THE ROLE OF A RECEPTOR-LIKE PROTEIN KINASE (At-RLK3) IN THE PERCEPTION OF CHEMICAL ACTIVATORS IN *Arabidopsis thaliana*

by

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LIST OF ABBREVIATIONS

A

APS	=	Ammonium persulphate
ASA	=	Acetylsalicylic acid
Avr	=	Avirulence

B

bp	=	Base pair
BTH	=	Benzo (1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester

C

CHRK	=	Chitinase-related receptor-like kinase
CRR	=	Cysteine-rich repeat

D

DEPC	=	Dimethyl pyrocarbonate
DEX	=	Dexamethazone
DMSO	=	Dimethylsulfoxide
dNTP	=	Deoxynucleotide triphosphate
DTT	=	Dithyothreitol
DUF	=	Domain of unknown function

E

EDTA	=	Ethylenediamine-tetraacetic acid

EGF	=	Epidermal growth factor
ET	=	Ethylene
H		
HR	=	Hypersensitive response
I		
INA	=	2,6-Dichloroisonicotinic acid
J		
JA	=	Jasmonic acid
K		
KAPP	=	kinase-associated protein phosphatase
kDa	=	Kilodalton
KI	=	Kinase interaction
L		
LRR	=	Leucine-rich repeat
LZ	=	Leucine zipper
M		
MS	=	Murashige and Skoog mineral salt mix
MAPK	=	Mitogen activated protein kinase

MAPKK	=	MAPK kinase
MAPKKK	=	MAPKK kinase
CDPK	=	Calcium-dependent protein kinase

N

NO	=	Nitric oxide
NBS	=	Nucleotide binding site

P

PAL	=	Phenylalanine ammonia lyase
PMSF	=	Phenylmethylsulfonyl-fluoride
PR	=	Pathogenesis-related
PK	=	Protein kinase
PAHBAH	=	ρ-hydroxybenzoic acid hydrazide
PPase	=	Protein phosphatase
PP2C	=	C-terminal type 2C serine/threonine protein phospathase

R

R-gene	=	Resistance gene
RLK	=	Receptor-like-protein kinase
ROS	=	Reactive oxygen species
RT-PCR	=	Reverse transcription polymerase chain reaction

S

SDS	=	Sodium dodecyl sulphate
SDS-PAGE	=	SDS polyacrylamide gel electrophoresis

SA	=	Salicylic acid
SAR	=	Systemic acquired resistance
S-RLK	=	S-domain RLK
SLG	=	Self-incompatibility-locus glycoprotein

T

TBS	=	Tris buffered saline
TBST	=	Tris buffer saline with Tween 20
TEMED	=	N,N,N',N'-Tetramethylethylenediamine
Tris	=	Tris(hydroxymethyl)-amino-methane
Tween 20	=	Polyoxyethylene sorbitanmonolaurate
TMV	=	Tobacco mosaic virus
TCV	=	Turnip crinkle virus
TCA	=	Trichloroacetic acid
TIR	=	Toll and interleukin-1 receptor
TNFR	=	Tumor-necrosis factor

W

WCI	=	Wheat chemically induced

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CHAPTER ONE

Introduction

Introduction

Plants are vital to the existence of man-kind. Learning more about the plant defense mechanism and the way they respond to pathogens, will contribute to our ability to manipulate resistance and hence control plant disease. A key element of the activation of the defense response, is the way that the plant recognizes and transduces signals that activate the defense response.

Plants have developed a range of defense mechanisms for protection against pathogens (Yalpani and Raskin, 1993). Some of these defenses are constitutively expressed and form part of the normal vegetative growth and development of the plant (Levine *et al.*, 1994). For example trichomes represent a structural constitutive defense barrier protecting the aerial regions of the plant. Other defense mechanisms are inducible, which is triggered when challenged by a pathogen. This response leads to the establishment of an activated defense state (Ryals *et al.,* 1996). These inducible mechanisms include the activation of existing pools of enzyme, such as the localized activation of callose synthase activity at the site of infection and the activation of glucanases and chitinases (Rathjen and Moffett, 2003). Inducible mechanisms also lead to the expression of a subset of defense genes (Ryals *et al.,* 1996). These defense genes encode proteins that can either function directly as deterrents, or enzymes that catalyse the synthesis of defense-related products such as phytoalexins (Chen *et al.,* 1995).

The hypersensitive response (HR) is a rapid, but localized death of plant cells in response to a challenge by pathogens (Levine *et al.,* 1994). This reaction is associated with both local and systemic acquired resistance (SAR) of the plant that may be mediated through the deposition of callose, lignin and hydroxyproline

rich glycoproteins as well as the synthesis of chitinases, glucanases and phytoalexins (Levine *et al.,* 1994). Salicylic acid, a natural plant compound, is the putative signaling molecule that initiates these multiple reactions (Gaffney *et al.*, 1993).

Many of the factors that regulate the defense response also play a role in plant development (Rathjen and Mofett, 2003). The same gene is often subjected to both developmental and environmental regulation (Thomma *et al.,* 1998).

One stimulus, such as the challenge by a pathogen or pest, leads to a broad spectrum of changes that must be coordinated within the plant for maximum efficiency (Levine *et al.,* 1994). It is now accepted that a stimulus triggers signaling events that affect the whole plant, not merely the locally affected area (Levine *et al.,* 1994). Therefore, the co-ordination of these local and systemic events must occur through extensive and integrated signal transduction pathways (Stone and Walker, 1995).

The onset of the defense response is associated with the recognition of the signal at the cell surface by an appropriate receptor, the initiation of phosphorylation cascades leading to e.g. the induction of pathogen-related (PR) gene expression and the accumulation of hydrogen peroxide as an early event in the signal transduction cascade (Ryals *et al.,* 1996).

Plant receptor-like protein kinases (RLKs) are classified into subfamilies based on the structural features of the extracellular domain and have been implicated in a wide range of signal transduction pathways in response to different signals (Walker, 1994). Salicylic acid (SA)–induced expression of plant RLK genes was first found in a member of the *Brassica S* gene family, *SFR2* (Pastuglia *et al.,* 1997). SA induced expression has also been reported for the *Arabidopsis thaliana At-RLK3* gene (Czernic *et al.,* 1999).

Since these genes are inducibly expressed after SA treatment, it is possible that they act as the initial binding site for SA, whereafter they activate a signaling cascade.

SAR is not exclusively induced by pathogenic elicitors, but certain plant activators could mimic the presence of the pathogen by activating the defense response in the plant after exogenous application. Plant activators such as salicylic acid (SA) (Ryals *et al.,* 1996), benzothiadiazole (BTH) (Görlach *et al.*, 1996), acetylsalicylic acid (ASA) (White, 1979) and 2,6-dichloroisonicotinic acid (INA) (Anfoka, 2000) are very effective in the induction of the plant defense against a wide range of different pathogens.

Plant RLKs have great potential in agriculture. If they play a role in the perception of plant activators, this ability could be exploited for benefit of crop production. Overexpression of such genes could make the crops much more sensitive for the presence of plant activators, allowing the farmer to artificially activate the defenses very effectively by spraying it with, for instance SA.

The aim of this study was three fold. The first objective was to determine the earliest time of defense activation after treatment of *Arabidopsis thaliana* with the different plant activators. This would allow us to establish how quickly the plants respond to the presence of the activators. Secondly, the aim was to determine whether At-RLK3 is involved in the perception process and whether higher levels of At-RLK3 showed any benefits for the plant on the activation of the defense response.

CHAPTER TWO

Literature Review

2.1 Introduction

Plants are continuously exposed to a wide range of abiotic (non-living) and biotic (living) factors in their environment. They must respond to these factors in an effort to accumulate nutrient supplies. These supplies are necessary for growth and reproductive maturity, but make the plant a prime target for pathogenic attack. In order to survive and adapt to pathogenic attack, plants have developed several survival strategies (Yalpani and Raskin, 1993; Ryals *et al.*, 1996).

The first step in discussing plant-pathogen interactions is to understand the terminology used to describe the host, the pathogen and the overall interaction between the two. The host plant can be either resistant or susceptible to either an avirulent or virulent pathogen (Levine *et al.*, 1994). The interaction between a susceptible host and a virulent pathogen is termed compatible, whereas, the interaction between a resistant host and an avirulent pathogen is termed incompatible. The other component in the interaction is favorable environmental conditions for disease development. Thus, it is a combination of the host, pathogen and the environment that determines whether disease will occur or not (Levine *et al.,* 1994).

When plants encounter pathogens, resistance mechanisms are activated that can prevent infection, aid recovery from disease and prevent future infection. This resistance mechanism occurs in three separate stages: recognition of the pathogen by the plant, signal transduction (both intra and intercellular) and the synthesis of the necessary factors that limit pathogen ingress. The pathogen recognition system relies on the gene-for-gene interaction and has been interpreted in terms of a receptor-ligand model (Rathjen and Moffett, 2003) and is

known as specific recognition. Plant defense responses can also be activated during non-specific recognition.

The resistance (*R*) genes in the plant have two basic properties, namely to recognize a specific pathogen and to transduce a signal to downstream response genes (Levine *et al.,* 1994). They are either transmembrane or intracellular proteins that recognize avirulence (Avr) products (elicitors or ligands) of an invading pathogen (Rathjen and Moffett, 2003). The pathogen-derived *avr* gene products are delivered indirectly to intercellular spaces or directly inside plant cells. R-gene mediated resistance then activates an internal signal upon ligand binding that triggers the early defense response (Mehdy, 1994). This defense response is termed the hypersensitive response (HR) and is associated with programmed cell death that involves rapid host cell necrosis at the site of recognition (Levine *et al.*, 1994). The cell death response benefits the plant by depriving pathogens of further access to nutrient sources and thus limits pathogen proliferation. Cells in and around the recognition site (locally) undergo several changes, including changes of phosphorylation state. They also experience large ion fluxes (especially of Ca^{2+}), the production of reactive oxygen species, cell wall strengthening near the infection site, release of secondary signal molecules such as nitric oxide (NO) and the synthesis of antimicrobial products including phytoalexins and pathogenesis-related (PR) proteins (Levine *et al.*, 1994).

Plant defense is not only limited to a local response, but can also generate a defense response in distal parts of the plant following the challenge by a pathogen (Levine *et al.,* 1994). An enhanced protection is systemically activated in uninfected parts of the plant against the plant pathogen that is called systemic acquired resistance (SAR) (Shah *et al.*, 1997). SAR is characterized by an increase in the signal molecule salicylic acid (SA) throughout the plant and the subsequent expression of PR genes (Chen *et al.*, 1995).

SAR can be induced after a hypersensitive response to an avirulent pathogen or by treatment with certain plant activators such as SA, 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole (BTH) (Yalpani *et al.*, 1993; Ryals *et al.*, 1996; Godard *et al.*, 1999; Anfoka, 2000).

However, little is known about the signal transduction pathway relaying the signal between the inducing stimulus and the onset of resistance. It is known that receptor-like-protein kinases (RLKs) may function as receptors for the extracellular signals involved in plant growth, development and defense responses (Walker, 1994). They are believed to transduce the extracellular information across the plasmamembrane into the cell through phosphorylation cascades after the binding of the signal ligand to an RLK, which leads to expression of appropriate target genes (Stone and Walker, 1995).

2.2 Signal molecules that elicits inducible defense responses

2.2.1 Pathogen derived elicitors

Pathogens are infectious, disease causing agents that invade target organisms and multiply inside or on them. Various types of pathogens can be distinguished by the way they parasitize the plant (Keller *et al.*, 1999).

Pathogens that are unable to survive on dead plant tissue are known as biotrophic pathogens. These include *Peronospora parasitica*, the causal agent of downy mildew (Godard *et al.*, 1999) and require living host tissue to complete their life cycle. Biotrophic pathogens are highly evolved with specialized feeding structures (haustoria) to retrieve nutrients from living plant cells. This type of pathogen can avoid the triggering of host defense responses by evading recognition or by producing suppressors of defense responses.

In contrast, other pathogens colonize dead tissue to extract nutrients. These pathogens are necrotrophic pathogens, such as *Botrytis cinerea* (Govrin and Levine, 2000). They secrete toxins or enzymes to kill and macerate plant tissue. They may try to kill host cells before the cells are able to switch on an efficient defense response (Keller *et al.*, 1999; Yang *et al.,* 1997).

In both pathogen types, disease will follow when the metabolic activities of the pathogen damages the plant tissue and interferes with the normal functioning of the plant. Therefore, a rapid and effective induction of defense responses to a particular pathogen appears to be the difference whether or not an invading microorganism succeeds in causing disease.

Before a defense response can be activated, a plant must be able to recognize the presence of the invading pathogen. This is possible through signal molecules known as elicitors (Gómez-Gómez and Boller, 2000). Elicitors have diverse chemical structures, such as polygalacturonides, β-glucans, chitosan, lipids and proteins (Boller, 1995). They can be placed into two classes, namely general and specific elicitors (Song *et al.,* 1995).

Specific elicitors are peptides can be subdivided into non-race and race-specific (Ji *et al.*, 1998). Non-race-specific elicitors can be either fungal or plant cell wall fragments that have been released during the infection process which can induce various defense responses in plants to minimize the effects of disease. In contrast to these, race-specific elicitors are molecules that are encoded by avirulence genes in the pathogen that trigger defense responses only in plant cultivars harboring the complementary *R* gene (Song *et al.*, 1995).

General elicitors on the other hand include oligosaccharides derived from the pathogen or plant, microbial proteins such as flagellin or nucleic acids (Felix *et al.*, 1999; Heath, 2000). Some elicitors, such as cellulolytic enzymes, can cause

transmembrane ion fluxes in artificial lipid bilayers. Other proteinaceous elicitors such as cryptogenin, have been shown to have binding sites on plant membranes, even on membranes of plant species in which they fail to induce a defense response (Bourque *et al.,* 1999).

