. This could lead to the activation of enzymes such as NPR1 whose activity depends on its reduced state (Després *et al.*, 2003). These defense reactions are however not sustained, indicating that At-RLK3 is essential for amplification of the plant defense response.

A similar scenario can be envisaged in the plants treated with SA (Fig. 6.2). In the wild type plants (Fig. 6.2a), the presence of SA is detected by SABP2 (Kumar and Klessig, 2003). The defense reaction of the plant is activated through the interaction of NPR1 and the TGA1 transcription factor (Després *et al.*, 2003) leading to the activation of plant defenses (Mou *et al.*, 2003) and the induced expression of *PR-2* 4 h after treatment (Fig. 5.12). Since the activation of the plant defense response was not via At-RLK3, it can explain why SA treatment did not lead to the activation of the enzyme through phosphorylation, as was found (4.4.6). *At-RLK3* expression was

however induced upon SA treatment, most likely as part of the induced defense reaction. Finally, similar to the scenario found when plants were treated with H_2O_2 (Fig. 6.1a), the defense reaction will be amplified through H_2O_2 production and recognition by At-RLK3 as seen with strongly induced *PR-2* expression 48 h after treatment (Fig. 5.12).

SA treatment of the C4 transgenic plants led to the activation of *PR-2* expression 4 h after treatment (Fig. 4.12). This is in agreement to the proposed hypothesis (Fig. 6.2b), since the initial induction of expression is At-RLK3 independent. H_2O_2 is then again produced as a result of increased NADPH oxidase and peroxidase activity, but as previously, with no At-RLK3 present to act as a receptor, it is thought that the H_2O_2 would enter the cell through diffusion, thereby transiently activating *PR-2* expression. Again, the activated defense response is not maintained 48 h after treatment.

Finally, the proposed role for At-RLK3 as redox sensor was further supported when the gene was overexpressed in transgenic *A. thaliana* plants and both the transgenic and wild type plants were subjected to SA treatment (S. Lategan, unpublished results). In addition to having an identical *PR-2* expression profile to SA treated wild type plants (Fig. 5.12), *PR-2* expression in the SA treated At-RLK3 overproducing transgenic plants was at least two times that of the wild type plants. This indicates that the presence of more At-RLK3 protein greatly facilitated an enhanced detection of H_2O_2 leading to a much stronger defense response.

It should also be noted that in the transgenic plants, the absence of At-RLK3 still led to the transient activation of *PR-2* expression, presumably due to the diffusion of H_2O_2 across the plasma membrane. This was found for both H_2O_2 (Fig. 5.11) and SA (Fig. 5.12) treatments. However, an equivalent activation between 8 and 24 h after treatment was absent from the treated wild type plants. Even though it is difficult to reach a definite conclusion from this result, one could argue that in the wild type plant, At-RLK3 is involved in the generation of a highly specific signal, possibly through a MAPK signaling pathway, leading to a tightly defined defense reaction involving specific enzymes. Eventually this led to an amplified and sustained activation of *PR-2* expression at 48 h after treatment. In the absence of At-RLK3, it

seems as if a general and non-specific defense reaction was induced that was not sustainable or amplified. The importance and relevance of this statement however needs to be verified through future experiments.

The final question that remains is whether At-RLK3 has the physical attributes to function as a redox sensitive receptor in *A. thaliana*. The answer to this question is yes. At-RLK3 is a typical RLK that is located on the plasma membrane of the cell with the extracellular domain ideally located to detect any changes in the extracellular environment. The intracellular protein kinase domain is active and can perform both auto- and trans phosphorylation of itself and other proteins. When plant cells were treated with H_2O_2, the protein was rapidly activated. In addition, both *At-RLK3* gene expression and At-RLK3 synthesis occurred within a very short time after treatment, indicating a very intimate relationship between H_2O_2 and At-RLK3.

As yet, no redox sensor has been described in plants. Yet, the key to the proposal of At-RLK3 acting as the redox sensor might lie in the C-X8-C-X2-C motif located on the extracellular domain of At-RLK3 (Fig. 3.2) which is thought to be involved in the formation of disulphide bridges (Ohtake *et al.*, 2000). The activation of NPR1 in response to exogenously applied SA depends on the reduction of the protein caused by changes in the redox potential within the cell (Mou *et al.*, 2003). The target of reduction is conserved cysteine residues that in the oxidised form are involved in both inter-and intra-molecular disulphide bonds, resulting in an inactive oligomeric complex. Upon SA treatment, a change in the redox state of the cell, probably via the accumulation of antioxidants, leads to the reduction of NPR1 into its active monomeric form.

The target of monomeric NPR1 is the TGA1 transcription factor (Després *et al.*, 2003). Similar to NPR1, in the oxidised state, TGA1 forms intra-molecular disulphide bonds between paired cysteine residues present in a conserved 30 amino acid sequence. In this form, the enzyme is not capable to bind to NPR1 or the *cis*-acting motif present on the promoter region. However, upon reduction, the disulphide bonds are broken and NPR1 and TGA1 can bind to each other, leading to the induced defense response.

Therefore, it is clear that cysteines present in a conserved amino acid sequence that are responsive to redox changes could play an important role in determining the activity of the protein. Since conserved cysteines are present on the extracellular domain of *A. thaliana* At-RLK3, future experiments will determine whether these cysteines are sensitive to changes in reduction potential occurring on the outside of the cell. This could make At-RLK3 a key component of the plant defense system during pathogen attack when extracellular ROS are produced as part of both HR and SAR (Grant and Loake, 2000). Further work will also involve the identification of At-RLK3 interacting proteins using the yeast two-hybrid system. Hopefully, an interaction between At-RLK3 and a MAPK signaling pathway could be established.

6.6 Conclusion

At-RLK3 shows great promise to either act as the elusive redox sensor in *A. thaliana* or at least play a key role as part of a bigger enzyme complex in the detection of H_2O_2. This statement is based on the expression and activation patterns of the gene and enzyme respectively, as well as to the unique phenotype displayed by the transgenic plants. Structurally, the enzyme is further well suited to fulfil this role. Future research must however be directed towards the confirmation of the role of At-RLK3 as a redox sensor. The initial aim stated at the beginning of the project, was therefore met since a putative function for At-RLK3 as redox sensor was finally proposed.

Chapter 7

Literature cited

Literature cited

Abe H, T Urao, T Ito, M Seki, K Shinozaki and K Yamaguchi-Shinozaki, 2003. *Arabidopsis* AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. The Plant Cell 15: 63 - 78.

Agatep R, RD Kirkpatrick, DL Parchaliuk, RA Woods and RD Gietz, 1998. Transformation of *Saccharomyces cerevisiae* by the lithium acetate/single-stranded carrier DNA/polyethylene glycol (LiAc/ss-DNA/PEG) protocol. Technical Tips Online (http://tto.trends.com)

Ahmed SU, M Bar-Peled and NV Raikhel, 1997. Cloning and subcellular location of an *Arabidopsis* receptor-like protein that shares common features of with protein-sorting receptors of eukaryotic cells. Plant Physiology 114: 325 – 336.

Allan AC and R Fluhr, 1997. Two distinct sources of elicited reactive oxygen species in tobacco epidermal cells. The Plant Cell 9: 1559 – 1572.

Allen RD, F Bernier, PA Lessard and RN Beachy, 1989. Nuclear factors interact with a soybean beta-conglycinin enhancer. The Plant Cell 1: 623 - 631.

Annen F and J Stockhaus, 1999. SbRLK1, a receptor-like kinase of *Sorghum bicolor* (L.) Moench that is expressed in mesophyll cells. Planta 208: 420 – 425.

Aoyama T and NH Chua, 1997. A glucocorticoid-mediated transcriptional induction system in transgenic plants. Plant Journal 11: 605 - 612.

Apel K and H Hirt, 2004. Reactive oxygen species: metabolism, oxidative stress and signal transduction. Annual Review of Plant Biology 55: 373 – 399.

Axelos M, C Curie, L Mazzolini, C Bardet and B Lescure, 1992. A protocol for transient expression in *Arabidopsis thaliana* protoplasts isolated from cell suspension cultures. Plant Physiology and Biochemistry 30: 123 – 128.

Baker MA and EW Orlandi, 1995. Active oxygen in plant pathogenesis. Annual Review of Phytopathology 33: 299 – 321.

Baker B, P Zambryski, B Staskawicz and SP Dinesh-Kumar, 1997. Signaling in plant-microbe interactions. Science 276: 726 – 733.

Baranowskij N, C Frohberg, S Prat and L Willmitzer, 1994. A novel DNA binding protein with homology to Myb oncoproteins containing only one repeat can function as a transcriptional activator. EMBO Journal 13: 5383 - 5392.

Bartosz G, 1997. Oxidative stress in plants. Acta Physiologiae Plantarum 19: 47 – 64.

Bassett CL, ML Nickerson, RA Cohen and MS Rajeevan, 2000. Alternative transcript initiation and novel post-transcriptional processing of a leucine-rich repeat receptor-like protein kinase gene that responds to short-day photoperiodic floral induction in morning glory (*Ipomoea nil*). Plant Molecular Biology 43: 43 – 58.

Bate N and D Twell, 1998. Functional architecture of a late pollen promoter: pollen-specific transcription is developmentally regulated by multiple stage-specific and co-dependent activator elements. Plant Molecular Biology 37: 859 - 869.

Bauer Z, L Gómez-Gómez, T Boller and G Felix, 2001. Sensitivity of different ecotypes and mutants of Arabidopsis thaliana toward the bacterial elicitor flagellin correlates with the presence of receptor-binding sites. Journal of Biological Chemistry 276: 45669 – 45676.

Baumann K, A De Paolis, P Costantino and G Gualberti, 1999. The DNA binding site of the Dof protein NtBBF1 is essential for tissue-specific and auxin-regulated expression of the rolB oncogene in plants. The Plant Cell 11: 323 - 333.

Baxter-Burrell A, Z Yang, PS Springer and J Bailey-Serres, 2002. RopGAP4-dependent Rop GTPase rheostat control of *Arabidopsis* oxygen deprivation tolerance. Science 296: 2026 – 2028.

Becraft PW, PS Stinard and DM McCarty, 1996. CRINKLY4: A TNFR-like receptor kinase involved in maize epidermal differentiation. Science 273: 1406 – 1409.

Beligni MV and L Lamattina, 2000. Nitric oxide stimulates seed germination and de-etiolation and inhibits hypocotyl elongation, three light –inducible responses in plants. Planta 210: 215 – 221.

Bleecker AB and H Kende, 2000. Ethylene: a gaseous signal molecule in plants. Annual Review of Cell and Developmental Biology 16: 1 -18.

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

Bolwell GP, 1999. Role of active oxygen species and NO in plant defense responses. Current Opinion in Plant Biology 2: 287 – 294.

Bolwell GP, LV Bindschedler, KA Blee, VS Butt, DR Davies, SL Gardner, C Gerrish and F Minibayeva, 2002. The apoplastic oxidative burst in response to biotic stress in plants: a three component system. Journal of Experimental Botany 53: 1367 – 1376.

Borges AA, HJ Cools and JA Lucas, 2003. Menadione sodium bisulphate, a novel plant defense activator which enhances local and systemic resistance to infection by *Leptosphaeria macilans* in oil seed rape. Plant Pathology 52: 429 – 436.

Borsani O, V Valpuesta and MA Botella, 2001. Evidence for a role of salicylic acid in the oxidative damage generated by NaCl and osmotic stress in *Arabidopsis* seedlings. Plant Physiology 126: 1024 – 1030.

Bower MS, DD Matias, E Fernandes Carvalho, M Mazzurco, T Gu, SJ Rothstein and DR Goring, 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.

Bowler C and R Fluhr, 2000. The role of calcium and activated oxygens as signals for controlling cross-tolerance. Trends in Plant Science 5: 241 – 246.

Boyle WJ, P van der Geer and T Hunter, 1991. Phosphopeptide mapping and phosphoamino acid analysis by two-dimensional separation on thin-layer cellulose plates. Methods in Enzymology 201: 110 – 149.