One example of a general elicitor is flagellin, which forms the main component of the flagellum filament of bacteria (Gómez-Gómez and Boller, 2000). It is one of the most studied proteinaceous elicitors. The flagellum is an extracellular propeller constructed of 11 protofilaments, each consisting of several thousand flagellin units (Gómez-Gómez and Boller, 2000). The secreted flagellin is usually assembled in the flagellum, but can also accumulate in the bacterial environment as a result of spillovers during the construction of flagellae. The flagellar motility allows the bacteria to respond to stimuli in their environment and is related to the infectivity of some pathogenic bacteria. In *Arabidopsis*, the application of the flg22 peptide acts as an elicitor leading to the induction of an oxidative burst, callose deposition, ethylene production and the induction of defense-related genes (Gómez-Gómez and Boller, 2000) but it never induces an HR type of necrosis (Felix *et al.,* 1999).

Another example of resistance elicitors is the fungal-derived proteinaceous molecules grouped under the generic name 'elicitins' (Benhamou *et al.*, 2001; Blein *et al.*, 2002). They were identified in the culture filtrate of *Phytophthora* and *Pythium* isolates. They are small cysteine-rich lipid-binding proteins that exhibit a sterol carrier activity. This sterol carrier activity is probably their main function, because *Phytophthora* and *Pythium* do not synthesize the sterols that are required for their reproduction. These elicitins act as shuttles, trapping the sterols from the host and then triggering active phases of sexual and asexual reproduction. Their ability to load sterols is crucial because the formation of a sterol-elicitin complex is required to trigger the biological responses.

These sterol loadings from the plant plasmamembrane allow elicitins to bind to their receptor, which then activates plant defense mechanisms. They induce a substantial level of protection against the oomycete fungus, *Phytophthora parasitica* and also induce systemic resistance to *Fusarium* crown and root rot in tomato plants (Benhamou *et al.,* 2001). These investigations of plant defense relationships show that lipid-binding proteins secreted by the plants play a key role in the lipid-mediated dialogue between the pathogen and the plant. Not only pathogen derived elicitors can lead to the activation of defense responses, but other natural plant signaling molecules can as well.

2.2.2 Plant activators

The phenylpropanoid pathway-derived natural product, SA, is an important signal molecule involved in local defense and SAR (Ryals *et al.*, 1996). It is synthesized from cinnamic acid, which is the product of phenylalanine ammonia lyase (PAL) activity. PAL is an enzyme that plays a key role in regulating the phenylpropanoid pathway. The central role of SA became apparent with the use of transgenic tobacco plants expressing the bacterial *nahG* gene encoding SA hydroxylase that converts SA to catechol (Gaffney *et al.*, 1993). Thus, when nahG plants were infected with pathogens that induced SAR, the plants failed to develop SAR and did not exhibit systemic expression of PR proteins (Gaffney *et al.,* 1993; Delaney *et al.*, 1994). These findings provided strong evidence for the involvement of SA in SAR.

Exogenous application of SA to tobacco (*Nicotiana tabacum*), tomato and *Arabidopsis* plants results in the induced expression of PR protein genes and acquired resistance similar to the activation upon pathogen attack. This suggests that SA is required for signaling (Ryals *et al.,* 1996).

In cucumber, SA treatment protects it against viral, bacterial and fungal pathogens by inducing SAR genes and enhanced resistance (Vernooij *et al.*, 1994).

Along with SA accumulation, plants often exhibit increased production of jasmonic acid (JA) upon infection by pathogens (Yang *et al.*, 1997). The function of JA has been attributed to the induction of defense-related proteins in plants (Niki *et al.,* 1998). This response is rapidly produced when a pathogen attacks the plant, particularly during necrotizing infection, leading to an increased accumulation of jasmonic acid levels (Yang *et al.*, 1997). It has also been postulated that JA might constitute lipid-derived messengers in the signal transduction chain preceding the activation of defense gene expression (Farmer *et al.*, 1998). Exogenous application of this signaling molecule induced a set of defense genes that were also activated upon pathogen infection (Epple *et al.,* 1997).

It has been reported that SA induced PR genes coding for acidic PR proteins whereas JA induce mostly the expression of the basic PR isoforms (Pieterse and Van Loon, 1999). Thus, at least two different signal transduction pathways can be distinguished, either SA- or JA-dependent, which are turned on in response to pathogen attack (Thomma *et al.*, 1998). Resistance conferred by biotrophic pathogens often requires SA signaling, whereas necrotrophic pathogens or wounding mainly activates a JA-dependent pathway.

The SAR was first described as a reaction to pathogen infection. It can also be activated by the exogenous application of natural compounds such as SA and by treatments with other plant activators such as acetylsalicylic acid (ASA, aspirin) which is a derivative of SA which is rapidly hydrolyzed to SA in biological tissues (White, 1979), BTH (Görlach *et al.*, 1996) and INA (Anfoka, 2000; Godard *et al.*, 1999; Schaffrath *et al.*, 1997; Schweizer *et al.*, 1999).

These plant activators share common structural features, including an aromatic ring system with a substituted carboxyl group (Fig. 2.1).

ASA induced PR protein synthesis and enhanced resistance to a number of pathogens, including tobacco mosaic virus (TMV) (Klessig and Malany, 1994) which correlated with results obtained in SA treatment.

BTH treatments have been shown to induce SAR in various plants, such as in tobacco against TMV that lead to the induced expression of SAR genes (Friedrich *et al.,* 1996). In wheat, BTH treatment resulted in the protection against powdery mildew *Erysiphe graminis* f.sp.*tritici* (Görlach *et al.*, 1996). Wheat chemical induced (WCI) genes are activated after treatment with BTH (Görlach *et al.*, 1996). When *Arabidopsis* plants are treated with BTH, PR protein accumulation was induced and the plants showed resistance to Turnip crinkle virus (TCV), the bacterium *Pseudomonas syringae* pv. 'tomato' and the fungus *Peronospora parasitica* (Lawton *et al.,* 1996). BTH treatment also results in protection of tomato plants against the root rot disease caused by *Fusarium oxysporum* f.sp. *radicis-lycospersici* (Benhamou and Belanger, 1998). These results demonstrate that BTH leads to the protection of different plant species against diseases caused by viral, bacterial and fungal pathogens.

INA is another plant activator of disease resistance being utilized to aid elucidation of the complex mechanisms of the defense response and to assess the potential of employing SAR commercially (Uknes *et al.*, 1992). In tobacco (Ward *et al.*, 1991a) and *A. thaliana* (Uknes *et al.*, 1992) INA treatments resulted in the induction of SAR with the accumulation of PR proteins. Another induced resistance response by INA was also described in barley (Kogel *et al.*, 1994).

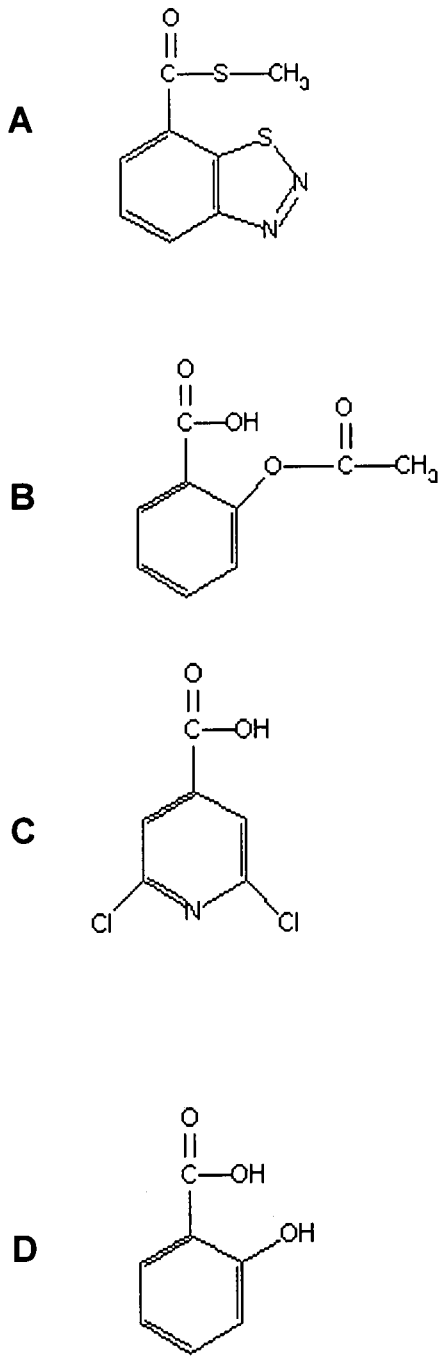


Fig. 2.1. Structures of SAR-inducing compounds. (A) BTH, (B) ASA, (C) INA, (D) SA (Wendehenne *et al.*, 1998).

Neither INA nor BTH treatment caused elevated levels of SA, nor could both compounds activate SAR when applied to NahG plants (Lawton *et al.*, 1996). This suggested that both INA and BTH act independently or downstream of SA in SAR signaling (Schweizer *et al.*, 1999). INA, BTH and SA were unable to activate SAR gene expression in the *nim1* mutant (noninducible immunity) of *Arabidopsis* suggesting that all three compounds activate the SAR signal transduction pathway through the same signaling cascade (Uknes *et al*, 1992; Lawton *et al.*, 1996; Wendehenne *et al.*, 1998).

2.3 Receptors for the recognition of pathogenic elicitors

The recognition system of plants for pathogenic derived elicitors includes a complex set of plant proteins. One class of genes encoding these proteins is known as plant *R* genes. The encoded R proteins can either be transmembrane or intracellular proteins. *R* genes encode proteins that determine the recognition of specific pathogen-derived *avr* proteins either directly (e.g. the protein) or indirectly (e.g. an enzymatic product), leading to the initiation of signal transduction pathways that activate a complex defense response. The interaction between the *avr* and *R* gene products is known as the gene-for-gene model. One interpretation of the gene-for-gene model is that pathogen *avr* gene product (i.e. the ligand) interacts directly with the host resistance gene product (i.e. the receptor).

Five classes of *R* genes were identified from various plant species according to the structural characteristics of their predicted protein products (Van Loon, 1997). These classes are the intracellular protein kinases (PKs), receptor-like protein kinases (RLKs) with an extracellular leucine-rich repeat (LRR) domain (Walker, 1994), intracellular LRR proteins with a nucleotide binding site (NBS) and a leucine zipper (LZ) motif, intracellular NBS-LRR proteins with a region with

similarity to the Toll and interleukin-1 receptor (TIR) proteins and LRR proteins that encode membrane-bound extracellular proteins (Van Loon, 1997).

The LRR region of R proteins serves as the specificity determinant for pathogen elicitors (Holt III *et al.*, 2000) and varies in both number and amino acid sequences. The NB site is a more highly conserved protein motif and is presumed to bind ATP/GTP. The LZ region is involved in protein-protein interactions similar to LRRs.

Other structural classes of *R* genes in plants encode membrane bound LRR proteins without an NB site, for instance *Cf*2/9 and *Cf*4/5 (Blumwald *et al.*, 1998). Some other *R* genes such as *Pto* in tomato (Tang *et al.*, 1999) and *Xa*21 in rice (Song *et al.*, 1995), encodes a serine/threonine kinase. *Xa*21 combines a Ser/Thr kinase domain with an LRR containing extracellular domain. These genes show variations in domain shuffling (NB, LRR, TIR, Ser/Thr kinase) to achieve the functional capabilities of the plant to activate a defense response.

2.4 RLKs

To date, the *Arabidopsis* genome contains genes encoding more than 600 members of the RLK family. RLKs is one of the *R* gene classes involved in the signal transduction pathway to coordinate plant development and to sense and respond to fluctuations in the surroundings. They perceive external stimuli, transduce the signals across the plasma membrane through phosphorylation cascades that ultimately lead to expression of appropriate target genes (Walker, 1994; Ohtake *et al.*, 2000).

The extracellular domain determines the potential binding of the RLK to an array of molecules, including carbohydrates, polypeptides, microbial cell-wall components and steroids.

Therefore, RLK signaling is thought to require the formation of complexes upon ligand binding, passing the signal across the plasma membrane through the signal complexes, leading to the activation of the protein kinase domain and associated proteins (Lease *et al.*, 1998).

RLKs have the following structural organization: an amino-terminal signal peptide followed by an extracellular domain whose amino acid sequence varies from protein to protein, a hydrophobic region predicted to be a membrane spanning region and a carboxy-terminal protein kinase domain that has serine/threonine protein kinase activity (Walker, 1994).

RLKs are identified according to this domain organization and classified into subfamilies according to the amino acid sequence of their extracellular domain (Walker, 1994). More than 21 different subfamilies have been identified up to date. These subfamilies include the S-domain class (S-RLK), LRR-class, TNFR-class (tumor-necrosis factor RLK), EGF-class (epidermal growth factor – RLK), PR-class, L-lectin motif class, CHRK1 class (chitinase-related receptor-like kinase), thaumatin domain class and a lysine domain class. Others that were identified include these with the DUF domain or also known as the CRR domain (cystein-rich repeat domain), proline-rich motif class, CrRLK1-like class (*Catharanthus roseus* RLK1) and LRK10-like class (Feuillet *et al.*, 1997).

These RLKs have been found to be involved in several different processes. The S-class possesses extracellular S-domains homologous to the self-incompatibility-locus glycoproteins (SLG) of *Brassica oleracea* involved in self-incompatibility recognition. One example of this family is *SRF2* from *Brassica* spp. involved in the defense response (Stein *et al.*, 1996). *SFR2* accumulated in response to wounding, pathogenic and non-pathogenic bacteria and was considered a part of the general stress response of the plant (Stein *et al.*, 1996).

The LRR classes comprise the largest class of the plant RLKs and possess tandem repeats of LRRs (Song *et al.*, 1995). Some RLKs of this class are implicated in the control of plant growth and development, such as CLV1 in controlling meristem development (Clark *et al.*, 1993), BRI1 in mediating brassinosteroid signaling (Li and Chory, 1997) and ERECTA in organ elongation (Torii *et al*, 1996). Other RLKs of this class are involved in plant-microbe interactions and stress responses, such as the rice *Xa21* that renders the plant resistant to *Xanthomonas* (Song *et al.*, 1995). Another example of such an *R* gene is the *LR10* locus that encodes LRK10 (Feuillet *et al.*, 1997) which gives wheat resistance against *Puccina triticina* that causes leaf rust.

The EGF, TNFR and lectin class of RLKs contain unique features such as the *Arabidopsis* WAK protein with an EGF-like motif that plays a role in protein-protein interactions and is induced upon bacterial infection (He *et al.*, 1996), the maize CR4 which has a TNFR-like motif required for normal epidermal patterning (Becraft *et al.*, 1998) and the *Arabidopsis* lecRK with a motif similar to the legume lectin-binding domain which may be involved in the perception of oligosaccharide-mediated signals (Herve *et al.*, 1996). FLS2 from *Arabidopsis* is involved in flagellin perception (Gómez-Gómez and Boller, 2000), while others RLKs are involved in nodulation (Endre *et al.*, 2002) and systemin signaling (Sheer and Ryan, 1999).

The predominant class of resistance genes contains a LRR domain, but there have been genes cloned that do not contain any LRR but encodes for only a protein kinase. The *Pto* gene of tomato is such an example (Song *et al.*, 1995). This *Pto* gene confers resistance in a gene-for-gene manner to bacteria expressing the *avrPto* gene and encodes a protein with similarity to protein kinases (Martin *et al.*, 1993).

Although *avrPto-Pto* is a classical gene-for-gene interaction, the resistance conferred by *Pto* is dependent on a second, tightly-linked *Prf* gene, which encodes a protein containing LRRs, NBS and LZ domains (Bent, 1996; Tang *et al.*, 1999; Oldroyd and Staskawicz, 1998).

The *Cf* proteins of tomato confers resistance to strains of the leaf mould *Cladosporium fulvum* carrying the appropriate *avr* genes (Blumwald *et al.*, 1998).

These genes encode glycoproteins with extracytoplasmic regions of LRRs attached to a transmembrane region and a small cytoplasmic tail (Cohn *et al.*, 2001). They lack a significant intracellular region that could constitute a signaling component (e.g. a protein kinase domain) (Ellis *et al.*, 2000). They interact with other gene products to form a complex that confer resistance. The binding of the AVR9 peptide to plant membranes did not correlate with the presence of the *Cf9* gene (Hammond-Kosack and Jones, 1996; Melchers and Stuiver, 2000). This suggested that the R gene product might not be exclusively involved in the recognition, but also in the coordination of membrane signaling.

2.5 Signal transduction

Although much effort has been devoted to understanding the signaling pathway of plants as well as in the identification of signaling components that activate the plant defense response (Gómez-Gómez and Boller, 2000). When a transmembrane protein recognizes an extracellular signal in the form of a polypeptide ligand, a signal transduction cascade is initiated through the initiation of protein phosphorylation. This reversible phosphorylation is catalyzed by protein kinases and phosphatases that play a central role in cellular signaling (Stone and Walker, 1995).

Protein kinases and phosphatases are classified into two groups based on their substrate specificity namely, serine/threonine kinases and phosphatases which act on both serine and threonine residues, and tyrosine kinases and phosphatases, which act on tyrosine residues (Stone and Walker, 1995). The serine/threonine protein phosphatase (PPases) specifically catalyzes the dephosphorylation of phosphoserine and phosphothreonine in protein substrates.

There are approximately 1000 protein kinase genes and 200 phosphatase genes, including 23 mitogen activated protein kinases (MAPKs), 9 MAPK kinases (MAPKKs), 25 MAPKK kinases (MAPKKKs) and 29 calcium-dependent protein kinases (CDPKs) within the *Arabidopsis* genome (Xing and Jordan, 2000).

Another protein phosphatase that acts as a downstream component that interacts with RLKs is the kinase-associated protein phosphatases (KAPP) (Li *et al.*, 1999). It possess an N-terminal type I signal anchor, the kinase interaction (KI) domain and a functional C-terminal type 2C serine/threonine protein phosphatases (PP2C) domain (Stone *et al.*, 1995). KAPP was discovered as a protein that interacted with the protein kinase catalytic domain of RLK5 (Stone *et al.*, 1995) as well as with CLV1 (Clark *et al.*, 1997). KAPP dephosphorylate autophosphorylated CLV1 (Lease *et al.*, 1998).

One of the known defense-related RLKs is the FLS2 gene that plays a role as a receptor for the bacterial elicitor, flg22 (Gómez-Gómez and Boller, 2000). It is composed of an extracellular LRR domain, a single membrane spanning domain and an intracellular serine/threonine protein kinase domain. The LRR domain of FLS2 is speculated to be part of a receptor for flg22 and flagellin. FLS2 is expressed in roots, stems, leaves and flowers of *A. thaliana* (Gómez-Gómez and Boller, 2000). The FLS2 promoter can also be activated by wounding, providing entry sites for potential pathogens and thereby enhancing the expression of FLS2 at the wound site.

Bacterial cells on the plant surface enter the plant via natural openings (Gómez-Gómez and Boller, 2000). The interaction of the flagellin with the FLS2 LRR domain activates the FLS2 kinase domain, possibly through dimerization of the receptor complex that then leads to autophosphorylation. This activation causes rapid phosphorylation-dependent activation of ion channels and NADPH oxidase.

The FLS2 kinase activity is directly or indirectly responsible for the phosphorylation and activation of AtMEKK1 which in turn phosphorylates AtMAKK 4/5, that activates MAPK 3/6 (a MAPK), which might further cause the activation of WRKY (Du and Chen, 2000) type of transcription factors. These transcription factors activate the expression of defense genes and also themselves, thereby amplifying the defense response (Kalde *et al.*, 2003).

2.6 Activation of plant defense responses

Inducible plant defense responses comprise of a complex of reactions that are activated not only upon plant-pathogen interaction, but also upon exposure to other elicitors such as both natural and plant activators (White, 1979; Görlach *et al.*, 1996; Anfoka, 2000; Godard *et al.*, 1999; Schaffrath *et al.*, 1997; Schweizer *et al.*, 1999). These responses are induced locally (HR response) at the site of recognition, as well as systemically (SAR response) in the uninfected tissue. The HR response includes programmed cell death to restrict pathogen spread, cell wall strengthening by lignification and cross-linking of cell wall compounds. In addition to the HR, the SAR response renders resistance to further infection by viral, bacterial and fungal pathogens (Shah *et al.*, 1997).

One of the earliest detectable cellular events associated with the HR is the rapid accumulation of reactive oxygen species (ROS), the so-called oxidative burst (Mehdy, 1994). The oxidative burst is a central component of a plants' defense machinery and is required for the activation of defense gene expression and the

production of antimicrobial metabolites, which may affect the attacking pathogen and the host cell at the site of infection (Shirasu and Schulze-Lefert, 2000). Superoxide is the first product of the oxidative burst and is rapidly converted to hydrogen peroxide (Huang *et al.*, 2002; Levine *et al.*, 1994; Finkel, 1998).

The burst of H_2O_2 production at the plant cell surface drives rapid peroxidase-mediated oxidative cross-linking of structural proteins in the cell wall, thereby reinforcing a physical barrier against pathogen ingress and limiting the spread of the pathogen (Mathieu *et al.*, 2002).

Activation of the HR is also associated with the synthesis of the signaling intermediates including SA, ethylene (ET) and JA, the synthesis of antimicrobial chemicals, such as phytoalexins and the activation of downstream defense genes that encode antimicrobial proteins (Popova *et al.*, 1997).

Phytoalexins normally accumulates around sites of pathogen infection and can be synthesized through key enzymes of phenylpropanoid metabolism, such as PAL (Hammond-Kosack and Jones, 1996). The purpose of phytoalexins synthesis is to reduce the severity of secondary infections or the overall growth rate of virulent pathogens.

The expression of a subset of PR genes encoding enzymes with antimicrobial activity occurs both locally and systemically (Ryals *et al.*, 1996). These encoded PR proteins are known as SAR proteins because they are involved in the onset of the SAR and are good molecular markers for a resistance response (Shah *et al.*, 1997).

Eleven pathogenesis-related protein families have been characterized from various plant species and classified according to sequence similarities ranging from PR-1 to PR-11 (Van Loon, 1997).

PR protein families include proteins with β-1,3-glucanase and chitinase activity, as well as proteinases and peroxidases. Most PR proteins have a detrimental effect on the pathogen. PR-1 and PR-5 interact with the plasmamembrane of the pathogen, whereas β-1,3-glucanases (PR-2) and chitinases (PR-3, PR-4, PR-8 and PR-11) attack β-1,3-glucans and chitin that are components of the cell walls of higher fungi. Chitinases can also display lysozyme activity to hydrolyze bacterial peptidoglycan (Melchers and Stuiver, 2000).

Plant β-1,3-glucanases are abundant proteins widely distributed among seed plant species and are involved in diverse physiological and developmental processes (Cheong *et al.*, 2000) including pollen germination, fertilization, seed germination and defense against pathogens. They are divided into three classes depending on their primary structure. Class I consists of the basic, vacuolar isoforms that are localized primarily in the epidermis of the lower leaves and in the roots of healthy plants (Keefe *et al.,* 1990). They undergo substantial post-translational modification including the removal of a carboxyl-terminal extension. Class II comprises three subgroups, namely the acidic, extracellular glucanases, the closely related isoforms with neutral or basic pI-values and the stylar-specific extracellular glucanase (Ward *et al.*, 1991b). Class III contains the pathogen-induced glucanase PR-Q, which is an acidic extracellular protein that differs in sequence from class I and II proteins (Payne *et al.,* 1990).

In *Arabidopsis*, SAR genes including PR-1 (encoding a protein of unknown function), PR-2 (β-1,3-glucanase) and PR-5 (thaumatin-like protein) (Lawton *et al.*, 1996; Uknes, *et al.*, 1992) were induced during the SAR. Therefore PR genes could be useful molecular markers for the expression of SAR.

2.7 A new receptor-like protein kinase, *At-RLK3*

At-RLK3, a putative RLK gene, was isolated from *A. thaliana* (Czernic *et al.*, 1999). The encoded protein shares all the regions characteristic of RLKs described in plants. Although it has 20 leucines and 12 cysteins within the extracellular domain it could not "fit" into any of the described groups of RLKs. It did however have two copies of the domain of unknown function (DUF) motive.

At-RLK3 gene expression was induced upon oxidative stress and salicylic acid treatment (Czernic *et al.*, 1999). During various plant-pathogen interactions, the *At-RLK3* transcripts accumulated transiently at higher levels during both compatible and incompatible plant-pathogen interactions. These results suggested that At-RLK3, as a new member of receptor-like protein kinases, could play an important role in the activation of the defense response in plants.

CHAPTER THREE

Material and Methods

3.1 Materials

3.1.1 Biological material

Wild-type *Arabidopsis thaliana* Columbia plants were used in all the studies as it is universally recognized as a model plant for research, easy and inexpensive to grow and produces many seeds.

Seed was surface sterilized for 30 sec in 75% (v/v) ethanol and thereafter in 1% (v/v) sodium hypochlorite, 0.05% (v/v) polyoxyethylene sorbitanmonolaurate (Tween-20) for 30 min. Seed was rinsed 6 times with sterile water to ensure that all the bleach had been removed. The seeds were germinated and grown on a growth medium in petridishes in a growth chamber. The growth medium consisted of half strength nutrient medium of Murashige and Skoog (MS) (Murashige and Skoog, 1962) pH 5.8, 1% (w/v) sucrose and 0.4% (w/v) plant agar. A constant temperature of 22°C and 16-h light/8-h dark cycles were maintained in the growth chamber. Approximately twenty seeds were planted on each plate.

Small seedlings were transplanted from the agar plates into soil. The soil mixture consisted of peat moss, potting compost, potting grit and vermiculite in a ratio of 2:2:2:1. To eliminate disease, the soil and pots were sterilized in an autoclave at 121°C for at least 20 min. The soil was moistened with water and transferred into the pots. *A. thaliana* plants were carefully transplanted to avoid damage to their roots and grown for 1 week under high humidity, before incubating it in the growth chamber.

Wax paper in the form of a cylinder was placed around the plants and the plants grown until siliques were formed.

To prevent the siliques from opening and releasing their seeds, the seeds were harvested when the siliques were totally dry. Seed were kept at 4°C for three days before sowing.

3.1.2 Bacterial strains

Agrobacterium tumefaciens pGV2260 was used for the transformation of *A. thaliana* to generate the *At-RLK3* overexpression transformants. The pTA7002 plasmid was used because it is a vector used for the inducible transcription of transgenes (Aoyama and Chua, 1997).

3.1.3 Chemicals

The ECL™ Western blotting system from Amersham Pharmacia Biotech was used in the western blot analysis to detect the bound primary antibody (rabbit antiserum raised against β-1,3-glucanase protein (Van der Westhuizen *et al.*, 1996) using a goat anti-rabbit-horseradish peroxidase-conjugated secondary antibody (Sigma-Aldrich).

DEPC (dimethyl pyrocarbonate) (Sigma) was used for the control of ribonuclease activity. The Titan one Tube RT-PCR kit (Roche) was used to quantify the levels of gene expression. PAHBAH (ρ-hydroxybenzoic acid hydrazide) (Sigma) is the most sensitive aroylhydrazine according to Lever (1972) and was used to determine the β-1,3-glucanase activity. Dexamethazone (DEX) (Sigma) is a synthetic glucocorticoid hormone that was used for the induction of expression of *At-RLK3* cloned into pTA7002. All the other chemicals were of the highest quality.

3.2 Methods

3.2.1 Application of plant activators

Plant activators used in the study were: benzothiadiazole (BTH), as Bion (Novartis, Switzerland) a 50% active ingredient, salicylic acid (SA) (BDH), acetylsalicylic acid (ASA) (BDH), and 2,6-dichloroisonicotinic acid (INA) (Sigma Aldrich). BTH, SA and ASA were dissolved in distilled water and the pH was adjusted to 6.5 with KOH (Du and Chen, 2000). INA was first dissolved in 500 µl acetone and then diluted in distilled water.

Two-week-old *A. thaliana* seedlings were sprayed with freshly prepared sterile solutions of 2 mM SA, 0.12 $mg.ml^{-1}$ BTH, 2 mM ASA (Du and Chen, 2000) and 0.1 mM INA. Application occurred on the same day, under the same conditions and at the same time, as a fine mist until the leaves were completely wet. The control plants were sprayed with sterile water except for INA where an acetone-water mixture (500 µl: 1 liter) was used. All plants were maintained in the growth chamber under conditions as described (3.1.1). In one experiment plant tissue were harvested 0, 2, 4, 8, 12, 24, 48, 72 and 96 h after treatment and in another experiment 0, 5, 15, 30, 60, 90, 120 and 240 min after treatment. Harvested plant material was immediately frozen in liquid nitrogen and ground to a fine white powder in liquid nitrogen using a mortar and pestle. The ground tissue was stored at –80°C prior to further analysis.