Bradford MM, 1976. A rapid sensitive method for the quantization of microgram quantities of proteins utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248 – 254.

Braun DM, JM Stone and JC Walker, 1997. Interaction of the maize and *Arabidopsis* kinase interaction domains with a subset of receptor-like protein kinases: implications for transmembrane signaling in plants. The Plant Journal 12: 83 – 95.

Bray EA, 1997. Plant responses to water deficit. Trends in Plant Science 2: 48 – 54.

Bray EA, 2002. Abscisic acid regulation of gene expression during water-deficit stress in the era of the *Arabidopsis* genome. Plant, Cell and Environment 25: 153 – 161.

Busk PK and M Pages, 1998. Regulation of abscisic acid-induced transcription. Plant Molecular Biology 37: 425 – 435.

Busk PK, AB Jensen and M Pages, 1997. Regulatory elements *in vivo* in the promoter of the abscisic acid responsive gene Rab17. The Plant Journal 11: 1285 – 1295.

Cao H, J Glazebrook, JD Clarke, S Volko and X Dong, 1997. The *Arabidopsis* NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell 88: 57 – 63.

Carrington J, T Bisseling, T Collmer and J Jones, 1999. Highlights from the ninth international congress on molecular plant-microbe interactions. The Plant Cell 11: 2063 – 2069.

Cassab GI, 1998. Plant cell wall proteins. Annual Review of Plant Physiology and Plant Molecular Biology 49: 281 – 309.

Chan CS, L Guo and MC Shih, 2001. Promoter analysis of the nuclear gene encoding the chloroplast glyceraldehyde-3-phosphate dehydrogenase B subunit of *Arabidopsis thaliana*. Plant Molecular Biology 46: 131 - 141.

Chang C, GE Schaller, SE Patterson, SF Kwok, EM Meyerowitz and AB Bleecker, 1992. The TMK1 gene from *Arabidopsis* codes for a protein with structural and biochemical characteristics of receptor protein kinase. The Plant Cell 4: 1263 – 1271.

Chang C, SF Kwok, AB Bleecker and EM Meyerowitz, 1993. *Arabidopsis* ethylene response gene *ETR1*: Similarity of product to two-component regulators. Science 262: 539 – 544.

Chen Z, 2001. A super family of proteins with novel cysteine-rich repeats. Plant Physiology 126: 473 – 476.

Chen Z, J Malamy, J Henning, U Conrath, P Sanchez-Casas, H Silva, J Ricigliano and F Klessig, 1995. Induction, modification and transduction of the salicylic acid signal in plant defense response. Proceedings of the National Academy of Sciences 92: 4134 – 4137.

Chen W, NJ Provart, J Glazebrook, F Katagiri, HS Chang, T Eulgem, F Mauch, S Luan, G Zou, SA Whitham, PR Budworth, Y Tao, Z Xie, X Chen, S Lam, JA

Kreps, JF Harper, A Si Ammour, B Mauch Mani, M Heinlein, K Kobayashi, T Hohn, JL Dangl, X Wang and T Zhu, 2002. Expression profile matrix of *Arabidopsis* transcription factor genes suggests their putative functions in response to environmental stresses. The Plant Cell 14: 559 - 574.

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

Chory J, N Aguilar and CA Peto, 1991. The phenotype of *Arabidopsis thaliana* det1 mutants suggests a role for cytokinins in greening. Symposium of the Society of Experimental Biology 45: 21 – 29.

Clark SE, 1997. Organ formation at the vegetative shoot meristem. The Plant Cell 9: 1067 - 1076.

Clark SE, RW Williams and EM Meyerowitz, 1997. The *Clavata1* gene encodes a putative receptor kinase that controls shoot and floral meristem size in *Arabidopsis*. Cell 89: 575 – 585.

Clarke MC, W Wei and K Lindsey, 1992. High frequency transformation of *Arabidopsis thaliana* by *Agrobacterium tumefaciens*. Plant Molecular Biology Reporter 10: 178 – 189.

Coello P, A Sassen, V Haywood, KR Davis and JC Walker, 1999. Biochemical characterization and expression of RLK4, a receptor-like kinase protein from *Arabidopsis thaliana*. Plant Science 142: 83 – 91.

Conrath U, Z Chen, JR Ricigliano and DF Klessig, 1995. Two inducers of plant defense responses, 2,4-dichloroisonicotinic acid and salicylic acid, inhibit catalase activity in tobacco. Proceedings of the National Academy of Sciences USA 92: 7143 – 7147.

Conrath U, H Silva and DF Klessig, 1997. Protein dephosphorylation mediates salicylic acid–induced expression of *PR-1* genes in tobacco. The Plant Journal 11: 747 – 757.

Creelman RA and JE Mullet, 1997. Biosynthesis and action of jasmonates in plants. Annual Review of Plant Physiology and Plant Molecular Biology 48: 355 – 381.

da Costa e Silva O, L Klein, E Schmelzer, GF Trezzini and K Hahlbrock, 1993. BPF-1, a pathogen-induced DNA-binding protein involved in the plant defense response. The Plant Journal 4: 125 – 135.

Dai Z and G An, 1995. Induction of nopaline synthase promoter activity by h202 that has no direct correlation with salicylic acid. Plant Physiology 109: 1191 – 1197.

Dangl JL, 1998. Innate immunity: Plants just say NO to pathogens. Nature 394: 525 – 526.

Dangl JL and JDG Jones, 2001. Plant pathogens and integrated defense responses to infection. Nature 411: 826 – 833.

Dat JF, H Lopez-Delgado, CH Foyer and IM Scott, 1998. Parallel changes in H_2O_2 and catalase during thermotolerance induced by salicylic acid or heat acclimation in mustard seedlings. Plant Physiology 116: 1351 – 1357.

Deeken R and R Kaldenhoff, 1997. Light repressible receptor protein kinase: a novel photo-regulated gene from *Arabidopsis thaliana*. Planta 202: 479 – 486.

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

Delorme V, JL Giranton, Y Hatzfield, A Friry, P Heizmann, MJ Ariza, C Dumas, T Gaude and JM Cock, 1995. Characterization of the S locus genes, *SLG* and *SRK*, of the Brassica S3 haplotype: identification of a membrane-localized protein encoded by the S locus receptor kinase gene. The Plant Journal 7: 429 – 440.

Dempsey DA, J Shah and DF Klessig, 1999. Salicylic acid and disease resistance in plants. Critical Reviews in Plant Sciences 18: 547 – 575.

Desikan R, EC Burnett, JT Hancock and SJ Neill, 1998. Harpin and hydrogen peroxide induce the expression of a homologue of gp-91-*phox* in *Arabidopsis thaliana* suspension cultures. Journal of Experimental Botany 49: 1767 – 1771.

Desikan R, A Clarke, JT Hancock and SJ Neill, 1999. H_2O_2 activates a MAP kinase-like enzyme in *Arabidopsis thaliana* suspension cultures. Journal of Experimental Botany 50: 1863 – 1866.

Desikan R, SA-H Mackerness, JT Hancock and SJ Neill, 2001. Regulation of the *Arabidopsis* transcriptome by oxidative stress. Plant Physiology 127: 159 – 172.

Deslandes L, F Pileur, L Liaubet, S Camut, C Can, K Williams, E Holub, J Beynon, M Arlat and Y Marco, 1998. Genetic characterization of *RRS1*, a recessive locus in *Arabidopsis thaliana* that confers resistance to the bacterial soil borne pathogen *Ralstonia solanacearum*. Molecular Plant-Pathogen Interactions 11: 659 – 667.

Després C, C Chubak, A Rochon, R Clark, T Bethune, D Desveaux and PR Fobert, 2003. The *Arabidopsis* NPR1 disease resistance protein is a novel cofactor that confer redox regulation of DNA binding activity to the basic domain/leucine zipper transcription factor TGA1. The Plant Cell 15: 2181 – 2191.

Dietz K-J, 1997. Functions and responses of the leaf apoplast under stress. Progress in Botany 58: 221 – 254.

Dietz K-J, 2001. The extracellular matrix of the plant cell: Location of signal perception, transduction and response. Progress in Botany 62: 215 – 237.

Dixon RA and LA Paiva, 1995. Stress-induced phenylpropanoid metabolism. The Plant Cell 7: 1085 – 1097.

Dixon MS, DA Jones, JS Keddie, CM Thomas, K Harrison and JDG Jones, 1996. The tomato *Cf-2* disease resistance locus comprises two functional genes encoding leucine-rich repeat proteins. Cell 84: 451 – 459.

Doares SH, T Syrovets, EW Weiler and CA Ryan, 1995. Oligogalacturonides and chitosan activate plant genes through the octadecanoid pathway. Proceedings of the National Academy of Sciences USA 93: 4095 – 4098.

Dong X, M Mindrinos, KR Davids and FM Ausubel, 1991. Induction of *Arabidopsis* defense genes by virulent and avirulent *Pseudomonas syringae* strains and by a cloned avirulence gene. The Plant Cell 3: 61 – 72.

Du L and Z Chen, 2000. Identification of genes encoding receptor-like protein kinases as possible targets of pathogen- and salicylic acid–induced WRKY DNA-binding proteins in *Arabidopsis*. The Plant Journal 24: 837 – 847.

Du H and DF Klessig, 1997. Role for salicylic acid in the activation of defense responses in catalase-deficient transgenic tobacco. Molecular Plant-Microbe Interactions 10: 922 – 925.

Durner J and DF Klessig, 1995. Inhibition of ascorbate peroxidase by salicylic acid and 2,4-dichloroisonicotinic acid, two inducers of plant defense responses. Proceeding of the National Academy of Sciences USA 92: 11312 – 11316.

Durner J, D Wendehenne and DF Klessig, 1998. Defense gene induction in tobacco by nitric oxide, cyclic GMP and cyclic ADP ribose. Proceedings of the National Academy of Sciences USA 95: 10328 – 10333.

Dwyer KG, K Muthugapatti, MK Kandasamy, DI Mahosky, J Acciai, BI Kudish, JE Miller, ME Nasrallah and JB Nasrallah, 1994. A super-family of *S* Locus-related sequences in *Arabidopsis*: diverse structures and expression patterns. The Plant Cell 6: 1829 – 1843.

Dykema PE, PR Sipes, A Marie, BJ Biermann, DN Crowell and SK Randall, 1999. A new class of proteins capable of binding transition metals. Plant Molecular Biology 41: 139 – 150.

Dzelzkalns VA, JB Nasrallah and M Nasrallah, 1992. Cell-cell communication in plants: self-incompatibility in flower development. Developmental Biology 153: 70 – 82.

Ellerstrom M, K Stalberg, I Ezcurra and L Rask, 1996. Functional dissection of a napin gene promoter: identification of promoter elements required for embryo and endosperm-specific transcription. Plant Molecular Biology 32: 1019 - 1027.

Elmayan T and M Tepfer, 1995. Evaluation in tobacco of the organ specificity and strength of the rol D promoter, domain A of the 35S promoter and the 35S^2 promoter. Transgenic Research 4: 388 - 396.

Endre G, A Kereszt, Z Kevel, S Milhacea, P Kalo and GB Kiss, 2002. A receptor kinase gene regulating symbiotic nodule development. Nature 417: 962 – 966.

Eulgem T, PJ Rushton, E Schmelzer, K Hahlbrock and IE Somssich, 1999. Early nuclear events in plant defense signaling: rapid gene activation by WRKY transcription factors. The EMBO Journal 18: 4689 – 4699.

Eulgem T, PJ Rushton, S Robatzek and IE Somssich, 2000. The WRKY super family of plant transcription factors. Trends in Plant Science 5: 199 - 206.

Fan W and X Dong, 2002. *In vivo* interaction between NPR1 and transcription factor TGA2 leads to salicylic acid –mediated gene activation in *Arabidopsis*. The Plant Cell 14: 1377 – 1389.

Faraday CD and RM Spanswick, 1992. Maize root plasma membranes isolated by aqueous polymer two-phase partitioning: assessment of residual tonoplast ATPase and pyrophosphatase activities. Journal of Experimental Botany 43: 1583 – 1590.