3.2.2 Preparation of the plant extract

Total protein was extracted by resuspending the frozen material in extraction buffer [50 mM Tris-HCl, pH 7.5, 2 mM ethylenediamine tetraacetic acid (EDTA),

10 mM mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride (PMSF)]. The ratio of plant material to buffer was 1:3. After 30 min on ice, the homogenate was centrifuged at 9 000 *g* for 15 min at 4°C. The supernatant was used for protein concentration determination, enzyme assays and immunoblotting.

3.2.3 Determination of total protein concentration

Protein concentration was determined spectrophotometrically at A_{595nm} according to Bradford (1976), using Biorad's protein assay solution and 0.5 $mg.ml^{-1}$ gamma-globulin as standard. Protein concentration was calculated using a standard curve which relates different protein concentrations. The protein concentration was expressed as $\mu g.ml^{-1}$.

3.2.4 β-1,3 glucanase activity

β-1,3-glucanase activity was assayed using a modified method of Lever (1972). The reaction mixture consisted of 40 µl of enzyme extract, 50 mM sodium acetate, pH 4.5, 0.25 $mg.ml^{-1}$ laminarin substrate in a total volume of 500 µl. A control was included containing 40 µl 50 mM NaAc with no enzyme extract. The test solutions were heated at 37°C for 30 min. To these solutions, 1.5 ml PAHBAH reagent was added and the tubes were incubated in a boiling water bath for 10 min to stop the reactions. The colored products were cooled and A_{410nm} values measured against the corresponding control. All the assays were performed in triplicate. The β-1,3-glucanase enzyme activity was calculated using a standard curve relating different glucanase concentrations to A_{410nm} of the colored products obtained from the reaction described above. The β-1,3-glucanase activity was expressed as mM glucose. μg^{-1} protein. h^{-1}.

3.2.5 Immunoblotting of β-1,3 glucanases

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli (1970) using a 12% (w/v) acrylamide separating gel [0.34 M Tris(hydroxymethyl)aminomethane (Tris) pH 8.8, 0.09% (w/v) sodium dodecyl sulfate (SDS), 0.03% (w/v) ammonium persulfate (APS), 12% (w/v) acrylamide: bis-acrylamide (30:1), 0.00016% (v/v) N,N,N',N' tetramethylethylene-diamine (TEMED)] with a 6% (w/v) stacking gel [0.12 M Tris pH 6.8, 0.09% (w/v) SDS, 0.03% (w/v) APS, 6% (w/v) acrylamide:bis-acrylamide (30:1), 0.00016% (v/v) TEMED]. To each 10 μg protein sample loading buffer was added to a final concentration of 1% (w/v) SDS, 4% (v/v) glycerol, 13 mM Tris-HCl pH 6.8, 50 mM dithyothreitol (DTT) and 0.04% (w/v) bromophenol blue. The samples were boiled for 5 min and loaded onto the gel. The gel was run at 200 V using a running buffer containing 0.1 M Tris, pH 6.8, 0.15 M glycine and 0.1% (w/v) SDS.

A low molecular weight standard was also separated on the gel. Polypeptide bands were visualized using a Coomassie staining solution consisting of 40% (v/v) methanol, 7% (v/v) acetic acid and 0.025% (w/v) Coomassie Brilliant blue R 250. The gels were stained for 30 min and then destained in 7% (v/v) acetic acid, 40% (v/v) methanol for 60 min. Finally, the gels were dried under vacuum.

For the immunoblotting, the separated polypeptides were transferred to a PVDF nitrocellulose membrane (Roche) for 1 h at 350 mA using a Biorad wet transfer cell system. The transfer buffer contained 25 mM Tris-HCl, pH 8.3, 192 mM glycine and 20% (v/v) methanol. After transfer, the membrane was stained for 2 min with Ponceau-S [0.2% (w/v) Ponceau-S, 3% (w/v) trichloroacetic acid (TCA)], to determine whether the transfer of proteins to the membrane was complete. The membrane was destained with TBST (10 mM Tris-HCl pH 8.3, 1.5 M NaCl, 0.1% (v/v) Tween-20) to remove the Ponceau-S and then blocked with 8% (w/v)

fat-free milk powder in TBST for 1 h at room temperature. The membranes were then incubated for 1 h in rabbit antiserum raised against β-1,3-glucanase protein (Van der Westhuizen *et al.*, 1996) diluted 1:9500 in TBST containing 8% (w/v) fat-free milk powder. The membrane was washed 4 times with TBST (first 15 min and thereafter 3 x 5 min). The membrane was then incubated in a goat anti-rabbit-horse radish peroxidase as a secondary antibody for 1 h. The antibody was diluted 1:2000 in TBST. Finally, the membrane was washed 4 times (first 15 min and thereafter 3 x 5 min) with TBST. The polypeptides were detected using the ECL Western blot detection reagents and the membrane exposed to an x-ray film.

3.2.6 Hydrogen peroxide concentration

Hydrogen peroxide levels were assayed in the treated *A. thaliana* plants according to Brennan and Frenkel (1977). Frozen plant material was homogenized in 5 ml acetone for 1 min. The extract was centrifuged at 5 520 *g* for 20 min at 0°C. The supernatant was recovered and 0.5 ml $TiCl_4$ (20% (v/v) $TiCl_4$ dissolved in concentrated HCl) was added. To this, 75% (v/v) NH_4OH (diluted in a ratio of 1:4 with NH_4OH and distilled water) was added dropwise, while shaking. The sample was centrifuged at 5 520 *g* and the precipitate washed repeatedly with 5 ml volumes of acetone until the supernatant was colorless. The precipitates were solubilized in 5 ml 2 N H_2SO_4. The A_{415nm} was determined using 2 *N* H_2SO_4 as a blank. The H_2O_2 concentrations were calculated using a standard curve. The hydrogen peroxide levels were expressed as mM $H_2O_2.g^{-1}$ fresh mass.ml^{-1}.

3.2.7 Total RNA extraction

All solutions used for RNA analyses, were treated overnight with 0.1% (v/v)

DEPC. The solutions were autoclaved the following morning to destroy the DEPC (Sambrook *et al.*, 1989). All the glassware used was baked at 280°C overnight to destroy all RNAses.

Total RNA was extracted from plant tissue according to Chomczynski and Sacchi (1987) by resuspending 0.1 g frozen tissue in 0.8 ml guanidine thiocyanate extraction buffer (4 M guanidine thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% (w/v) N-lauroyl sarcosine, 0.1 M β-2-mercaptoethanol) and keeping it on ice for 10 min. To this was added 0.5 ml phenol:chloroform:isoamylalcohol (25:24:1) pH 8.0 and the tubes were briefly vortexed. The aqueous phase was transferred to a new eppendorf and 50 µl 3 M sodium acetate, pH 4.8, 500 µl acidic phenol and 100 µl chloroform were added. The mixture was centrifuged at 9 000 *g* for 5 min. The aqueous phase was transferred into a new eppendorf and 500 µl chloroform was added, mixed and the tube recentrifuged. RNA precipitation was accomplished by the addition of 500 µl 2-propanol to the aqueous phase and incubation at –20°C for 30 min. The RNA was pelleted by centrifugation for 5 min at 9 000 *g*. To wash the pellet, 1 ml 75% (v/v) ethanol was added, after which the tubes were centrifuged for 5 min at 9 000 *g*. The supernatant was discarded, the pellet air dried for 10 min and the RNA dissolved in 100 µl DEPC water. The concentrations were determined and expressed as $\mu g.ml^{-1}$ (Sambrook *et al.*, 1989).

To confirm the quality and concentration, 1 µg RNA of each sample was separated on agarose gel. The gel used was a 1% (w/v) agarose gel in 0.5 x TAE (0.02 M Tris-HCl, pH 8.0, 0.28% (v/v) glacial acetic acid and 0.5 mM EDTA (pH 8.0), containing 0.5 $\mu g.ml^{-1}$ ethidium bromide (Sambrook *et al.*, 1989).

Loading buffer (0.08% (w/v) bromophenolblue, 0.08% (w/v) xylene cyanol and 5% (v/v) ficoll) was added to each RNA sample where after the samples were

loaded on the gel and separated at 120 V until the dye migrated an appropriate distance. The RNA was visualized under UV light and the gels photographed.

3.2.8 RT-PCR analysis

In order to study the expression pattern of the *At-RLK3* gene, the amount of mRNA transcripts in a specific sample at a specific time was analyzed by multiplex one-step RT-PCR. The RT-PCR reaction consisted of 2 steps, a reverse transcription step and a normal PCR reaction step.

Different primer sets were specifically designed on the basis of known sequences of the both *At-RLK3* and 18S rDNA genes, using a web based web primer program (http://genome-www2.stanford.edu/cgi-bin/SGD/web-primer). The first set was designed in order to amplify the gene portion coding for the extracellular domain of At-RLK3 and the second set to amplify a portion of the 18S rDNA gene (Table 3.1). Bovis 26 and 27 yielded a 226 bp rDNA fragment that acted as an internal amplification control. All primers were synthesized by IDT (Integrated DNA Technologies, Inc).

The RT-PCR reactions were carried out in a final volume of 50 µl using the Titan one tube RT-PCR system. Each reaction contained 10 ng total RNA, 5% (v/v) dimethylsulphoxide (DMSO), 5 mM DTT, 0.25 mM deoxynucleotide triphosphate mix (dNTP's), 1 x RT-PCR buffer, 1 µl enzyme mixture and 25 pmol of each primer. The primer sets used are indicated in Table 3.1. RT-PCR was carried out on a Hybaid OmniGene system using the following program regime: one cycle of 50°C for 30 min and 94°C for 2 min, 35 cycles of 94°C for 10 sec, 56°C for 1 min, 68°C for 4 min and a final single 4 min extension at 68°C.

Table 3.1. Nucleotide sequences of primers used to amplify the DNA sequences encoding the extracellular domain of At-RLK3, a portion of the 18S rDNA gene and a portion of the PR-2 protein of *A. thaliana*.

Primer	Oligonucleotide sequence (5'→3')		Amplified regions	Product size (bp)
Bovis 26	CAACTTTCGATGGTAGGATAGT	Forward primer		
Bovis 27	CTCGTTAAGGGATTTAGATTG	Reverse primer	A portion of the 18S rDNA gene	226
Bovis 12	CTCCAGCTGAGAATTCTTTGCTATC	Forward primer		
Bovis 3	TACTCTAGAAATGAAGCAGAGGAGTTTGTTTTC	Reverse primer	A portion of the *At-RLK3* gene	789
Bovis 37	TAGGCGATACCTTGCCAA	Forward primer		
Bovis 38	ACTTCATACTTAGACTGTCGA	Reverse primer	A portion of the *PR-2* gene	891

The amplified DNA fragments were separated on a 1% (w/v) agarose gel (3.2.7) and visualized.

3.2.9 The overexpression of *At-RLK3* in *A. thaliana*

3.2.9.1 Preparation of the recombinant plasmid

The pTA7002 binary plasmid vector was used for the overexpression of the *At-RLK3* gene in *A. thaliana*. The recombinant plasmid contained the full length *At-RLK3* gene which was inserted downstream of the inducible promoter using the *Xho*I and *Spe*I restriction sites. Both the initiation and termination codons were included in the coding sequence.

3.2.9.2 The transformation of *A. thaliana* using floral dipping

Transformation of *A. thaliana* floral tissue with *A. tumefaciens* was done according to the floral dipping method described by Clough and Bent (1998). *A. thaliana* plants were planted on MS medium (3.1.1), grown for 2 weeks and then transferred to soil. To obtain more floral buds per plants, the primary bolt was clipped, relieving apical dominance and encouraging synchronized emergence of multiple secondary bolts. Plants were dipped when most secondary inflorescences were about 1 – 10 cm tall (4 – 8 days after clipping).

The transformation was mediated by *A. tumefaciens* pGV2260 carrying the recombinant plasmid (3.2.9.1). The *A. tumefaciens* was grown to stationary phase at 30°C in 5 ml LB [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl with 50 $mg.ml^{-1}$ kanamycin].

After 24 hours the culture was re-inoculated in 100 ml LB containing 50 $\mu g.ml^{-1}$ kanamycin. After two days the cells were collected by centrifugation for 20 min at 5500 *g*. The pellet was resuspended in 5% (w/v) sucrose solution to an A_{600nm} of 0.8. Before dipping, 0.05% (v/v) Silwet L-77 (OSi Specialties) was added to the culture. The Silwet L-77 reduces surface tension to allow entry of the bacteria into relatively inaccessible plant tissues.

The above-ground parts of the plants were dipped into the *A. tumefaciens* solution for 2-3 seconds, until a film of liquid coating the leaves could be seen. The dipped plants were kept under high humidity for 16-24 h and then transferred to a growth cabinet and grown as described (3.1.1).

The harvested seed were replanted on MS-medium (3.1.1) containing 50 $\mu g.ml^{-1}$ hygromycin for the selection of transformants. Putative transformants were transplanted to soil and seeds were harvested (3.1.1).

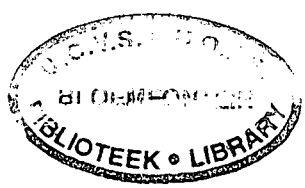
3.2.10 RT-PCR analysis of *At-RLK3* expression in transgenic plants

The induction of *At-RLK3* expression in the transgenic and wild-type plants was analysed by spraying two-week old plants with 0.03 M dexamethazone (DEX) containing 0.01% (v/v) Tween 20 until micro droplets were visible on the plants. They were then grown in the growth chamber as described (3.1.1). The plant tissue was collected at 0 and 3 h after DEX treatment.

It was immediately frozen in liquid nitrogen and stored at –80°C. Total RNA was extracted as described (3.2.7) and used in a RT-PCR reaction to analyze the induced expression of *At-RLK3* after treatment with DEX (3.2.8).