Fath A, PC Bethke and RL Jones, 2001. Enzymes that scavenge reactive oxygen species are down-regulated prior to gibberellic acid-induced programmed cell death in barley aleurone. Plant Physiology 126: 156 – 166.

Felix G, JD Duran, S Volko and T Boller, 1999. Plants recognize bacteria through the most conserved domain of flagellin. The Plant Journal 18: 265 – 276.

Feys BJ, LJ Moisan, M-A Newman and JE Parker, 2001. Direct interaction between the *Arabidopsis* disease resistance signaling proteins, EDS1 and PAD4. EMBO Journal 20: 5400 – 5411.

Feuillet C, G Schachermayr and B Keller, 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.

Feuillet C, C Reuzeau, P Kjellbom and B Keller, 1998. Molecular characterization of a new type of receptor-like kinase (*wlrk*) gene family in wheat. Plant Molecular Biology 37: 943 – 953.

Finkel T, 2000. Redox-dependent signal transduction. FEBS letters 476: 52 – 54.

Fish L and A Jagendorf, 1982. High rates of protein synthesis in isolated chloroplasts. Plant Physiology 70: 1107 – 1114.

Fletcher JC, U Brand, MP Running, R Simon and EM Meyerowitz, 1999. Signaling of cell fate decisions by *CLAVATA3* in *Arabidopsis* shoot meristems. Science 283: 1911 – 1914.

Flor HH, 1971. Current status of the gene-for-gene concept. Annual Review of Phytopathology 9: 275 – 296.

Foyer CH and G Noctor, 2003. Redox sensing and signaling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. Physiologia Plantarum 119: 355 – 364.

Frank W, T Munnik, K Kerkmann, F Salamini and D Bartels, 2000. Water deficit triggers phospholipase D activity in the resurrection plant *Craterostigma plantagineum*. The Plant Cell 12: 111 – 124.

Friedrichsen DM, CAP Joazeiro, J Li, T Hunter and J Chory, 2000. Brassinosteroid-insensitive-1 is a ubiquitously expressed leucine-rich repeat receptor serine/threonine kinase. Plant Physiology 123: 1247 – 1255.

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

Garretón V, J Carpinelli, X Jordana and L Holuigue, 2002. The as-1 promoter element is an oxidative tress-responsive element and salicylic acid activates it via oxidative species. Plant Physiology 130: 1516 – 1526.

Giranton J-L, MJ Ariza, C Dumas, JM Cock and T Gaude, 1995. The S locus receptor kinase gene encodes a soluble glycoprotein corresponding to the SKR extracellular domain in *Brassica oleracea*. The Plant Journal 8: 827 - 34.

Giranton J-L, C Dumas, JM Cock and T Gaude, 2000. The integral membrane S-locus receptor kinase of *Brassica* has serine/threonine activity in a membranous environment and spontaneously forms oligomers *in planta*. Proceedings of the National Academy of Sciences USA 97: 3759 – 3764.

Glazebrook J, WJ Chen, B Estes, H-S Chang, C Nawrath, J-P Métraux, T Zhu and F Katagiri, 2003. Topology of the network integrating salicylate and jasmonate signal transduction derived from global expression phenotyping. Plant Journal 34: 217 – 228.

Godiard L, L Sauviac, N Dalbin, L Liaubet, D Callard, P Czernic and Y Marco, 1998. *CYP76C2*, an *Arabidopsis thaliana* cytochrome P450 gene expressed during hypersensitive and developmental cell death. FEBS letters 438: 245 – 249.

Gómez-Gómez L and T Boller, 2000. FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. Molecular Cell 5: 1003 – 1011.

Gómez-Gómez L and T Boller, 2002. Flagellin perception: a paradigm for innate immunity. Trends in Plant Science 7: 251 – 256.

Gómez-Gómez L, G Felix and T Boller, 1999. A single locus determines sensitivity to bacterial flagellin in *Arabidopsis thaliana*. The Plant Journal 18: 277 – 284.

Goring DR and SJ Rothstein, 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.

Grant JJ and GJ Loake, 2000. Role of reactive oxygen intermediates and cognate redox signaling in disease resistance. Plant Physiology 124: 21 – 29.

Green PJ, SA Kay and N-H Chua, 1987. Sequence-specific interactions of a pea nuclear factor with light-responsive elements upstream of the rbcs3A gene. EMBO Journal 6: 2543 - 2549.

Gu Y-Q, C Yang, VK Thara, J Zhou and GB Martin, 2000. Pti4 is induced by ethylene and salicylic acid and its product is phosphorylated by the Pto kinase. The Plant Cell 12: 771 – 785.

Guan LM, J Zhao and JG Scandalios, 2000. Cis-elements and trans-factors that regulate expression of the maize Cat1 antioxidant gene in response to ABA and osmotic stress: H_2O_2 is the likely intermediary signaling molecule for the response. Plant Journal 22: 87 – 95.

Gustin MC, J Albertyn, M Alexander and K Davenport, 1998. MAP kinase pathways in the yeast *Saccharomyces cerevisiae*. Microbiology and Molecular Biology Reviews 62: 1264 – 1300.

Hajouj T, R Michelis and S Gepstein, 2000. Cloning and characterization of a receptor-like protein kinase gene associated with senescence. Plant Physiology 124: 1305 – 1314.

Hammond-Kosack KE and JDG Jones, 1996. Resistance gene-dependent plant defense responses. The Plant Cell 8: 1773 – 1791.

Hammond-Kosack KE and JDG Jones, 1997. Plant disease resistance genes. Annual Review of Plant Physiology and Plant Molecular Biology 48: 575 - 607.

Hammond-Kosack KE and JDG Jones, 2000. Response to plant pathogens. *In* BB Buchanan, W Gruissem and RL Jones (eds). Biochemistry and Molecular Biology of plants. ASPP Press, Rockville, MD, pp 1102 – 1156.

Hancock JT, R Desikan, A Clarke, RD Hurst and SJ Neill, 2002. Cell signaling following plant/pathogen interactions involves the generation of reactive oxygen and reactive nitrogen species. Plant Physiology and Biochemistry 40: 611 – 617.

Hanks SK and AM Quinn, 1991. Protein kinase catalytic domain sequence database: identification of conserved features of primary structure and classification of family members. Methods in Enzymology 200: 38 – 62.

Hardie DG, 1999. Plant protein serine / threonine kinases: Classification and functions. Annual Review of Plant Physiology and Plant Molecular Biology 50: 97 – 131.

Harlow E and D Lane, 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp 298 – 290.

He Z-H, M Fujiki and BD Kohorn, 1996. A cell-wall associated receptor-like protein kinase. The Journal of Biological Chemistry 271: 19789 – 19793.

He Z-H, D He and BD Kohorn, 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.

He Z, Z-Y Wang, J Li, Q Zhu, C Lamb, P Ronald and J Chory, 2000. Perception of brassinosteroids by the extracellular domain of the receptor kinase BRI1. Science 288: 2360 – 2363.

Heldin CH and O Ostman, 1996. Ligand-induced dimerization of growth factor receptors: variations on the theme. Cytokine Growth Factor Reviews 7: 3 - 10.

Henzler T and E Steudle, 2000. Transport and metabolic degradation of hydrogen peroxide in *Chara corallina*: model calculations and measurements with the pressure prove suggest transport of H_2O_2 across water channels. Journal of Experimental Botany 51: 2053 – 2066.

Herrin DL and GW Schmidt, 1988. Rapid reversible staining of Northern blots prior to hybridization. Bio-Techniques 6: 196.

Hervé C, P Dabos, J-P Galaud, P Rougé and B Lescure, 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.

Higo K, Y Ugawa, M Iwamoto and T Korenaga, 1999. Plant *cis*-acting regulatory DNA elements (PLACE) database. Nucleic Acids Research 27: 297 - 300.

Hodges TK and RT Leonard, 1974. Purification of a plasma membrane-bound adenosine triphosphate from plant roots. Methods in Enzymology 32: 392 – 406.

Holt III BF, DC Boyes, M Ellerström, N Siefers, A Wiig, S Kauffman, MR Grant and JL Dangl, 2002. An evolutionary conserved mediator of plant disease resistance gene function is required for normal *Arabidopsis* development. Developmental Cell 2: 807 – 817.

Hong SW, JH Jon, JM Kwak and HG Nam, 1997. Identification of a receptor-like kinase gene rapidly induced by abscisic acid, dehydration, high salt and cold treatments in *Arabidopsis thaliana*. Plant Physiology 113: 1203 – 1212.

Horn MA and JC Walker, 1994. Biochemical properties of the autophosphorylation of RLK5, a receptor-like protein kinase from *Arabidopsis thaliana*. Biochimica et Biophysica Acta 1208: 65 – 74.

Huang X, E Kiefer, U van Rad, D Ernst, I Foissner and J Durner, 2002. Nitric oxide burst and nitric oxide-dependent gene induction in plants. Plant Physiology and Biochemistry 40: 625 – 631.

Hughes M and M Dunn, 1996. The molecular biology of plant acclimation to low temperature. Journal of Experimental Botany 47: 291 – 305.

Hunter T, 1995. Protein kinases and phosphatases: the ying and yang of protein phosphorylation and signaling. Cell 80: 225 – 236.

Hwang I, H-C Chen and J Sheen, 2002. Two-component signal transduction pathways in *Arabidopsis*. Plant Physiology 129: 500 – 515.

Inoue H, H Nojima and H Okayama, 1990. High efficiency transformation of *Escherichia coli* with plasmids. Gene 96: 23 – 28.

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

Jenkins GI, 1999. Signal transduction networks and the integration of responses to environmental stimuli. Advances in Botanical Research 29: 53 – 73.

Jensen AB, J Mundy, M Pages and A Leone, 1996. Expression of the maize *rab17* gene in response to abscisic acid and osmotic stress. *In* S Grillo (ed). Physical stresses in plants: genes and their products for tolerance. Springer-Verlag, Berlin, pp 123 – 130.

Jeong S, AE Trotochaud and SE Clark, 1999. The *Arabidopsis CLAVATA2* gene encodes a receptor-like protein required for the stability of the CLAVATA1 receptor-like kinase. The Plant Cell 11: 1925 – 1933.

Jinn T-L, JM Stone and JC Walker, 2000. *HAESA*, an *Arabidopsis* leucine-rich repeat receptor kinase, controls floral organ abscission. Genes and Development 14: 108 – 117.

Johnson LN, MEM Noble and DJ Owen, 1996. Active and inactive protein kinases: structural basis for regulation. Cell 85: 149 – 158.

Jonak C, L Ökresz, L Bögre and H Hirt, 2002. Complexity, cross-talk and integration of plant MAP kinase signaling. Current Opinion in Plant Biology 5: 415 – 424.

Jones DA and JDG Jones, 1997. The role of leucine-rich repeats in plant defenses. Advances in Botanical Research incorporating Advances in Plant Pathology 24: 89 – 167.

Jones DA, CM Thomas, KE Hammond-Kosack, PJ Balint-Kurti and JDG Jones, 1994. Isolation of the tomato Cf-9 gene for resistance to *Cladosporium fulvum* by transposon tagging. Science 266: 789 – 793.

Kachroo P, J Shanklin, J Shah, EJ Whittle and DF Klessig, 2001. A fatty acid desaturase modulates the activation of defense signaling pathways in plants. Proceedings of the National Academy of Sciences USA 98: 9448 – 9453.

Kagaya Y, K Ohmiya and T Hattori, 1999. RAV1, a novel DNA-binding protein, binds to bipartite recognition sequence through two distinct DNA-binding domains uniquely found in higher plants. Nucleic Acids Research 27: 470 - 478.

Kajava AV, 1998. Structural diversity of leucine-rich repeat proteins. Journal of Molecular Biology 277: 519 – 527.

Kakimoto T, 1996. CKI1, a histidine kinase homolog implicated in cytokinin signal transduction. Science 274: 982 – 985.