Transgenic and wild-type plants sprayed with 0.03 M DEX, were treated with 2 mM SA (3.2.1). Tissue was harvested after 0, 4, 8, 12, 24 and 48 h of treatment and directly frozen in liquid nitrogen and stored at –80 °C. RNA was extracted from the tissue as described (3.2.7). The expression of the *At*-RLK3, β-1,3-glucanase and 18S rDNA genes was determined using the indicated primers (Table 3.1) in RT-PCR reactions (3.2.8).

The bands on the gel were integrated to quantify the RT-PCR amplified products using the Molecular Analyst Software (Bio-Rad). The amplification product of both the β-1,3- glucanase and *At-RLK3* genes together with the portion of the 18S rDNA gene of each time interval was selected and the volume of each measured separately. The values obtained for *At-RLK3* and β-1,3-glucanase respectively at each time point was divided by that of the 18S rDNA gene. This value of each time point was then divided by the value obtained for time 0 to indicate either an increase or decrease of expression.



CHAPTER FOUR

Results

4.1 Standard curves

The standard curves used for the determination of protein concentration, ß-1,3-glucanase activity and the hydrogen peroxide content curve (Fig. 4.1) show in each case a linear relationship between the levels and absorption values at the specific wavelengths. These linear relationships allow us to perform comparisons within the indicated ranges.

4.2 The activation of defense responses with different plant activators

The activation of the defense reaction in *A. thaliana* in response to treatment with different plant activators was studied on various levels. This included β-1,3-glucanase activity, the expression of β-1,3-glucanases on protein level, hydrogen peroxide levels and the expression of *At-RLK3*. These results will give more insight into the role of At-RLK3 in the perception of these plant activators.

All treatments were evaluated in two separate experiments over different time intervals after treatment. The first experiment was over a 96 h period while the other was over a shorter 4 h time period with the 96 h period, the activation of the defense response was confirmed, while the shorter 4 h period allowed us to determine the earliest time after treatment that the plants reacted to the presence of the plant activator. For each β-1,3-glucanases activity determination, the reaction was done in triplicate.

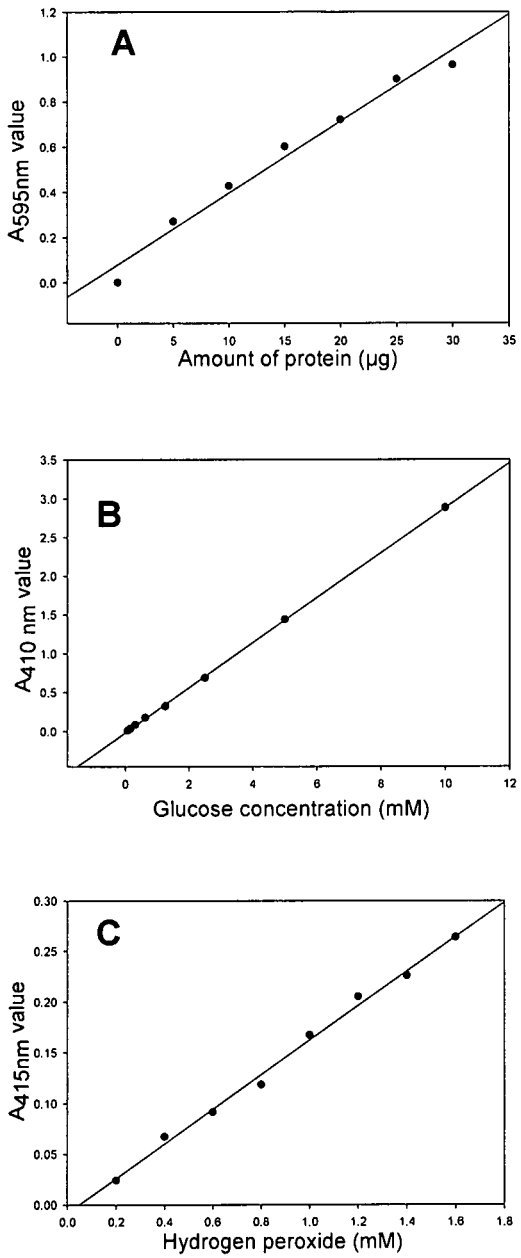


Fig. 4.1. Standard curves relating absorption values at specific wavelengths to (A) protein (R^2 = 0.9779839311), (B) glucose (R^2 = 0.9998099319) and (C) hydrogen peroxide concentrations (R^2 = 0.9901187836).

4.2.1 Control treatment

The effect of the water and water/acetone mixture treatments of *A. thaliana* plants on the different defense related parameters was included as controls in a comparative study using different plant activators.

The β-1,3-glucanase activity changes during the 96 h and 4 h period after water and water/acetone mixture treatments were relatively small (Fig. 4.2 A and B; Fig. 4.3 A and B).

The expression of the β-1,3-glucanase genes in *A. thaliana* plants treated with water and water/acetone mixture was examined on protein level by means of Western blot analysis. Four β-1,3-glucanase cross-reacting polypeptides with molecular masses of 53, 52, 43 and 37 kDa could be distinguished (Fig. 4.4 A and B; Fig. 4.5 A and B). The single band visible in the coomassie stained gels (Fig. 4.4 A (ii) and B (ii); Fig. 4.5 A (ii) and B (ii)) represented the most prominent polypeptide namely, the large subunit of Rubisco with a molecular mass of 56.0 kDa. According to the levels of the polypeptide bands (Fig. 4.4 A and B; Fig. 4.5 A and B), the proteins were expressed constitutively and at unchanged levels over both the studied time intervals after water and water/acetone mixture treatments. The decrease at 2 and 4 h of the water/acetone mixture treatments was most probably the result of unequal loading.

4.2.2 The effect of different plant activators

The induction of the β-1,3-glucanase activity was expressed in terms of percentage relative to the water controls for the SA, ASA and BTH treatments and the water/acetone mixture for INA treatment.

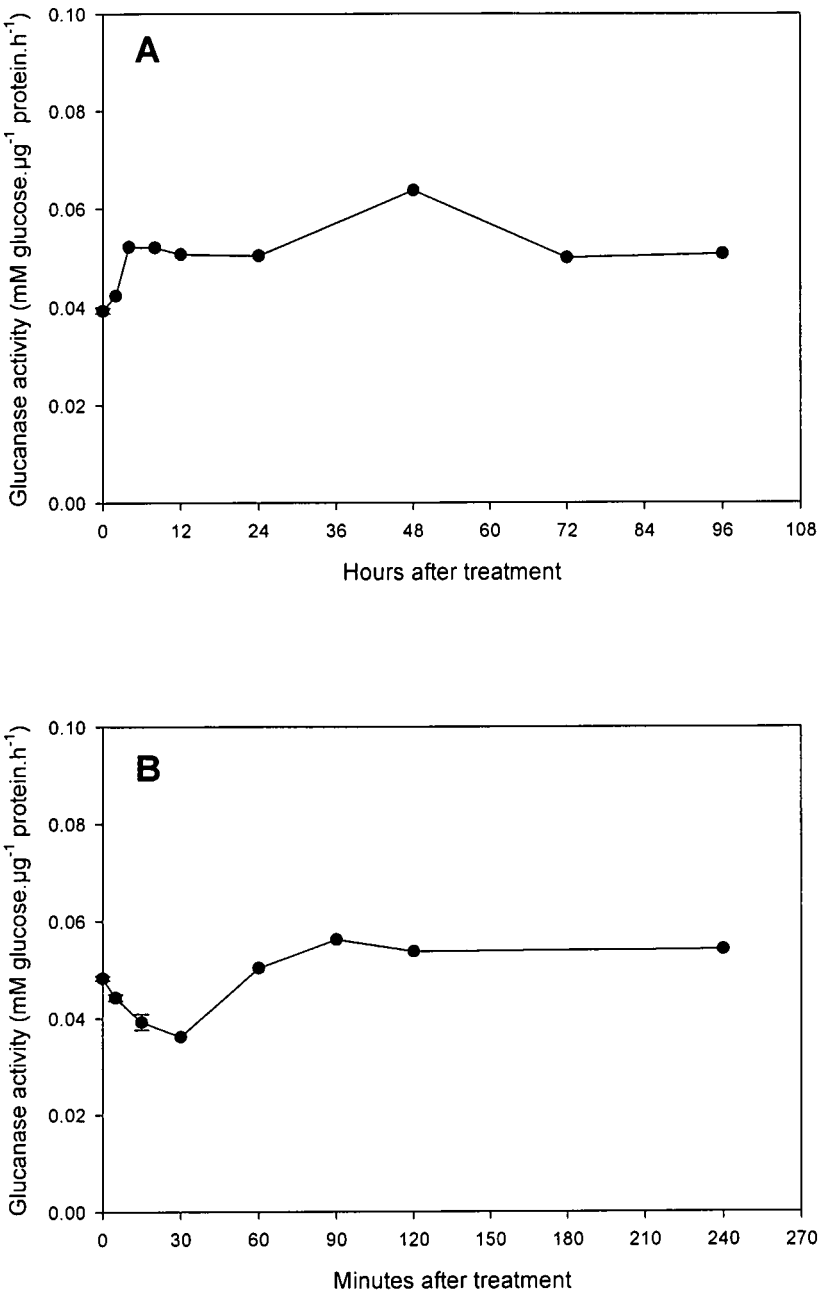


Fig. 4.2. The effect of water treatment of *A. thaliana* plants on β-1,3-glucanase activity at longer (A) and shorter (B) time intervals after treatment. Error bars indicate standard deviation, n=3.

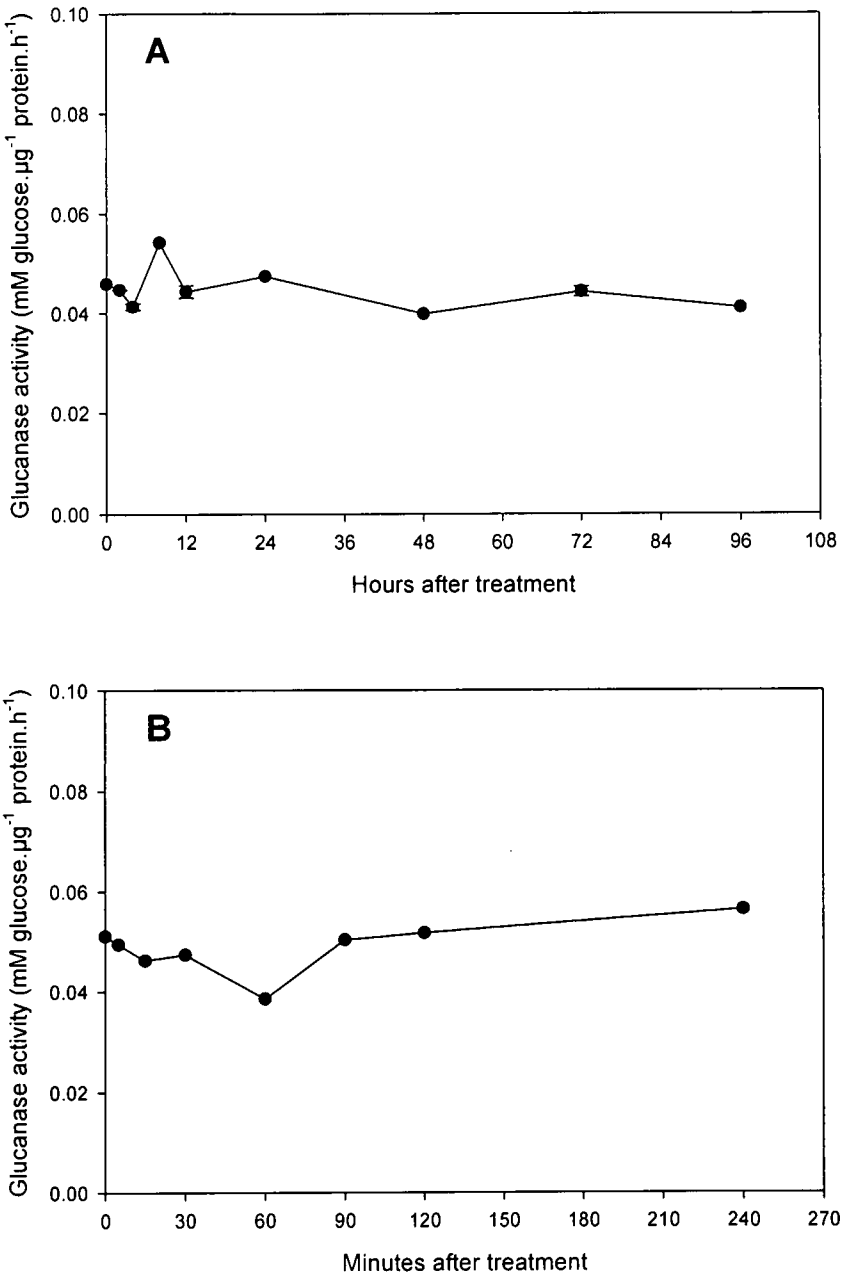


Fig. 4.3. The effect of a water/acetone mixture treatment of *A. thaliana* plants on β-1,3-glucanase activity at longer (A) and shorter (B) time intervals after treatment. Error bars indicate standard deviation, n=3.