Kim SY, HJ Chung and TL Thomas, 1997. Isolation of a novel class of bZIP transcription factors that interact with ABA-responsive and embryo-specification elements in the Dc3 promoter using a modified yeast one-hybrid system. The Plant Journal 11: 1237 - 1251.

Kim C, D-H Jeong and G An, 2000a. Molecular cloning and characterization of OsLRK1 encoding a receptor-like protein kinase from *Oryza sativa*. Plant Science 152: 16 – 26.

Kim YS, JH Lee, GM Yoon, HS Cho, S-W Park, MC Suh, D Choi, HJ Ha, JR Liu and H-S Pai, 2000b. CHRK1, a chitinase-related receptor-like kinase in tobacco. Plant Physiology 123: 905 – 915.

Kinkema M, W Fan and X Dong, 2000. Nuclear localization of NPR1 is required for activation of PR gene expression. The Plant Cell 12: 2339 – 2350.

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

Knight H, AJ Trewavas and MR Knight, 1997. Calcium signaling in *Arabidopsis thaliana* responding to drought and salinity. The Plant Journal 12: 1067 – 1078.

Kobe B and J Deisenhofer, 1994. The leucine-rich repeat – a versatile binding motif. Trends in Biochemical Sciences 19: 415 – 412.

Kohorn BD, S Lane and TA Smith, 1992. An *Arabidopsis* serine / threonine kinase homologue with an epidermal growth factor repeat selected in yeast for its specificity for a thylakoid membrane protein. Proceedings of the National Academy of Sciences 89: 10989 – 10992.

Komjanc M, S Festi, L Rizzotti, L Cattivelli, F Cervone and G De Lorenzo, 1999. A leucine-rich repeat receptor-like protein kinase (LRPKm1) gene is induced in *Malus x domestica* by *Venturia inaequalis* infection and salicylic acid treatment. Plant Molecular Biology 40: 945 – 957.

Kovtun Y, W-L Chiu, G Tena and J Sheen, 2000. Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. Proceedings of the National Academy of Sciences USA 97: 2940 – 2945.

Kowyama Y, K Kakeda, K Kondo, T Imada and T Hattori, 1996. A putative receptor protein kinase gene in *Ipomoea trifida*. Plant Cellular Physiology 37: 681 - 685.

Krusell L, LH Madsen, S Sato, G Aubert, A Genua, K Szczyglowski, G Duc, T Kaneko, S Tabata, F de Bruijn, E Pajuelo, N Sandal and J Stougaard, 2002. Shoot control of root development and nodulation is mediated by a receptor-like kinase. Nature 420: 422 – 425.

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

Kunkel BN, AF Bent, D Dahlbeck, RW Innes and BJ Staskawicz, 1993. *RPS2*, an *Arabidopsis* disease resistance locus specifying recognition of *Pseudomonas syringae* strains expressing the avirulence gene *avrRpt2*. The Plant Cell 5: 865 – 875.

Laemmli UK, 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680 – 685.

Laloi C, D Mestres-Ortega, Y Marco, Y Meyer and JP Reichheld, 2004. The *Arabidopsis* cytosolic thioredoxin *h*5 gene induction by oxidative stress and its W-Box-mediated response to pathogen elicitor. Plant Physiology 134: 1006 – 1016.

Lam E and NH Chua, 1989. ASF-2: A factor that binds to the cauliflower mosaic virus 35S promoter and a conserved GATA motif in cab promoters. The Plant Cell 1: 1147-1156.

Lamb CJ and RA Dixon, 1997. The oxidative burst in plant disease resistance. Annual Review in Plant Physiology and Plant Molecular Biology 48: 251 – 275.

Landschulz WH, PF Johnson and SL McKnight, 1988. The leucine zipper: a hypothetical structure common to new DNA binding proteins. Science 240: 1759 – 1764.

Lange J, Z-P Xie, WJ Broughton, R Vögeli-Lange and T Boller, 1999. A gene encoding a receptor-like protein kinase in the roots of common bean is differentially regulated in response to pathogens, symbionts and nodulation factors. Plant Science 142: 133 – 145.

Langebartels C, M Schraudner, W Heller, D Ernst and H Sandermann, 2000. Oxidative stress and defense reactions in plants exposed to air pollutants and UV-B radiation. *In* D Inze and M van Montagu (eds). Oxidative stress in plants. Harwood Academic Publishers, London, pp 105 – 135.

Larsson C, 1985. Plasma membranes. *In* HF Linskens and JF Jackson (eds). Modern Methods of Plant Analysis, Volume 1. Cell Components. Springer Verlag, Berlin, pp 85 – 104.

Laudert D and EW Weiler, 1998. Allene oxide synthase: a major control point in *Arabidopsis thaliana* octadecanoid signaling. The Plant Journal 15: 675 – 684.

Lecain E, X Chenivesse, R Spagnoli and D Pompon, 1996. Cloning by metabolic interference in yeast and enzymatic characterization of *Arabidopsis thaliana* sterol Δ7-reductase. Journal of Biochemical Chemistry 271: 10866 – 10873.

Leckie F, B Mattei, C Capodicasa, A Hemmings, L Nuss, B Aracri, G de Lorenzo and F Cervone, 1999. The specificity of polygalacturonase-inhibiting protein (PGIP): a single amino acid substitution in the solvent exposed β-strand/β-turn region of the leucine-rich repeats (LRRs) confers a new recognition capability. EMBO Journal 18: 2352 – 2363.

Lee H-S, B Karunanandaa, A McCubbin, S Gilroy and T Kao, 1996a. PRK1, a receptor-like kinase of *Petunia inflata*, is essential for postmeiotic development of pollen. The Plant Journal 9: 613 – 624.

Lee Y, YB Choi, S Suh, J Lee, SM Assmann, CO Joe, JF Kelleher and RC Crain, 1996b. Abscisic acid-induced phosphoinositide turnover in guard cell protoplasts of *Vicia faba*. Plant Physiology 110: 987 – 996.

Lee HS, YY Chung, C Das, B Karunanandaa, JL Vanwent, C Mariana and TH Kao, 1997. Embryo sac development is affected in *Petunia inflata* plants transformed with an antisense gene encoding the extracellular domain of receptor kinase PRK1. Sexual Plant Reproduction 10: 341 – 350.

Lee S, H Hirt and Y Lee, 2001. Phosphatidic acid activates a wound-activated MAPK in *Glycine max*. The Plant Journal 26: 479 – 486.

Lemmon MA and J Schlessinger, 1994. Regulation of signal transduction and signal diversity by receptor oligomerization. Trends in Biochemical Sciences 19: 459 - 463.

Leon J, E Rojo and JJ Sanchez-Serrano, 2001. Wound signaling in plants. Journal of Experimental Botany 52: 1 – 9.

Leung J and J Giraudat, 1998. Abscisic acid signal transduction. Annual Review of Plant Physiology and Plant Molecular Biology 49: 199 – 222.

Leung J, M Bouvier-Durand, P-C Morris, D Guerrier, F Chefdor and J Giraudat, 1994. *Arabidopsis* ABA response gene *ABI1*: features of a calcium-modulated protein phosphatase. Science 264: 1448 – 1452.

Li J, 2003. Brassinosteroids signal two receptor-like kinases. Current Opinion in Plant Biology 6: 494 – 499.

Li J and J Chory, 1997. A putative leucine-rich repeat receptor kinase involved in brassinosteroid signal transduction. Cell 90: 929 – 938.

Li H-Y and JE Gray, 1997. Pollination enhanced expression of a receptor-like protein kinase related gene in tobacco styles. Plant Molecular Biology 33: 653 – 665.

Li Z and ET Wurtzel, 1998. The *ltk* gene family encodes novel receptor-like kinases with temporal expression in developing maize endosperm. Plant Molecular Biology 37: 749 – 761.

Li J, P Nagpal, V Vitart, TC McMorris and J Chory, 1996. A role for brassinosteroids in light-dependent development of *Arabidopsis*. Science 272: 398 – 401.

Li J, GP Smith and JC Walker, 1999. Kinase interaction domain of kinase-associated phosphatase, a phospho-binding protein. Proceedings of the National Academy of Sciences USA 96: 7821 – 7826.

Li J, KA Lease, FE Tax and JC Walker, 2001. BRS1, a serine carboxypeptidase, regulates BRI1 signaling in *Arabidopsis thaliana*. Proceedings of the National Academy of Sciences USA 98: 5916 – 5921.

Li L, C Li and GA Howe, 2002. Genetic analysis of wound signaling in tomato. Evidence for a dual role of jasmonic acid in defense and female fertility. Plant Physiology 127: 1414 – 1417.

Lin CC and CH Kao, 2001. Abscisic acid induced changes in cell wall peroxidase activity and hydrogen peroxide level in roots of rice seedlings. Plant Science 5: 323 - 329.

Lin HY, XF Wang, E Ng-Eaton, RA Weinberg and HF Lodish, 1992. Expression cloning of a TGF-type II receptor, a functional transmembrane serine/threonine kinase. Cell 68: 775 – 785.

Loetscher H, Y-CE Pan, H-W Lahm, R Gentz, M Brockhaus, H Tabuchi and W Lesslauer, 1990. Molecular cloning and expression of the human 55 kD tumor necrosis factor receptor. Cell 61: 351 – 359.

Loh Y-T and GB Martin, 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.

Lozoya E, A Block, R Lois, K Hahlbrock and D Scheel, 1991. Transcriptional repression of light-induced flavonoid biosynthesis by elicitor treatment of cultured parsley cells. The Plant Journal 1: 227 – 234.

Main GD, S Reynolds and JS Gartland, 1995. Electroporation protocols for *Agrobacterium*. Methods in Molecular Biology 44: 405 – 412.

Maleck K and RA Dietrich, 1999. Defense of multiple fronts: how do plants cope with diverse enemies? Trends in Plant Sciences 4: 215 – 219.

Mariano AC, MO Andrade, AA Santos, SMB Carolino, ML Oliveira, MC Baracat-Pereira, SG Brommonshenkel and EPB Fontes, 2004. Identification of a novel receptor-like protein kinase that interacts with a geminivirus nuclear shuttle protein. Virology 318: 24 – 31.

Martin GB, S Brommonschenkel, J Chunwongse, A Frary, MW Ganal, R Spivey, T Wu, ED Earle and SD Tanksley, 1993. Map-based cloning of a protein kinase gene conferring disease resistance in tomato. Science 262: 1432 – 1436.

Martin GB, A Frary, T Wu, S Brommonschenkel, J Chunwongse, ED Earle and SD Tanksley, 1994. A member of the Pto gene family confers sensitivity to fenthion resulting in rapid cell death. The Plant Cell 6: 1543 – 1552.

Matsubayashi Y and Y Sakagami, 1996. Phytosulfokine, sulfated peptides that induce the proliferation of single mesophyll cells of *Asparagus officinalis* L. Proceedings of the National Academy of Sciences USA 93: 7623 – 7627.

Matsubayashi Y, M Ogawa, A Morita and Y Sakagami, 2002. An LRR receptor kinase involved in perception of a peptide plant hormone, phytosulfokine. Science 296: 1470 – 1472.

Mauch-Mani B and AJ Slusarenko, 1996. Production of salicylic acid precursors is a major function of phenylalanine ammonialyase in the resistance of *Arabidopsis* to *Peronospora parasitica*. The Plant Cell 8: 203 – 212.

Minet M, M-E Dufour and F Lacroute, 1992. Complementation of *Saccharomyces cerevisiae* auxotrophic mutants by *Arabidopsis thaliana* cDNAs. The Plant Journal 2: 417 – 422.

Mol J, GI Jenkins, E Schäfer and D Weiss, 1996. Signal perception, transduction and gene expression involved in anthocyanins biosynthesis. Critical Reviews in Plant Science 15: 525 – 557.

Montoya T, T Nomura, K Farrar, T Kaneta, T Yokota and GJ Bishop, 2002. Cloning the tomato *Curl3* gene highlights the putative dual role of the leucine-rich repeat receptor kinase tBRI1/SR160 in plant steroid hormone and peptide hormone signaling. The Plant Cell 14: 3163 – 3176.