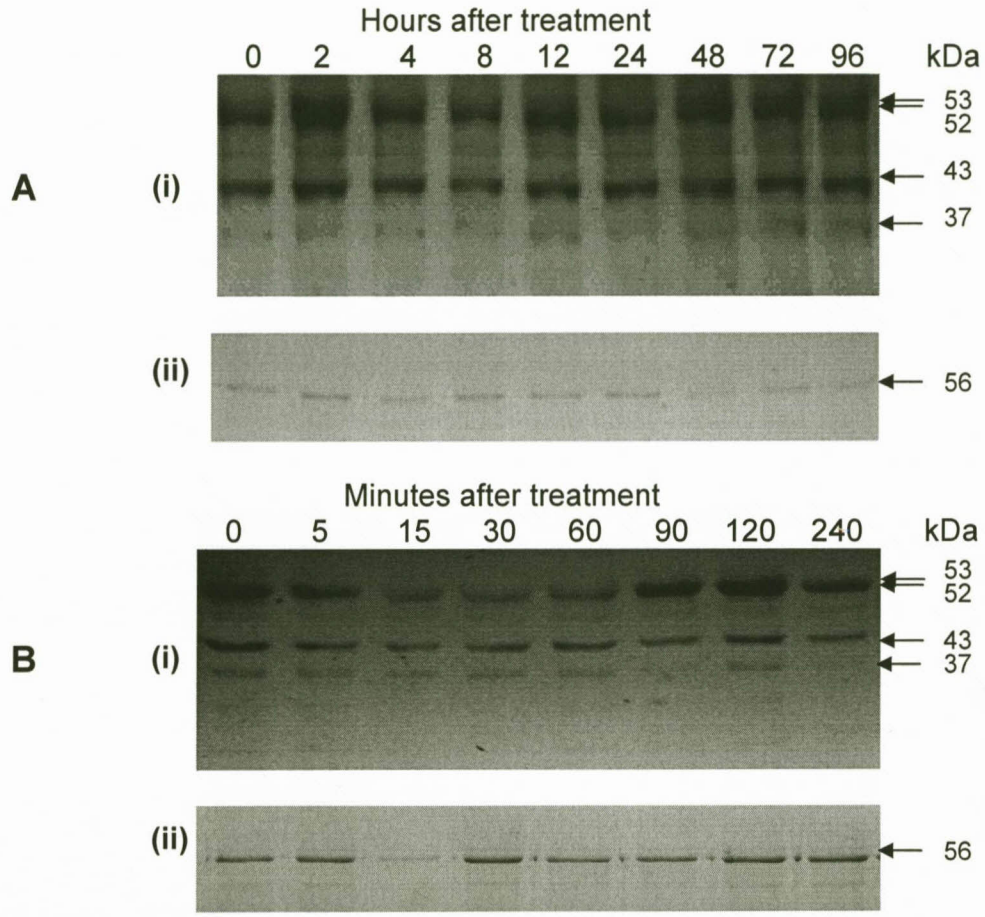


Fig. 4.4. Immunoblot analysis of β-1,3-glucanase polypeptides (i) from *A. thaliana* seedlings treated with water. (A) represents the longer time intervals and (B) the shorter time intervals after treatment. Coomassie stained gels (ii) were included to verify that equal quantities of proteins were loaded.

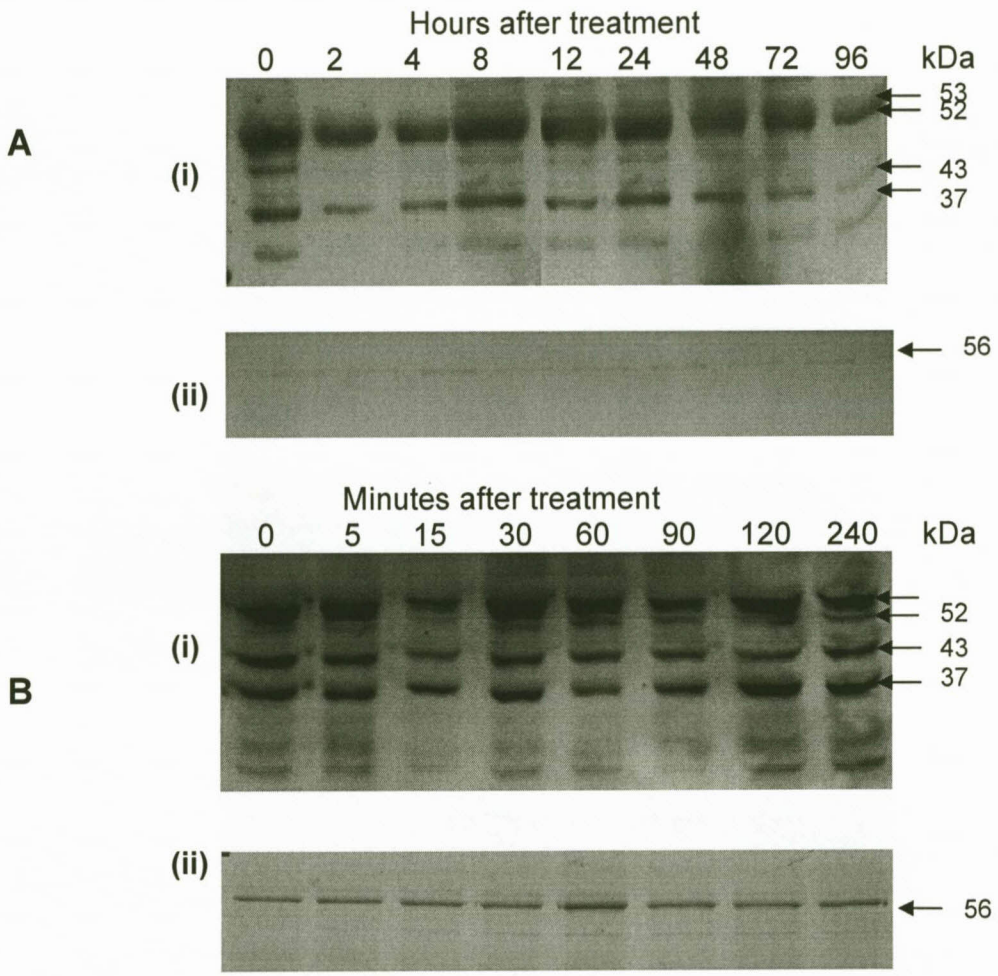


Fig. 4.5. Immunoblot analysis of β-1,3-glucanase polypeptides (i) from *A. thaliana* seedlings treated with water/acetone mixture after treatment. (A) represents the longer time intervals and (B) the shorter time intervals. Coomassie stained gels (ii) were included to verify that equal quantities of proteins were loaded.

4.2.2.1 Salicylic acid (SA)

The activation of defense responses in *A. thaliana* was analyzed after spraying the plants with 2 mM SA.

During the 96 h period after SA treatment the β-1,3-glucanase activity was transiently induced to a peak level before 48 h after treatment. A second much lower induction occurred 72 h after treatment (Fig. 4.6 (A)). The increase in β-1,3-glucanase activity indicated that the defense response was activated within a short time after spraying and remained high for at least 24 h after treatment. The early transient induction of β-1,3-glucanase activity was confirmed with the results of the second experiment (Fig. 4.6 (B)). A clear activation was seen within 15 min after treatment.

The expression of β-1,3-glucanase polypeptides in *A. thaliana* after SA treatment was illustrated by means of Western blots (Fig. 4.7). Six β-1,3-glucanase cross-reacting polypeptides could be distinguished from 72 h after SA treatment. Their molecular masses were 53, 52, 45, 43, 37 and 20 kDa (Fig. 4.7 (A)). Two unique peptides (20 and 43 kDa) appeared from 48 h (Fig. 4.7 (A)). The 43 kDa polypeptide increased up to 96 h, but the 20 kDa polypeptide levels decreased after 72 h. During the first 4 h after SA treatment only four isozymes, with molecular masses of 53, 52, 43 and 37 kDa were visible.

Another parameter measured as a very early indicator of the induction of the defense response was the levels of the reactive oxygen species, H_2O_2. According to Fig. 4.8, SA transiently stimulated the accumulation of H_2O_2 30 min after application. The H_2O_2 level however decreased to its minimum value 2 h after SA application.

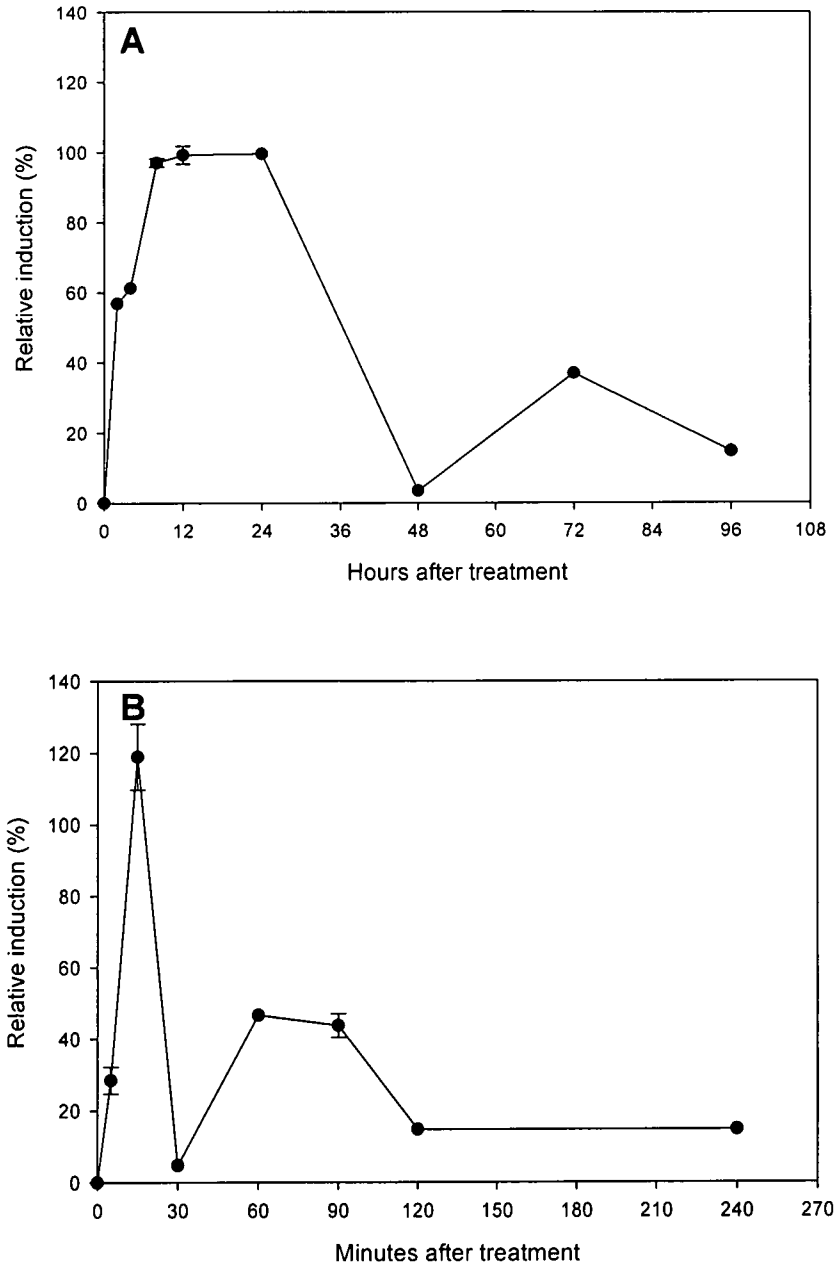


Fig. 4.6. The effect of 2 mM SA treatment of *A. thaliana* plants on β-1,3-glucanase activity at longer (A) and shorter (B) time intervals after treatment. Error bars indicate standard deviation, n=3.

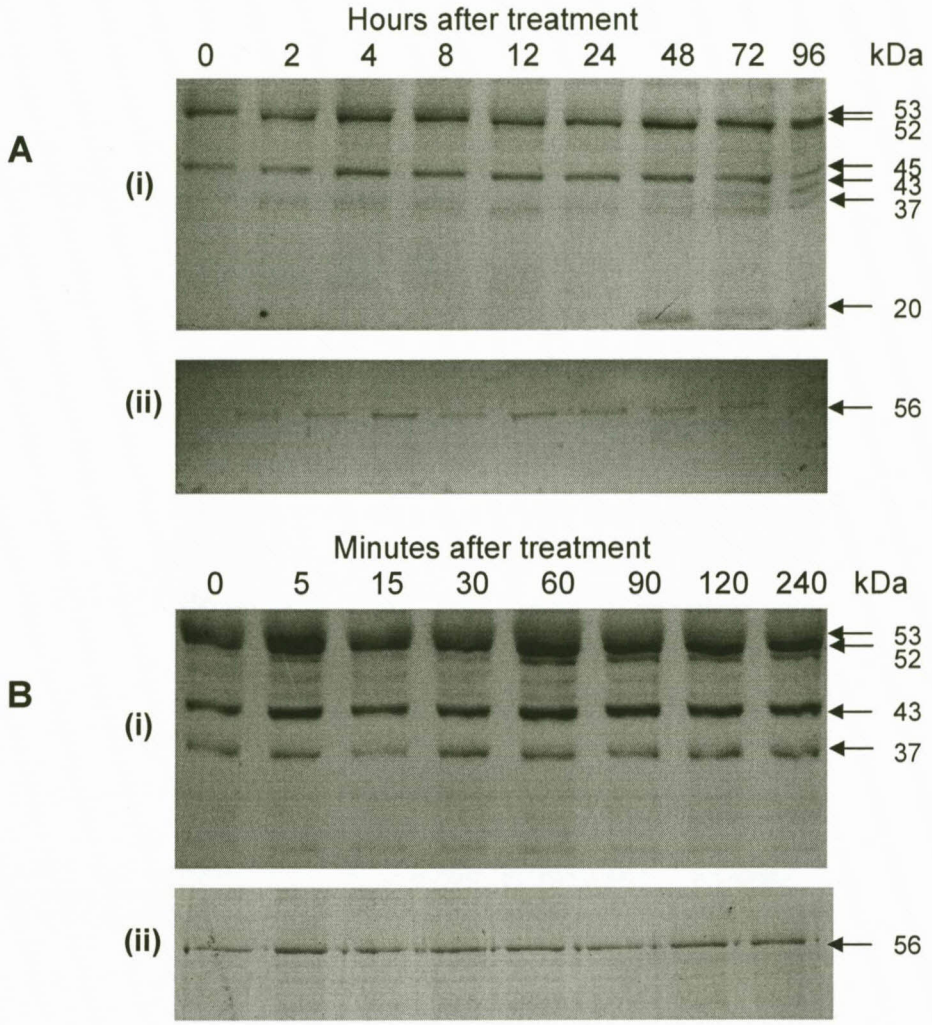


Fig. 4.7. Immunoblot analysis of β-1,3-glucanase polypeptides (i) from *A. thaliana* seedlings treated with 2 mM SA. (A) represents the longer time intervals and (B) the shorter time intervals. Coomassie stained gels (ii) were included to verify that equal quantities of proteins were loaded.