Morris PC, 2001. MAP kinase signal transduction pathways in plants. New Phytologist 151: 67 – 89.

Morris ER and JC Walker, 2003. Receptor-like protein kinases: the keys to response. Current Opinion in Plant Biology 6: 339 – 342.

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

Mu J-H., H-S. Lee and T-H. Kao, 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.

Mudd JB, 1997. Biochemical basis for the toxicity of ozone. *In* M Yunus and M Iqbal (eds). Plant response to air pollution. John Wiley and Sons, New York, pp 267 – 284.

Mühling KH, C Plieth, UP Hansen and B Sattelmacher, 1995. Apoplastic pH of intact leaves of *Vicia faba* as influenced by light. Journal of Experimental Botany 46: 377 – 382.

Munnik T, 2001. Phosphatidic acid: an emerging plant lipid second messenger. Trends in Plant Sciences 6: 227 – 233.

Munnik T and HJG Meijer, 2001. Osmotic stress activated distinct lipid and MAPK signaling pathways in plants. FEBS Letters 498: 172 – 178.

Munnik T, W Ligterink, II Meskiene, O Calderini, J Beyerly, A Musgrave and H Hirt, 1999. Distinct osmo-sensing protein kinase pathways are involved in signaling moderate and severe hyper-osmotic stress. The Plant Journal 20: 381 - 388.

Murata Y, Z-M Pei, IC Mori and J Schroeder, 2001. Abscisic acid activation of plasma membrane Ca^{2+} channels in guard cells requires cytosolic NAD(P)H and is differentially disrupted upstream and downstream of reactive oxygen species production in abi1-1 and abi2-1 protein phosphatase 2C mutants. The Plant Cell 13: 2513 – 2523.

Muschietti J, Y Eyal and S McCormick, 1998. Pollen tube localization implies a role in pollen-pistil interactions for the tomato receptor-like protein kinases LePRK2 and LePRK2. The Plant Cell 10: 319 – 330.

Mustilli A-C, S Merlot, A Vavasseur, F Fenzi and J Giraudat, 2002. *Arabidopsis* OST1 protein kinase mediated the regulation of stomatal aperture by abscisic acid and acts upstream of reactive oxygen species production. The Plant Cell 14: 3089 – 3099.

Nakagawa H, K Ohmiya and T Hattori, 1996. A rice bZIP protein, designated OSBZ8, is rapidly induced by abscisic acid. The Plant Journal 9: 217 – 227.

Nam KH and J Li, 2002. BRI1/BAK1, a receptor kinase pair mediating brassinosteroid signaling. Cell 110: 203 – 212.

Nasrallah JB and ME Nasrallah, 1993. Pollen-stigma signaling in the sporophytic self-incompatibility response. The Plant Cell 5: 1325 – 1335.

Nasrallah JB, T Nishio and ME Nasrallah, 1991. The self-incompatibility genes of *Brassica*: expression and use in genetic ablation of floral tissues. Annual Review of Plant Physiology and Plant Molecular Biology 42: 393 - 422.

Nasrallah JB, S-M Yu and ME Nasrallah, 1988. Self-incompatibility genes of *Brassica oleracea*: expression, isolation and structure. Proceedings of the National Academy of Sciences USA 85: 5551 – 5555.

Neill SJ, R Desikan, A Clarke, RD Hurst and JT Hancock, 2002. Hydrogen peroxide and nitric oxide as signaling molecules in plants. Journal of Experimental Botany 53: 1237 – 1247.

Neuenschwander U, B Vernooij, L Friedrich, S Uknes, H Kessmann and J Ryals, 1995. Is hydrogen peroxide a second messenger of salicylic acid in systemic acquired resistance? The Plant Journal 8: 227 – 233.

Nielsen H, J Engelbrecht, S Brunak and G von Heijne, 1997. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. Protein Engineering 10: 1 - 6.

Ninnemann H and J Maier, 1996. Indications for the occurrence of nitric oxide synthases in fungi and plants and the involvement in photoconidation of *Neurospora crassa*. Photochemistry and Photobiology 64: 393 – 398.

Nishiguchi M, K Yoshida, T Sumizono and K Tazaki, 2002. A receptor-like protein kinase with a lectin-like domain from Lombardy poplar: gene expression in response to wounding and characterization of phosphorylation activity. Molecular Genetics and Genomics 267: 506 – 514.

Noritake T, K Kawakita and N Doke, 1996. Nitric oxide induces phytoalexin accumulation in potato tuber tissue. Plant Cellular Physiology 37: 113 – 116.

O'Donnell PJ, C Calvert, R Atzorn, C Wasternack, HMO Leyser and DJ Bowles, 1996. Ethylene as a signal mediating the wound response of tomato plants. Science 274: 1914 – 1917.

Ohtake Y, T Takahashi and Y Komeda, 2000. Salicylic acid induces the expression of a number of receptor-like kinase genes in *Arabidopsis thaliana*. Plant and Cell Physiology 41: 1038 – 1044.

Pasternak D, 1987. Salt tolerance and crop production: a comprehensive approach. Annual Review of Phytopathology 25: 271 – 291.

Pastuglia M, D Roby, C Dumas and JM Cock, 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.

Pei Z-M, Y Murata, G Benning, S Thomine, B Klusener, GJ Allen, E Grill and JI Schroeder, 2000. Calcium channels activated by hydrogen peroxide mediate abscisic acid signaling in guard cells. Nature 406: 731 – 734.

Penninckx IAMA, BPHJ Thomma, A Buchala, J-P Metraux and WF Broekaert, 1998. Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in *Arabidopsis*. The Plant Cell 10: 2103 – 2113.

Petch LA, J Harris, VW Raymond, A Blasband, DC Lee and HS Earp, 1990. A truncated secreted form of the epidermal growth factor receptor is encoded by an alternatively spliced transcript in normal rat tissue. Molecular and Cellular Biology 10: 2973 – 2892.

Peumans WJ and EJ van Damme, 1995. Lectins as plant defense proteins. Plant Physiology 109: 347 – 352.

Pieterse CMJ and LC van Loon, 2004. NPR1: the spider in the web of induced resistance signaling pathways. Current Opinion in Plant Biology 7: 456 – 464.

Pieterse CMJ, SCM van Wees, JA van Pelt, M Knoester, R Lann, H Gerrits, PJ Weisbeek and LC van Loon, 1998. A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. The Plant Cell 10: 1571 – 1580.

Pieterse CMJ, J Ton and LC van Loon, 2001. Cross-talk between plant defense signaling pathways: boost or burden? AgBiotechNet 3: ABN 068.

Porankiewicz-Asplund J, B Nilsson, A-S Höglund and L-G Josefsson, 2000. Removal of cross-reactive anti-carrier (keyhole limpit haemocyanin, KLH) from rabbit serum using immobilized KLH. Life Science News 5: 16 – 17.

Prasad TK, MD Anderson, BA Martin and CR Stewart, 1994. Evidence for chilling-induced oxidative stress in maize seedlings and a regulatory role for hydrogen peroxide. The Plant Cell 6: 65 – 74.

Price AH and GAF Hendry, 1991. Iron-catalysed oxygen radical formation and its possible contribution to drought damage in nine native grasses and three cereals. Plant Cell and Environment 14: 477 – 484.

Price A, M Knight, H Knight, T Cuin, D Tomos and T Ashenden, 1996. Cytosolic calcium and oxidative plant stress. Biochemical Society Transactions 24: 479 – 483.

Ranjeva R and AM Boudet, 1987. Phosphorylation of proteins in plants: regulatory effects and potential involvement in stimulus/response coupling. Annual Review of Plant Physiology 38: 73 – 93.

Rao MV and KR Davis, 2001. The physiology of ozone induced cell death. Planta 312: 682 – 690.

Rao MV, H-I Lee, RA Creelman, JE Mullet and KR Davis, 2000. Jasmonic acid signaling modulates ozone-induced hypersensitive cell death. The Plant Cell 12: 1633 – 1646.

Rathjen JP, JH Chang, BJ Staskawicz and RW Michelmore, 1999. Constitutively active *Pto* induces a *Prf*-dependent hypersensitive response in the absence of avrPto. The EMBO Journal 18: 3232 – 3240.

Rebay I, RJ Fleming, RG Fehon, L Cherbas, P Cherbas and S Artavanis Tsakonas, 1991. Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. Cell 67: 687 – 699.

Redman J, J Whitcraft, C Jhonson and J Arias, 2002. Abiotic and biotic stress differentially stimulate as-1 element activity in *Arabidopsis*. Plant Cell Reports 21: 180 – 185.

Reuzeau C and RF Pont-Lezica, 1995. Comparing plant and animal extracellular matrix-cytoskeleton connections. Are they alike? Protoplasma 186: 113 – 121.

Rieping M and F Schoffl, 1992. Synergistic effect of upstream sequences, CCAAT box elements, and HSE sequences for enhanced expression of chimeric heat shock genes in transgenic tobacco. Molecular and General Genetics 231: 226 - 232.

Riou C, C Hervé, V Pacquit, P Dabos and B Lescure, 2002. Expression of an *Arabidopsis* lectin kinase receptor gene, *lecRK-a1*, is induced during senescence, wounding and in response to oligogalacturonic acids. Plant Physiology and Biochemistry 40: 431 – 438.

Rogers HJ, N Bate, J Combe, J Sullivan, J Sweetman, C Swan, DM Lonsdale and D Twell, 2001. Functional analysis of cis-regulatory elements within the promoter of the tobacco late pollen gene g10. Plant Molecular Biology 45: 577 - 585.

Rogl H, K Kosemund, W Kühlbrand and I Collinson, 1998. Refolding of *Escherichia coli* produced membrane protein inclusion bodies immobilized by nickel chelating chromatography. FEBS Letters 432: 21 – 26.

Romeis T, 2001. Protein kinases in the plant defense response. Current Opinion in Plant Biology 4: 407 – 414.

Romeis T, P Piedras, S Zhang, DF Klessig, H Hirt and JDG Jones, 1999. Rapid Avr9 and Cf-9 dependent activation of MAP kinases in tobacco cell cultures and leaves: convergence of resistance gene, elicitor, wound and salicylate responses. The Plant Cell 11: 273 – 287.

Rushton PJ, JT Torres, M Parniske, P Wernert, K Hahlbrock and IE Somssich, 1996. Interaction of elicitor-induced DNA-binding proteins with elicitor response elements in the promoters of parsley PR1 genes. The EMBO Journal 15: 5690 – 5700.

Ryals JA, UH Neuenschwander, MG Willits, A Molina, H-Y Steiner and MD Hunt, 1996. Systemic acquired resistance. The Plant Cell 8: 1809 – 1819.

Ryan CA and G Pearce, 1998. Systemin, a polypeptide signal for plant defense genes. Annual Review of Cellular and Developmental Biology 14: 1 – 17.

Sagi M and R Fluhr, 2001. Superoxide production by plant homologues of the $gp91^{phox}$ NADPH oxidase. Modulation of activity by calcium and by tobacco mosaic virus infection. Plant Physiology 126: 1281 – 1290.

Salmeron JM, GED Oldroyd, CMT Rommens, SR Scofield, H-S Kim, DT Lavelle, D Dahlbeck and BJ Staskawicz, 1996. Tomato *Prf* is a member of the leucine-rich repeat class of plant disease resistance genes and lies embedded within the *Pto* kinase gene cluster. Cell 86: 123 – 133.

Sambrook J, EF Fritsch and T. Maniates, 1989. Molecular cloning, a laboratory manual (2^{nd} edition). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp A1 – E16.

Samuel MA, GP Miles and BE Ellis, 2000. Ozone treatment rapidly activates MAP kinase signaling in plants. The Plant Journal 22: 367 – 376.

Satterlee JS and MR Sussman, 1998. Unusual membrane-associated protein kinases in plants. Journal of Membrane Biology 164: 205 – 212.