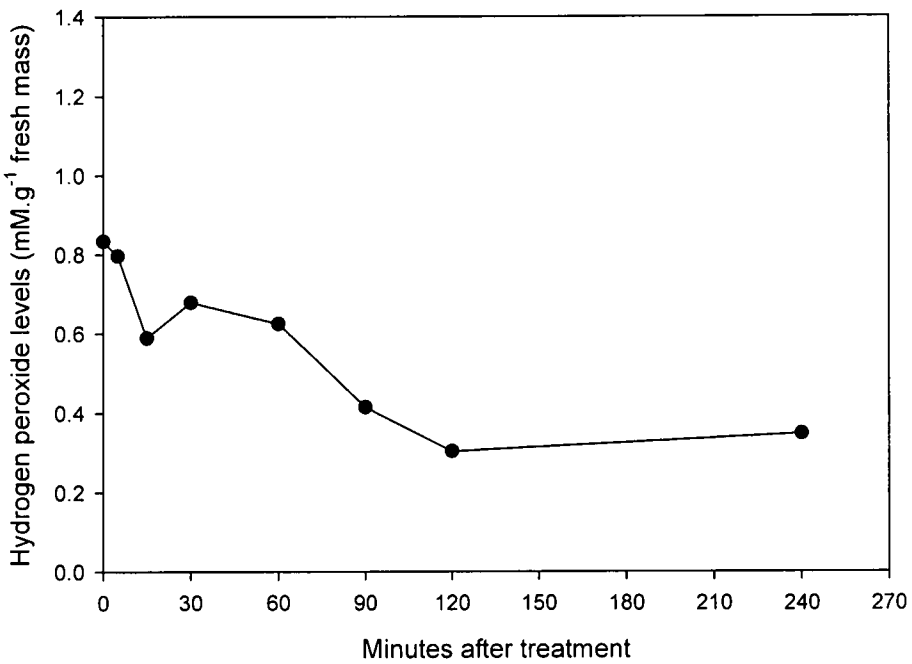


Fig. 4.8. The effect of 2 mM SA on the accumulation of H_2O_2 in *A. thaliana* plants.

To determine the effect of SA application on *At-RLK3* expression, *At-RLK3* cDNA transcripts were separated by gel electrophoresis. According to Fig. 4.9 (A) the *At-RLK3* transcripts reached a maximum level within 12 h after treatment. However, *At-RLK3* transcripts began to accumulate within 15 min after SA treatment (Fig. 4.9 (B).

4.2.2.2 Acetyl salicylic acid (ASA)

ASA is a chemical derivative of SA and was found to be rapidly hydrolyzed to SA in biological tissue (Klessig and Malany, 1994).

ASA treatment resulted in two β-1,3-glucanase activity peaks over a period of 96 h after treatment (Fig. 4.10 (A)). The β-1,3-glucanase activity increased 2 h after treatment, reached the first transient peak at 12 h and remained high for another 36 h. The second peak occurred 96 h after treatment. It thus appeared that over the longer time interval ASA treatment gave a very similar induction profile than SA time interval. Further dissemination of the first 2 h period after SA treatment showed a very early transient increase in β-1,3-glucanase activity which reach a maximum 30 min after treatment (Fig. 4.10 (B)).

Three β-1,3-glucanase cross-reacting polypeptides with molecular masses of 54, 52 and 43 kDa were visible during all time intervals up to 96 h after ASA treatment (Fig. 4.11 (A)). On the other Western blot (Fig.4.11 (B)) obtained from proteins of plants at shorter time intervals after treatment, five β-1,3-glucanase cross-reacting polypeptides with molecular masses, 54, 52, 43, 37 and 20 kDa could be distinguished. During the longer time intervals, glucanase polypeptide levels decreased 72 h after treatment. In all other cases, the polypeptides remained constant.

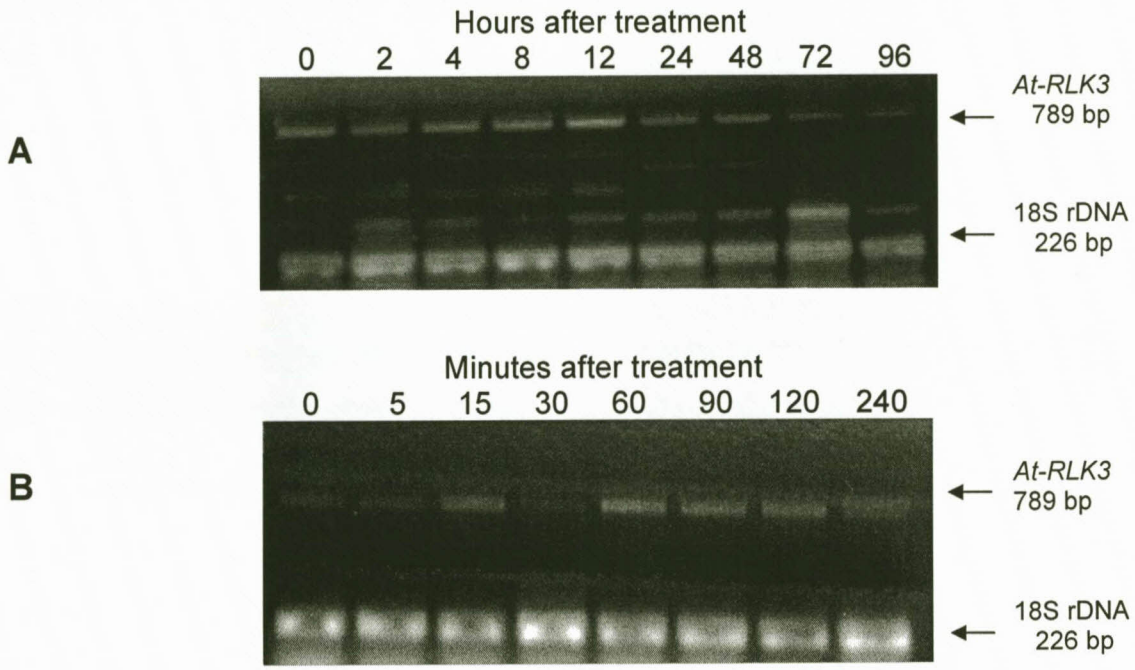


Fig. 4.9. Analysis of *At-RLK3* expression in *A. thaliana* plants treated with 2 mM SA over a longer (A) and shorter (B) time interval. The 18S rDNA was included to verify that equal quantities of RNA were used for the RT-PCR reaction.

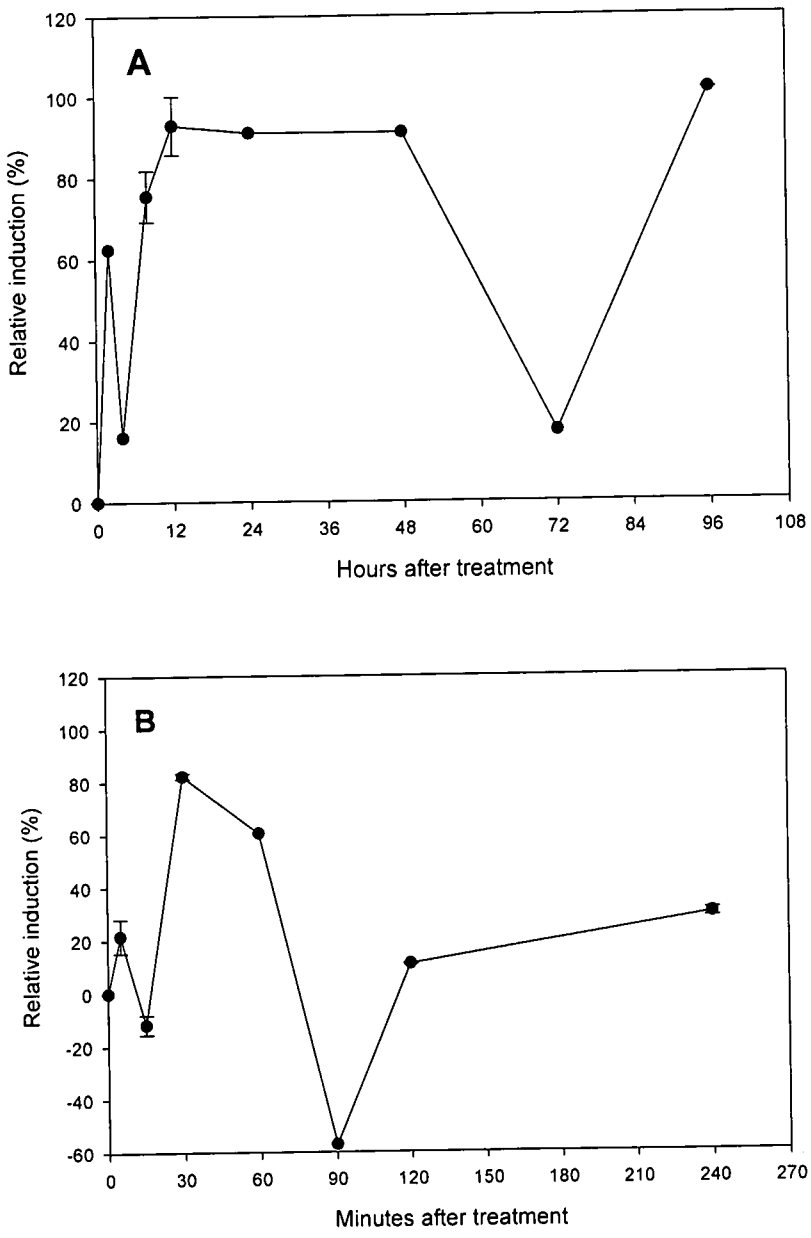


Fig. 4.10. The effect of 2 mM ASA treatment of *A. thaliana* plants on ß-1,3-glucanase activity at longer (A) and shorter (B) time intervals after treatment. Error bars indicate standard deviation, n=3.

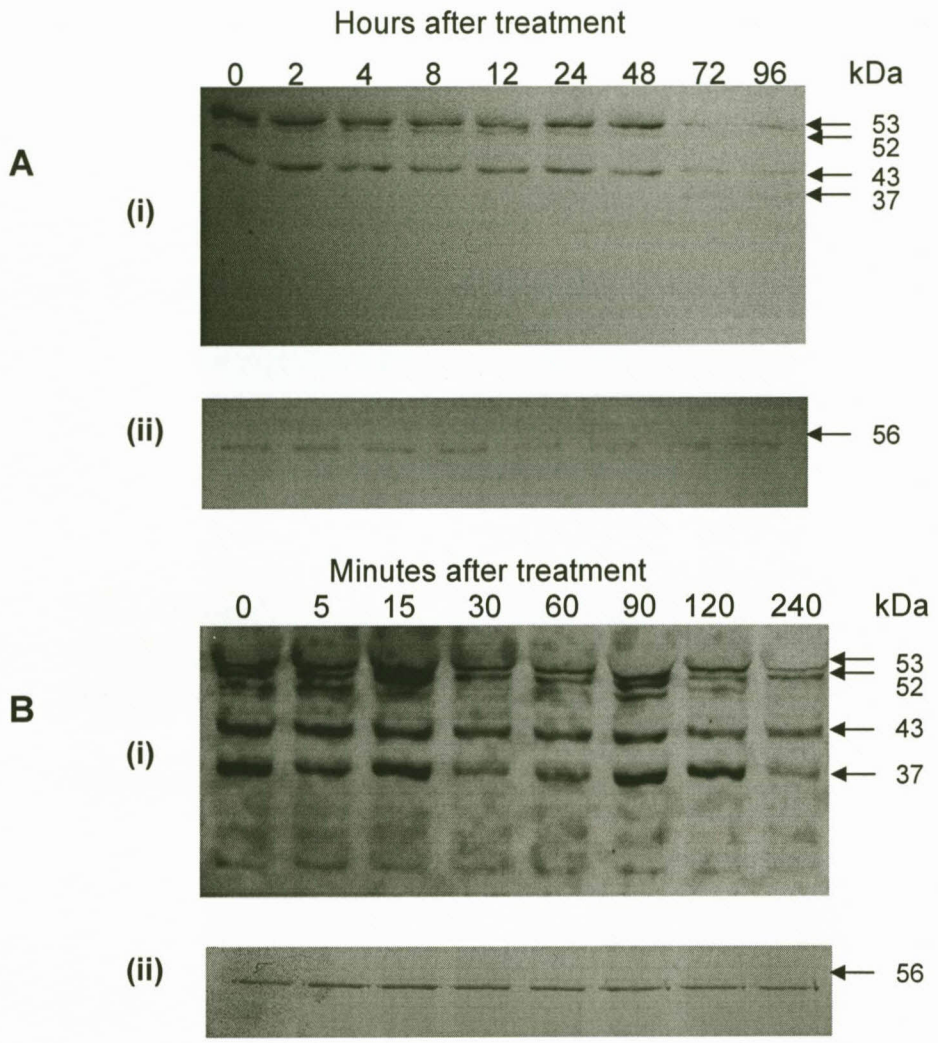


Fig. 4.11. Immunoblot analysis of β-1,3-glucanase polypeptides (i) from *A. thaliana* seedlings treated with 2 mM ASA. (A) represents the longer time intervals and (B) the shorter time intervals. Coomassie stained gels (ii) were included to verify that equal quantities of proteins were loaded.

After an initial decline the H_2O_2 level increased sharply from 60 min after ASA treatment with a maximum level at 120 min. The increase was again transient. Although somewhat later the trends of change in H_2O_2 levels corresponded with the SA treatment (Fig. 4.12).

Figure 4.13 (A) shows that *At-RLK3* transcripts began to accumulate 2 h after ASA treatment. From 4 h after treatment the expression was inhibited until 96 h where expression again increased. During the short time intervals (Fig. 4.13 (B)) *At-RLK3* transcripts remained constant up to 4 hrs after treatment.

4.2.2.3 Benzothiadiazole (BTH)

Since it was suggested that BTH is a functional analogue of SA (Görlach *et al.*, 1996) by inducing the same set of responses as SA in *A. thaliana* and tobacco (Wendehenne *et al.*, 1998), the effects of BTH on *A. thaliana* were studied after spraying the plants with 0.12 $mg.ml^{-1}$ BTH.

BTH application effectively induced β-1,3-glucanase activity from 8 h after application to a maximum level at 12 h (Fig. 4.14(A)). It seemed that a very early transient peak 15 min after treatment (Fig. 4.14(B)) and after 240 min a second more prolonged induction correlating with that in Fig. 4.14 (A) occurred.