Savouré A, X-J Hua, N Bertauche, M Van Montagu and N Verbruggen, 1997. Abscisic acid-independent and abscisic acid-dependent regulation of proline biosynthesis following cold and osmotic stresses in *Arabidopsis thaliana*. Molecular and General Genetics 254: 104 - 109.

Schaller GE and AB Bleecker, 1993. Receptor-like kinase activity in membranes of *Arabidopsis thaliana*. FEBS Letters 333: 306 – 310.

Scheer JM and CA Ryan, 1999. A 160-kD systemin receptor on the surface of *Lycopersicon peruvianum* suspension-cultured cells. The Plant Cell 11: 1525 – 1535.

Scheer JM and CA Ryan, 2002. The systemin receptor SR160 from *Lycopersicon peruvianum* is a member of the LRR receptor kinase family. Proceedings of the National Academy of Sciences USA 99: 9585 – 9590.

Schmidt EDL, F Guzzo, MAJ Toonen and SC de Vries, 1997. A leucine-rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos. Development 124: 2049 – 2062.

Schneider R and M Schweiger, 1991. A novel modular mosaic of cell adhesion motifs in the extracellular domains of the neurogenic trk and trkB tyrosine kinase receptors. Oncogene 6: 1807 – 1811.

Schopfer C, M Nasrallah and J Nasrallah, 1999. The male determinant of self-incompatibility in *Brassica*. Science 286: 1697 – 1700.

Schopfer P, C Plachy and G Frahry, 2001. Release of reactive oxygen intermediates (superoxide radicals, hydrogen peroxide, and hydroxyl radicals) and peroxidase in germinating radish seeds controlled by light, gibberellin, and abscisic acid. Plant Physiology 125: 1591 - 1602.

Schulze-Muth P, S Irmler, G Schröder and J Schröder, 1996. Novel type of receptor-like protein kinase from a higher plant (*Catharanthus roseus*). Journal of Biological Chemistry 271: 26684 – 26689.

Scofield SR, CM Tobias, JP Rathjen, JH Chang, DT Lavelle, RW Michelmore and BJ Staskawicz, 1996. Molecular Basis of Gene-for-Gene Specificity in Bacterial Speck Disease of Tomato. Science 274: 2063 - 2065.

Searle IR, AE Men, TS Laniya, DM Buzas, I Iturbe-Ormaetxe, BJ Carroll and PM Gresshoff, 2003. Long-distance signaling in nodulation directed by a CLAVATA1-like receptor kinase. Science 299: 109 – 112.

Senaratna T, D Touchell, T Bunn and K Dixon, 2000. Acetyl salicylic acid (aspirin) and salicylic acid induce multiple stress tolerance in bean and tomato plants. Plant Growth Regulation 30: 157 – 161.

Sessa G and GB Martin, 2000. Protein kinases in the plant defense response. Advances in Botanical Research 32: 379 – 404.

Sessa G, M D'Ascenzo, Y-T Loh and GB Martin, 1998. Biochemical properties of two protein kinases involved in disease resistance signaling in tomato. Journal of Biological Chemistry 273: 15860 – 15865.

Sessa G, M D'Ascenzo and GB Martin, 2000. Thr38 and Ser198 are Pto autophosphorylation sites required for the Avr-Pto-Pto-mediated hypersensitive response. The EMBO Journal 10: 2257 – 2269.

Shah J, 2003. The salicylic acid loop in plant defense. Current Opinion in Plant Biology 6: 365 – 371.

Shah J, F Tsui and DF Klessig, 1997. Characterization of a salicylic acid insensitive mutant (*sai1*) of *Arabidopsis thaliana*, identified in a selective screen utilizing the SA-inducible expression of the *tms2* gene. Molecular Plant and Microbe Interaction 10: 69 – 78.

Shah J, PK Kachroo and DF Klessig, 1999. The *Arabidopsis ssi1* mutation restores pathogenesis-related gene expression in *npr1* plants and renders defensin gene expression salicylic acid dependent. The Plant Cell 11: 191 – 206.

Shah J, PK Kachroo, A Nandi and DF Klessig, 2001. A recessive mutation in the *Arabidopsis* SSI2 gene confers SA- and NPR1-independent expression of PR genes

and resistance against bacterial and oomycete pathogens. The Plant Journal 25: 563 – 574.

Sharma YK, J León, I Raskin and KR Davis, 1996. Ozone-induced responses in *Arabidopsis thaliana*: the role of salicylic acid in the accumulation of defense-related transcripts and induced resistance. Proceeding of the National Academy of Sciences 93: 5099 – 5104.

Shinozaki K and K Yamaguchi-Shinozaki, 1997. Gene expression and signal transduction in water stress response. Plant Physiology 115: 327 – 334.

Shirano Y, P Kachroo, J Shah and DF Klessig, 2002. A gain-of-function mutation in an *Arabidopsis* toll interleukin 1 receptor-nucleotide binding site- leucine rich repeat type *R* gene triggers defense responses and results in enhanced disease resistance. The Plant Cell 14: 3149 – 3162.

Shirasu K, H Nakajima, K Rejashekar, RA Dixon and C Lamb, 1997. Salicylic acid potentiates an agonist-dependent gain control that amplifies pathogen signals in the activation of defense mechanisms. The Plant Cell 9: 261 – 270.

Shirsat A, N Wilford, R Croy and D Boulter, 1989. Sequences responsible for the tissue specific promoter activity of a pea legumin gene in tobacco. Molecular and General Genetics 215: 326 - 331.

Shiu S-H and AB Bleecker, 2003. Expansion of the receptor-like kinase / Pelle gene family and receptor-like proteins in *Arabidopsis*. Plant Physiology 132: 530 – 543.

Showalter AM, 1993. Structure and function of plant cell wall proteins. The Plant Cell 5: 9 - 23.

Shulaev V, J León and I Raskin, 1995. Is salicylic acid a translocated signal of systemic acquired resistance in plants? The Plant Cell 7: 1691 – 1701.

Shulaev V, P Silverman and I Raskin, 1997. Airborne signaling by methyl salicylate in plant pathogen resistance. Nature 385: 718 – 721.

Silva NF and DR Goring, 2002. The proline-rich, extensin-like receptor kinase-1 (PERK1) gene is rapidly induced by wounding. Plant Molecular Biology 50: 667 – 685.

Simpson SD, K Nakashima, Y Narusaka, M Seki, K Shinozaki and K Yamaguchi-Shinozaki, 2003. Two different novel cis-acting elements of erd1, a clpA homologous *Arabidopsis* gene function in induction by dehydration stress and dark-induced senescence. The Plant Journal 33: 259 - 270.

Singer SJ, 1990. The structure and insertion of integral proteins in the membrane. Annual Review of Cell Biology 6: 247 – 296.

Singh KK, 2000. The *Saccharomyces cerevisiae* SLN1P-SSK1P two-component system mediates response to oxidative stress and in an oxidant-specific fashion. Free Radical Biology and Medicine 29: 1043 – 1050.

Smirnoff N, 1993. The role of active oxygen in the response of plants to water deficit and desiccation. New Phytologist 125: 27 – 58.

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

Spoel SH, A Koornneef, SM Claessens, JP Korzelius, JA van Pelt, MJ Mueller, AJ Buchala, J-P Metraux, R Brown, K Kazan, LC van Loon, X Dong and CMJ Pieterse, 2003. NPR1 modulates crosstalk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. The Plant Cell 15: 760 – 770.

Stalberg K, M Ellerstom, I Ezcurra, S Ablov and L Rask, 1996. Disruption of an overlapping E-box/ABRE motif abolished high transcription of the napA storage-protein promoter in transgenic *Brassica napus* seeds. Planta 199: 515 - 519.

Stein JC and JB Nasrallah, 1993. A plant receptor-like gene, the S-locus receptor kinase of *Brassica oleracea* L., encodes a functional serine/threonine kinase. Plant Physiology 101: 1103 – 1106.

Stein JC, B Howlett, DC Boyes, ME Nasrallah and JB Nasrallah, 1991. Molecular cloning of a putative receptor protein kinase encoded at the self-incompatibility locus of *Brassica oleracea*. Proceedings of the National Academy of Sciences USA 88: 8816 – 8820.

Stein JC, R Dixit, ME Nasrallah and JB Nasrallah, 1996. SRK, the stigma specific S locus receptor kinase of *Brassica*, is targeted to the plasma membrane in transgenic tobacco. The Plant Cell 8: 429 – 445.

Stone JM, MA Collinge, RD Smith, MA Horn and JC Walker, 1994. Interaction of a protein phosphatase with an *Arabidopsis* serine-threonine receptor kinase. Science 266: 793 – 795.

Stone JM, AE Trotochaud, JC Walker and SE Clark, 1998. Control of meristem development by CLAVATA1 receptor kinase and kinase-associated protein phosphatase interactions. Plant Physiology 117: 1217 - 1225.

Stone S, M Arnoldo and D Goring, 1999. A breakdown of *Brassica* self-incompatibility in *ARC1* antisense transgenic plants. Science 286: 1729 – 1731.

Stracke S, C Kistner, S Yoshida, L Mulder, S Sato, T Kaneko, S Tabata, N Sandal, J Stougaard, K Szczyglowski and M Parniske, 2002. A plant receptor-like kinase required for both bacterial and fungal symbiosis. Nature 417: 959 – 962.

Suzuki G, M Watanabe, N Kai, N Matsuda, K Toriyama, S Takayama, A Isogai and K Hinata, 1997. Three members of the *S* multigene family are linked to the *S* locus of *Brassica*. Molecular and General Genetics 256: 257 – 264.

Szekeres M, 2003. Brassinosteroid and systemin: two hormones perceived by the same receptor. Trends in Plant Science 8: 102 – 104.

Takahashi T, J-H Mu, A Gasch and N-H Chua, 1998. Identification by PCR of receptor-like protein kinases from *Arabidopsis* flowers. Plant Molecular Biology 37: 587 – 596.

Takaiwa F and K Oono, 1990. Interaction of an immature seed-specific trans-acting factor with the 5' upstream region of a rice glutelin gene. Molecular and General Genetics 224: 289 - 293.

Takemoto D, M Hayashi, N Doke, M Nishimura and K Kawakita, 2000. Isolation for the gene for EILP, an elicitor-inducible LRR receptor-like protein from tobacco by differential display. Plant and Cellular Physiology 41: 458 – 464.

Tanaka N, F Che, N Watanabe, S Fujiwara, S Takayama and A Isogai, 2003. Flagellin form an incompatible strain of *Acidovorax avenae* mediates H_2O_2 generation accompanying hypersensitive cell death and expressing of *PAL*, *Cht-1* and *PBZ1*, but not of *LOX* in rice. Molecular Plant Pathogen Interactions 16: 422 – 428.

Tang W and SE Perry, 2003. Binding site selection for the plant MADS-domain protein AGL15: an *in vitro* and *in vivo* study. Journal of Biological Chemistry 278: 28154 – 28159.

Tang X, RD Frederick, J Zhou, DA Halterman, Y Jia and GB Martin, 1996. Initiation of plant disease resistance by physical interaction of AvrPto and Pto kinase. Science 274: 2060 – 2063.

Tang X, M Xie, YJ Kim, J Zhou, DF Klessig and GB Martin, 1999. Overexpression of *Pto* activates defense responses and confers broad resistance. The Plant Cell 11: 15 – 29.

Taylor DP, J Slattery and AC Leipold, 1996. Apoplastic pH in corn root gravitropism: a laser scanning confocal microscopy measurement. Physiology Plantarum 97: 35 – 38.

Tepass U, C Theres and E Knust, 1990. Crumbs encodes an EGF-like protein expressed on apical membranes of Drosophila epithelial cells and required for organization of epithelia. Cell 61: 787 – 799.

Terzaghi WB and AR Cashmore, 1995. Light-regulated transcription. Annual Review of Plant Physiology and Plant Molecular Biology 46: 445 - 474.

Thomas CM, DA Jones, M Parniske, K Harrison, PJ Balint-Kurti, K Hatzixanthis and JDG Jones, 1997. Characterization of the tomato *Cf-4* gene for resistance to *Cladosporium fulvum* identified sequences that determine recognitional specificity in Cf-4 and Cf-9. The Plant Cell 9: 2209 – 2224.

Thomma BPHJ, K Eggermont, KFM-J Tierens and WF Broekaert, 1999. Requirement of functional *EIN2* (ethylene insensitive 2) gene for efficient resistance of *Arabidopsis thaliana* to infection by *Botrytis cinerea*. Plant physiology 121: 1093 – 1101.

Timoshenko AV, LV Dubovskaya, OD Karvatskaya, VV Zharkov, S Andre and HJ Gabius, 1999. NO-dependent regulation of lectin- and menadione-induced H_2O_2 production by cells from pleural effusions of lung cancer patients and by immune cells. International Journal of Oncology 14: 793 - 798.

Tiwari BS, B Belenghi and A Levine, 2002. Oxidative stress increased respiration and generation of reactive oxygen species, resulting in ATP depletion, opening of mitochondrial permeability transition and programmed cell death. Plant Physiology 128: 1271 – 1281.

Tjaden G, JW Edwards and GM Coruzzi, 1995. *cis* elements and *trans*-acting factors affecting regulation of a non-photosynthetic light-regulated gene for chloroplast glutamine synthetase. Plant Physiology 108: 1109 - 1117.

Tobias CM and JB Nasrallah, 1996. An *S*-locus-related gene in *Arabidopsis* encodes a functional kinase and produces two classes of transcripts. The Plant Journal 10: 523 – 531.

Tobias CM, B Howlett and JB Nasrallah, 1992. An *Arabidopsis thaliana* gene with sequence similarity to the S-locus receptor kinase of *Brassica oleracea*. Plant Physiology 99: 284 – 290.

Ton J, JA van Pelt, LC van Loon and CMJ Pieterse, 2002. Differential effectiveness of salicylate-dependent and jasmonate/ethylene-dependent induced resistance in *Arabidopsis*. Molecular Plant-Microbe Interaction 15: 27 – 34.

Torii KU, 2000. Receptor kinase activation and signal transduction in plants: an emerging picture. Current Opinion in Plant Biology 3: 361 – 367.

Torii KU and SE Clark, 2000. Receptor-like kinases in plant development. Advances in Botanical Research 32: 225 – 267.

Torii KU, N Mitsukawa, T Oosumi, Y Matsuura, R Yokoyama, RF Whittier and Y Komeda, 1996. The *Arabidopsis ERECTA* gene encodes a putative receptor protein kinase with extracellular leucine-rich repeats. The Plant Cell 8: 735 – 746.

Trewavas AJ and HG Jones, 1991. An assessment of the role of ABA in plant development. *In* WJ Davies and HG Jones (eds). Abscisic Acid: Physiology and Biochemistry. BIOS Scientific Publishers, Oxford, pp 169 – 188.

Trotochaud AE, T Hao, G Wu, Z Yang and SE Clark, 1999. The *Arabidopsis* CLV1 receptor-like kinase requires CLV3 for its assembly into a signaling complex that includes KAPP and a Rho GTPase. The Plant Cell 11: 393 – 405.

Trotochaud AE, S Jeong and SE Clark, 2000. CLAVATA3, a multimeric ligand for the CLAVATA1 receptor-kinase. Science 289: 613 – 617.

Turner JG, C Ellis and A Devoto, 2002. The jasmonate signal pathway. The Plant Cell 14 Supplement: 153 – 164.

Uknes S, A Winter, T Delaney, B Vernooij, A Morse, L Friedrich, G Nye, S Potter, E Ward and J Ryals, 1993. Biological induction of systemic acquired resistance in *Arabidopsis*. Molecular Plant-Microbe Interaction 6: 692 – 698.

Ullrich A and J Schlessinger, 1990. Signal transduction by receptors with tyrosine kinase activity. Cell 61: 203 – 212.

Urao T, K Yamaguchi-Shinozaki, S Urao and K Shinozaki, 1993. An *Arabidopsis* myb homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. The Plant Cell 5: 1529 - 1539.

Urao T, B Yakubov, R Satoh, K Yamaguchi-Shinozaki, M Seki, T Hirayama and K Shinozaki, 1999. A transmembrane hybrid-type histidine kinase in *Arabidopsis* functions as an osmosensor. The Plant Cell 11: 1743 – 1754.

Urao T, K Yamaguchi-Shinozaki and K Shinozaki, 2000. Two-component systems in plant signal transduction. Trends in Plant Science 5: 67 – 73.

Valon C, J Smalle, HM Goodman and J Giraudat, 1993. Characterization of an *Arabidopsis thaliana* gene (TMKL1) encoding a putative transmembrane protein with an unusual kinase-like domain. Plant Molecular Biology 23: 415 – 421.

Van der Ackerveken GFJM, JAL van Kan and PJGM de Wit, 1992. Molecular analysis of the avirulence gene *Avr9* of the fungal pathogen *Cladosporium fulvum* fully supports the gene-for-gene hypothesis. The Plant Journal 2: 359 – 366.

Van der Knaap E, W-Y Song, D-E Ruan, M Sauter, PC Ronald and H Kende, 1999. Expression of a gibberellin-induced Leucine-rich repeat receptor-like protein kinase in deepwater rice and its interaction with kinase-associated protein phosphatase. Plant Physiology 120: 559 – 569.

Van Loon LC, 1997. Induced resistance in plants and the role of PR proteins. European Journal of Plant Pathology 103: 753 – 765.

Van Loon LC and EA van Strien, 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.

Van Montfort RL, M Congreve, D Tisi, R Carr and H Jhoti, 2003. Oxidation state of the active-site cysteine in protein tyrosine phosphatase 1B. Nature 423: 773 – 777.

Verberne MC, RAB Muljono and R Verpoorte, 1999. Salicylic acid biosynthesis. *In* PJJ Hooykaas, MA Hall and KR Libbenga (eds). Biochemistry and molecular biology of plant hormones. Amsterdam: Elsevier Science Publications pp 295–312.

Verberne MC, R Verpoorte, JF Bol, J Mercado-Blanco, and HJ Linthorst, 2000. Overproduction of salicylic acid in plants by bacterial transgenes enhances pathogen resistance. National Biotechnology 18: 779 – 783.

Verbruggen N, R Villarroel and M van Montagu, 1993. Osmoregulation of a pyrroline-5-carboxilate reductase gene in *Arabidopsis thaliana*. Plant Physiology 103: 771 – 781.

Vernooij B, L Friedrich, A Morse, R Reist, R Kolditzjawhar, E Ward, S Uknes, H Kessmann and J Ryals, 1994. Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance, but is required in signal transduction. The Plant Cell 6: 959 – 969.

Vidal S, I Ponce de León, J Deneke and TE Palva, 1997. Salicylic acid and the plant pathogen *Erwinia carotovora* induce defense genes via antagonistic pathways. The Plant Journal 11: 115 – 123.

von Heijne G, 1990. The signal peptide. Journal of Membrane Biology 115: 195 – 201.

Walker JC, 1993. Receptor-like protein kinase genes of *Arabidopsis thaliana*. The Plant Journal 3: 451 – 456.

Walker JC, 1994. Structure and function of the receptor-like kinases of higher plants. Plant Molecular Biology 26: 1599 – 1609.

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

Wang X, P Zafian, M Choudhary and M Lawton, 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 USA 93: 2598 - 2602.

Wang G-L; D-L Ruan, W-Y Song, S Sideris, L Chen, L-Y Pi, S Zhang, Z Zhang, C Fauquet, BS Gaut, MC Whalen and PC Ronald, 1998. *Xa21D* encodes a receptor-like molecule with a leucine-rich repeat domain that determines Race-specific recognition and is subject to adaptive evolution. The Plant Cell 10: 765 – 779.

Wang ZY, H Seto, S Fujioka, S Yoshida and J Chory, 2001. BRI1 is a critical component of as a plasma membrane receptor for plant steroids. Nature 410: 380 – 383.

Wang W, B Vinocur and A Altman, 2003. Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta 218: 1 – 14.

Washida H, CY Wu, A Suzuki, U Yamanouchi, T Akihama, K Harada and F Takaiwa, 1999. Identification of cis-regulatory elements required for endosperm expression of the rice storage protein glutelin gene GluB-1. Plant Molecular Biology 40: 1 - 12.

Weigel D and J Glazebrook, 2002. *Arabidopsis*: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Weil M, S Krausgrill, A Schuster and T Rausch, 1994. A 17 kD *Nicotiana tabacum* cell-wall peptide acts as an *in vitro* inhibitor of the cell wall isoform of acid invertase. Planta 193: 438 – 445.

Weinstein JN, R Blumenthal, J van Renswoude, C Kempf and RD Klausner, 1982. Charge clusters and the orientation of membrane proteins. Journal of Membrane Biology 66: 203 – 212.

Whalen MC, RW Innes, AF Bent and BJ Staskawicz, 1991. Identification of *Pseudomonas syringae* pathogens of *Arabidopsis* and a bacterial locus determining avirulence on both *Arabidopsis* and soybean. The Plant Cell 3: 49 – 59.

Wildermuth MC, J Dewdney, G Wu and FM Ausubel, 2001. Isochorismate synthase is required to synthesize salicylic acid for plant defense. Nature 414: 562 – 565.

Williams RW, JM Wilson and EM Meyerowitz, 1997. A possible role for kinase associated protein phosphatase in the *Arabidopsis* CLAVATA signaling pathway. Proceedings of the National Academy of Sciences USA 94: 10467 – 10472.

Wobbe KK and DF Klessig, 1996. Salicylic acid, an important signal in plants. *In* ES Dennis, B Hahn, T Hohn, F Meins Jr, J Schell and DPS Verma (eds). Plant Gene Research: Signal transduction and Development. Springer, New York, pp 167 – 196.

Wu C, H Washida, Y Onodera, K Harada and F Takaiwa, 2000. Quantitative nature of the Prolamin-box, ACGT and AACA motifs in a rice glutelin gene promoter: minimal cis-element requirements for endosperm-specific gene expression. The Plant Journal 23: 415-421.

Xu Y, PFL Chang, D Liu, ML Narasimhan, KG Raghothama, PM Hasegawa and RA Bressan, 1994. Plant defense genes are synergistically induced by ethylene and methyl jasmonate. The Plant Cell 6: 1077 – 1085.

Xu N, G Hagen and T Guilfoyle, 1997. Multiple auxin response modules in the soybean SAUR 15A promoter. Plant Science 126: 193 - 201.

Yalpani N, AJ Enyedi, J Leon and I Raskin, 1994. Ultraviolet light and ozone stimulate accumulation of salicylic acid ant pathogenesis-related proteins and virus-resistance in tobacco. Planta 193: 373 – 376.

Yamaguchi-Shinozaki K and K Shinozaki, 1993. Characterization of the expression of a desiccation-responsive rd29 gene of *Arabidopsis thaliana* and analysis of its promoter in transgenic plants. Molecular and General Genetics 236: 331 – 340.

Yamamuro C, Y Ihara, X Wu, T Noguchi, S Fujioka, S Takatsuto, M Ashikari, H Kitano and M Matsuoka, 2000. Loss of function of a rice brassinosteroid insensitive1 homologue prevents internode elongation and bending of the lamina bend. The Plant Cell 12: 1591 – 1606.

Yanagisawa S, 2000. Dof1 and Dof2 transcription factors are associated with expression of multiple genes involved in carbon metabolism in maize. The Plant Journal 21: 281 - 288.

Yardin Y and A Ullrich, 1988. Growth factor receptor tyrosine kinases. Annual Review of Biochemistry 57: 443 – 478.

Youl JJ, A Bacic and D Oxley, 1998. Arabinogalactan proteins from *Nicotiana alata* and *Pyrus communis* contain glycosylphosphatidyl inositol membrane anchors. Proceedings of the National Academy of Sciences USA 95: 7921 – 7926.

Yu D, Y Liu, B Fan, DF Klessig and Z Chen, 1997. Is the high basal level of salicylic acid important for disease resistance in potato? Plant Physiology 115: 343 – 349.

Yu D, C Chen and Z Chen, 2001. Evidence for an important role of WKRY DNA binding proteins in the regulation of the *NPR1* gene. The Plant Cell 13: 1527 – 1539.

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

Zhang S and DF Klessig, 1997. Salicylic acid activates a 48 kD MAP kinase in tobacco. The Plant Cell 9: 809 – 824.

Zhang R and JC Walker, 1993. Structure and expression of the S locus-related genes of maize. Plant Molecular Biology 21: 1171 – 1174.

Zhang Y, W Fan, M Kinkema, X Li and X Dong, 1999. Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the *PR-1* gene. Proceedings of the National Academy of Sciences USA 96: 6523 – 6528.

Zhang YL, MJ Tessaro, M Lassner and X Li, 2003. Knockout analysis of *Arabidopsis* transcription factors TGA2, TGA5 and TGA6 reveals their redundant and essential roles in systemic acquired resistance. The Plant Cell 15: 2647 – 2653.

Zhao Y, X-H Feng, JC Watson, PJ Bottino and S-D Kung, 1994. Molecular cloning and biochemical characterization of a receptor-like serine/threonine kinase from rice. Plant Molecular Biology 26: 791 – 803.

Zhao L, G Chen and C Zhang, 2001. Interaction between reactive oxygen species and nitric oxide in drought-induced abscisic acid systhesis in root tips of wheat seedlings. Australian Journal of Plant Physiology 28: 1055 – 1061.

Zhou DX, 1990. Regulatory mechanism of plant gene transcription by GT-elements and GT-factors. Trends in Plant Science 4: 210 - 214.

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

Zhou J, X Tang and GB Martin, 1997. The Pto kinase conferring resistance to tomato bacterial speck disease interacts with proteins that bind a *cis*-element of pathogenesis related genes. The EMBO Journal 16: 3207 – 3218.

Zhu J-K, 2001. Cell signaling under salt, water and cold stresses. Current Opinion in Plant Biology 4: 401- 406.

– ———————————————————— –

Chapter 8

Summaries

Summary

An attempt was made during this study to assign a putative role for At-RLK3 in *Arabidopsis thaliana*.

Receptor-like protein kinases (RLKs) are a group of proteins that was described in various plants. These proteins have a very characteristic structure. They are integral plasma membrane proteins where a hydrophobic transmembrane domain links an extracellular ligand binding domain with an intracellular protein kinase domain. RLKs are involved in various plant processes including development, disease resistance and hormonal signaling.

At-RLK3 is a single copy gene in *A. thaliana* that is expressed during various stress conditions. Oxidative and osmotic stress, infection with pathogens and exposure to salicylic acid (SA) all led to the induced expression of the gene. A similar increase in At-RLK3 protein levels was found after the respective treatments. The kinase domain shares high homology with several other protein kinases and has all 11 conserved sub-domains characteristic of plant protein kinases. It was proven that At-RLK3 is an active protein kinase that specifically phosphorylates serine and threonine amino acids within its active domain. It preferentially uses Mg^{2+} as cofactor. The enzyme is located at the plasma membrane and is rapidly activated upon treatment with hydrogen peroxide (H_2O_2), menadione and osmotic stress conditions, but not after SA treatment.

The extracellular domain has two conserved cysteine rich regions that form part of two "domain of unknown function" (DUF) regions. These cysteines are thought to be involved in the formation of disulphide bridges and to be responsive to changes in the redox conditions surrounding the cell.

Plants expressing an antisense copy of the gene showed an altered response to treatments with H_2O_2 and SA compared to the wild type plants. Whereas the wild type plants activated the plant defense shortly after treatment with both H_2O_2 and SA, only SA managed a similar activation in the transgenic plants. The transgenic plants treated with H_2O_2 did not show this initial activation. In addition, after both treatments the wild type plants exhibited an amplified and sustained activation of the defense reaction 48 h after treatment which was completely absent in the transgenic plants.

This led to the hypothesis that At-RLK3 is responsible to detect changes in extracellular H_2O_2 levels in *A. thaliana* most probably due to a change in redox conditions. This then leads to the activation of the enzyme and the subsequent activation of the plant defense response.

Key words: RLK, *At-RLK3*, oxidative stress, osmotic stress, redox sensor.

Opsomming

Die doel van die studie was om ‘n moontlike funksie vir At-RLK3 in *Arabidopsis thaliana* voor te stel.

Reseptorproteïenkinases (RLKs) is ‘n groep proteïene wat in verskillende plante beskryf is. Die proteïene vorm die groep as gevolg van hulle kenmerkende struktuur. Hulle is integrale plasmamembraanproteïene wat in die membraan deur ‘n hidrofobiese transmembraan domein geanker is. Die domein verbind twee ander gedeeltes van die proteïen met mekaar, naamlik ‘n intrasellulêre proteïen kinase domein en ‘n ekstrasellulêre domein wat in staat is om ligande te bind. RLKs is betrokke by ‘n verskeidenheid van sellulêre prosesse soos plantontwikkeling, die weerstand van die plant teen siektes en die oordrag van hormonale seine.

At-RLK3 is ‘n enkelkopie geen in die *A. thaliana* genoom. Die uiting van die geen word deur ‘n verskeidenheid faktore aangeskakel. Dit sluit in oksidatiewe en osmotiese strestoestande, infeksies deur patogene en die blootstelling van die plant aan salisielsuur (SA). Die aktivering van geenuiting was gevolg deur ‘n soortgelyke toename in proteïenkonsentrasie binne die sel. Die kinasedomein toon homologie met verskeie ander plant proteïenkinases. Verder is al 11 gekonserveerde subdomeins wat kenmerkend is van aktiewe proteïen kinases, teenwoordig. At-RLK3 is ‘n aktiewe proteïenkinase wat serien en treonien aminosure in sy kinasedomein spesifiek fosforileer. Die ensiem gebruik ook by voorkeur Mg^{2+} as kofaktor. Die ensiem is plasmamembraan gebonde en word vinnig na waterstofperoksied (H_2O_2), menadioon en osmotiese stres behandelings geaktiveer. SA behandeling aktiveer die ensiem egter glad nie.

Die ekstrasellulêre domein van At-RLK3 besit twee gekonserveerde sisteïenryke gebiede wat deel vorm van twee ander gebiede wat bekend staan as “domein met onbekende funksie” (DUFs). Dit is moontlik dat die gekonserveerde sisteïene by die

vorming van disulfiedbrûe betrokke kan wees en dat dit verder sensitief vir enige veranderings in die redokstoestande buite die sel is.

Transgeniese plante waarin ‘n teensinkopie van die geen tot uiting kom, het ‘n ander fenotipe as die wilde tipe plante getoon nadat dit met H_2O_2 en SA behandel is. Die wilde tipe plante het in beide gevalle ‘n aktivering van die plantverdedigingsreaksie kort na behandeling getoon. Terwyl SA behandelde transgeniese plante ook ‘n soortgelyke aktivering getoon het, het die transgeniese plante wat met H_2O_2 behandel is, geen aktivering getoon nie. Verder het die wildetipe plante op die langer duur ‘n sterk voortgesette aktivering van plantverdediging getoon, wat totaal in die transgeniese plante afwesig was.

Dit het daartoe gelei dat ‘n hipotetiese funksie van At-RLK3 voorgestel is waar die ensiem verantwoordelik is vir die deteksie van verhoogde H_2O_2-vlakke buite die sel. Die aktivering van die ensiem is bes moontlik die gevolg van veranderinge in die redokskondisies, wat uiteindelik dan tot die aktivering van die plantverdedigingsrespons lei.

Sleutelwoorde: RLK, *At-RLK3*, oksidatiewe stres, osmotiese stres, redoks sensor.

Appendix

Appendix 1. List of oligonucleotides used

Name	Sequence	Palindrome	Application
Bovis 1	5'-TGC-**TCT-AGA-**CTA-CAG-ATT-CAT-TGG-TAT-ACG-3'	*Xba*1	Amplification of portion of *At-RLK3* encoding the kinase domain for cloning into pKYLX71:35S
Bovis 2	5'-CCG-**GAG-CTC-**GAC-TTG-CTT-GTA-TAC-TGA-TCA-GAC-3'	*Sac*1	Amplification of portion of *At-RLK3* encoding the kinase domain for cloning into pKYLX71:35S
Bovis 3	5'- TGC **TCT AGA** AAT GAA GCA GAG GAG TTT GTT TTC-3'	*Xba*1	Amplification of full length *At-RLK3* gene for cloning into pTA7002
Bovis 4	5'- CCG **GTC GAC** GAC TTG CTT GTA TAC TGA TCA GAC -3'	*Sal*1	Amplification of full length *At-RLK3* gene for cloning into pTA7002
Bovis 5	5'- TGC **TCT AGA** CTA CAG ATT CAT TGG TAT ACG-3'	*Xba*1	Amplification of portion of *At-RLK3* encoding the kinase domain for cloning into pTA7002
Bovis 6	5'- CCG **GTC GAC** GAC TTG CTT GTA TAC TGA TCA G-3'	*Sal*1	Amplification of portion of *At-RLK3* encoding the kinase domain for cloning into pTA7002
Bovis 7	5'-GC**G-AAT-TC**A-ATG-AAG-CAG-AGG-AGT-TTG-TT-3'	*Eco*R1	Upstream primer for cloning of full-length At-RLK3 gene from a cDNA library
Bovis 10	5'-TAT-AT**C-CAT-GG**A-TGC-AAT-GCA-CGC-CGG-3'	*Nco*1	Upstream primer for expression of full-length and extracellular domain of *At-RLK3* in yeast and bacteria
Bovis 11	5'-GAC-TTG-CTT-**GAA-TTC**-TGA-TCA-GAC-3'	*Eco*RI	Downstream primer for expression of full-length *At-RLK3* cDNA in *E. coli*
Bovis 12	5'-CTC-CAG-CTG-A**GA-ATT-C**TT-TGC-TAT-C-3'	*Eco*R1	Downstream primer for expression of extracellular domain of *At-RLK3* in *E. coli*
Bovis 13	5'-CTA-CAG-ATT-**CCA-TGG**-TAT-ACG-ATT-TTA-AGA-C-3'	*Nco*1	Upstream primer for expression of kinase domain of *At-RLK3* in *E. coli*

Bovis 14	5'-GAC-TTG-CTT-**GTC-GAC**-TGA-TCA-GAC-3'	*Sal*1	Downstream primer for expression of full-length *At-RLK3* in yeast
Bovis 15	5'-TGC-**TCT-AGA**-AAT-GAA-GCA-GAG-GAG-TTT-GTT-TTC-3'	*Xba*1	Amplification of full length *At-RLK3* gene for cloning into pKYLX71:35S
Bovis 16	5'-CCG-**GAG-CTC**-GAC-TTG-CTT-GTA-TAC-TGA-TCA-GAC-TC-3'	*Sac*1	Amplification of full length *At-RLK3* gene for cloning into pKYLX71:35S
Bovis 26	5'-CAA-CTT-TCG-ATG-GTA-GGA-TAG-3'	-	Amplification of the 18S rRNA fragment
Bovis 27	5'-CTC-GTT-AAG-GGA-TTT-AGA-TTG-3'	-	Amplification of the 18S rRNA fragment
Bovis 30	5'-ACT-GAT-CAG-ACT-CTG-ATCCTTC-3'	-	Amplification of antisense transcripts of the portion of *At-RLK3* encoding the kinase domain in transgenic plants
Bovis 31	5'-CAG-ATT-CAT-TGG-TAT-ACG-ATT-3'	-	Amplification of antisense transcripts of the portion of *At-RLK3* encoding the kinase domain in transgenic plants
Bovis 37	5'-TAG-GCG-ATA-CCT-TGC-CAA-3'	-	Amplification of a portion of the *PR-2* gene
Bovis 38	5'-ACT-TCA-TAC-TTA-GAC-TGT-CG-3'	-	Amplification of a portion of the *PR-2* gene