To establish whether the BTH induced glucanase activities were the result of increased β-1,3-glucanase protein levels, Western blots were prepared. Five β-1,3-glucanase cross-reacting proteins were detected with molecular masses, 53, 52, 43, 37 and 20 kDa (Fig. 4.15). The 53, 52 and 43 kDa β-1,3-glucanase isozymes occurred prominently at all time intervals (Fig. 4.15). Also apparent, was that the expression of the 37 kDa polypeptide was repressed after 4 h, only

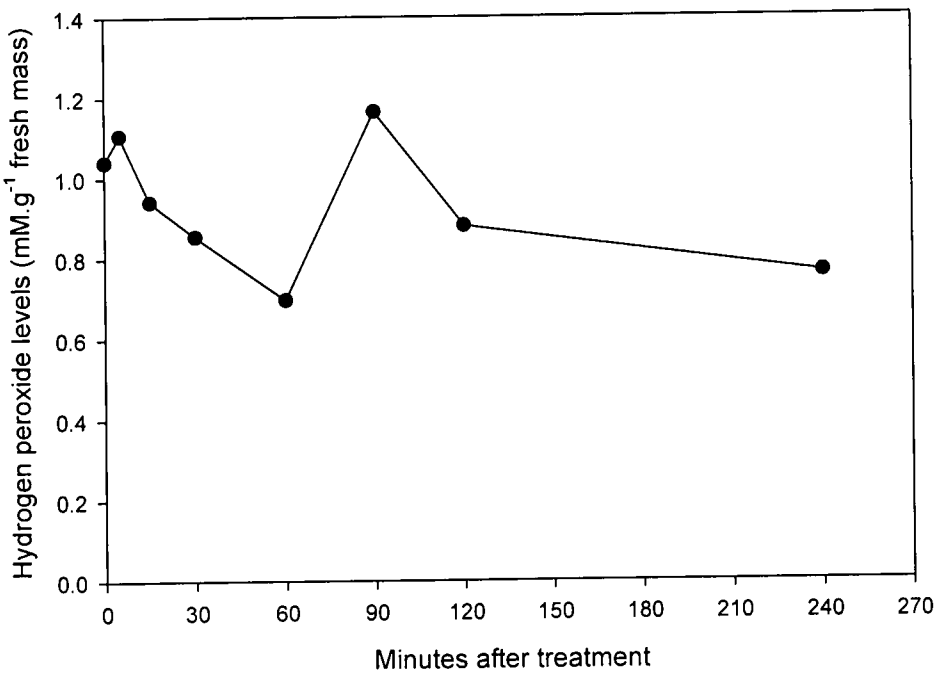


Fig. 4.12. The effect of 2 mM ASA on the accumulation of H_2O_2 in *A. thaliana* plants.

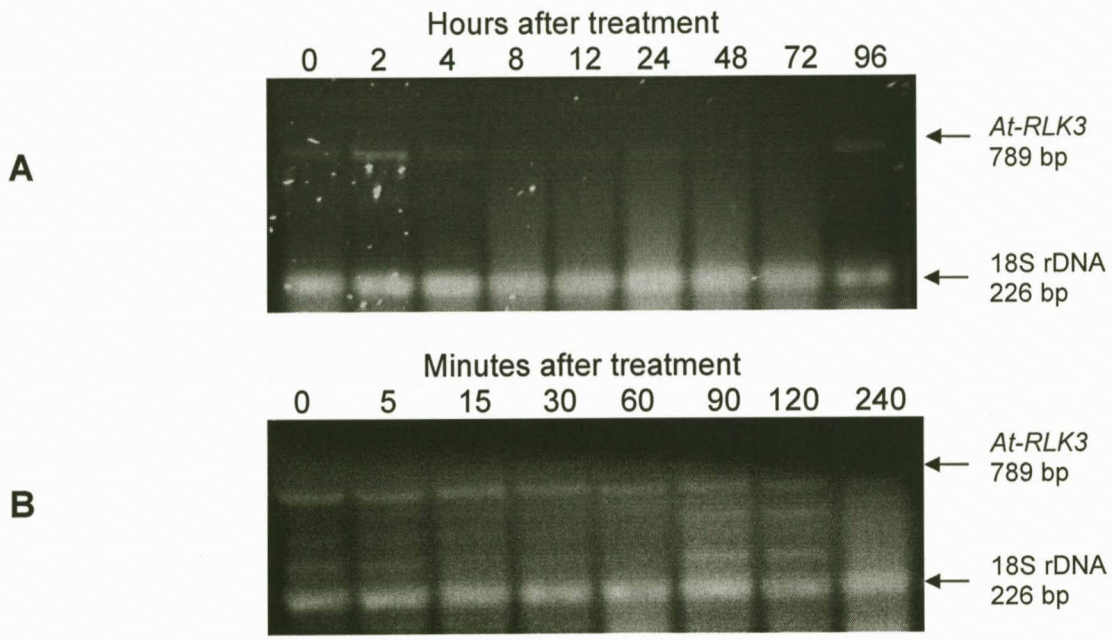


Fig. 4.13. Analysis of *At-RLK3* expression in *A. thaliana* plants treated with 2 mM ASA over a longer (A) and shorter (B) time interval. The 18S rDNA was included to verify that equal quantities of RNA were used for the RT-PCR reaction.

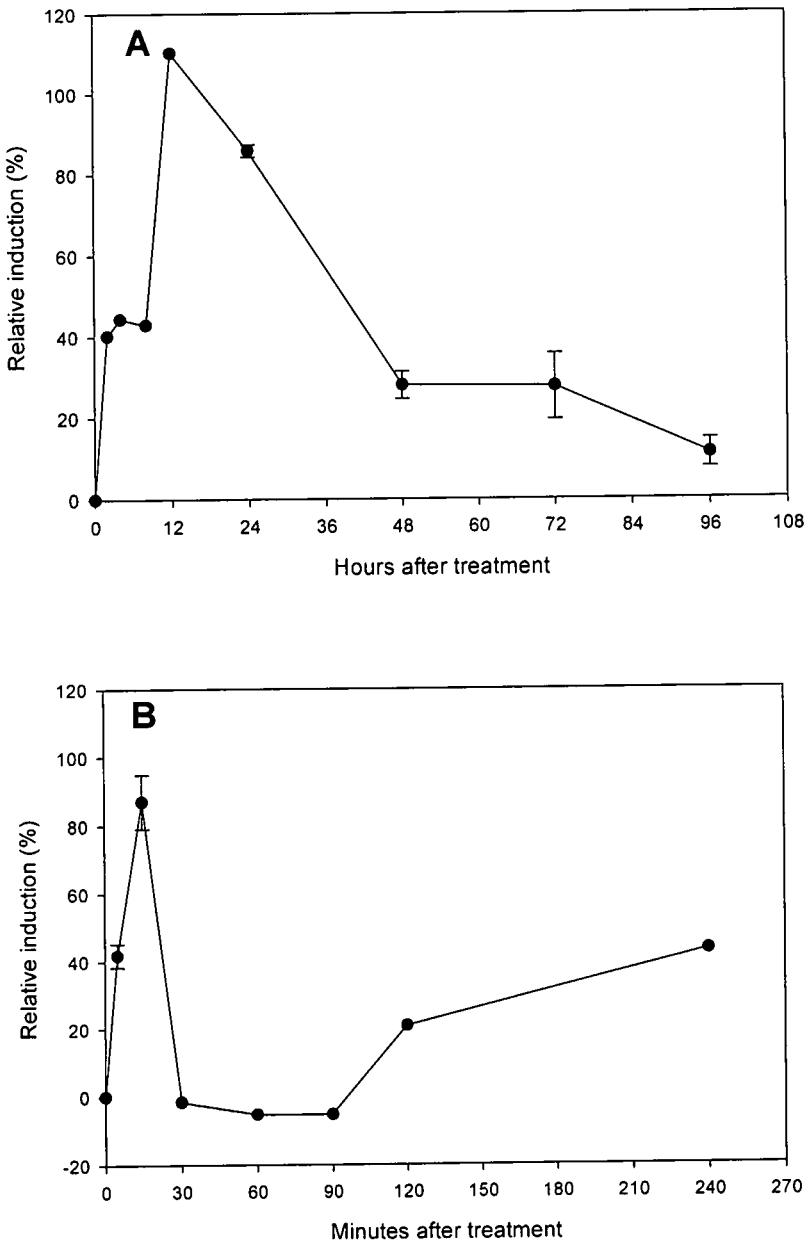


Fig. 4.14. The effect of 0.2 mg.ml^{-1} BTH treatment of *A. thaliana* plants on β-1,3-glucanase activity at longer (A) and shorter (B) time intervals after treatment. Error bars indicate standard deviation, n=3.

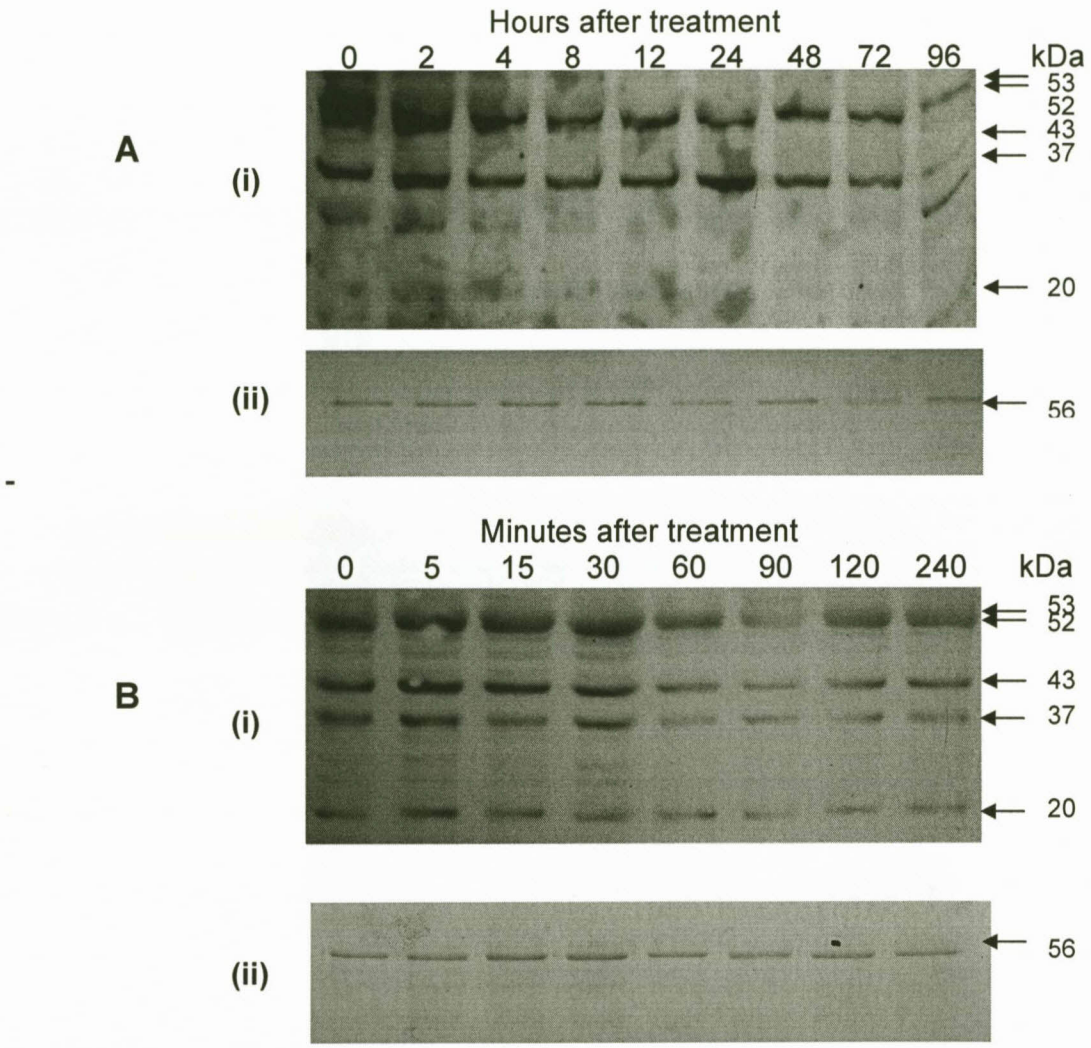


Fig. 4.15. Immunoblot analysis of β-1,3-glucanase polypeptides (i) from *A. thaliana* seedlings treated with 0.12 mg.ml^{-1} BTH. (A) represents the longer time intervals and (B) the shorter time intervals. Coomassie stained gels (ii) were included to verify that equal quantities of proteins were loaded.

to reappear at 96 h after treatment and the 20 kDa polypeptide could only be detected at 96 hr (Fig. 4.15 (A)). During the shorter time intervals, a decrease in polypeptide levels found was between 60 min and 240 min (4 h) after treatment.

BTH treatment resulted in a rapid accumulation of H_2O_2 (Fig. 4.16) with the highest level reached 30 min after treatment. This increase was transient with the levels decreased again thereafter.

Even though very little *At-RLK3* transcripts were present at time 0, *At-RLK3* mRNA started to accumulate 15 min after BTH application and remained high for at least two hours whereafter it decreased (Fig. 4.17 (A) and (B)). The *At-RLK3* transcript expression increase again 96 h after BTH treatment.

4.2.2.4 2,6-Dichloroisonicotinic acid (INA)

For the fact that INA is regarded as a functional analogue of SA, ASA and BTH (Uknes *et al.*, 1992; Vernooij *et al.*, 1994; Wendehenne *et al.*, 1998) it was decided to study the effect of INA on the activation of plant defenses.

A transient increase in β-1,3-glucanase activity occurred within 4 h after INA treatment (Fig. 4.18 (A)). This is reflected also in Fig. 4.18 (B). A second more prolonged increase was observed from 24 h to 48 h after treatment (Fig. 4.18 (A)).

The expression of the β-1,3-glucanase protein was determined by Western blot analysis. The anti-β-1,3-glucanase antibody recognized four β-1,3-glucanase polypeptides (Fig. 4.19 (A)). The molecular masses of these four isozymes were 53, 52, 43 and 37 kDa. In addition to these polypeptides, 20 kDa polypeptide

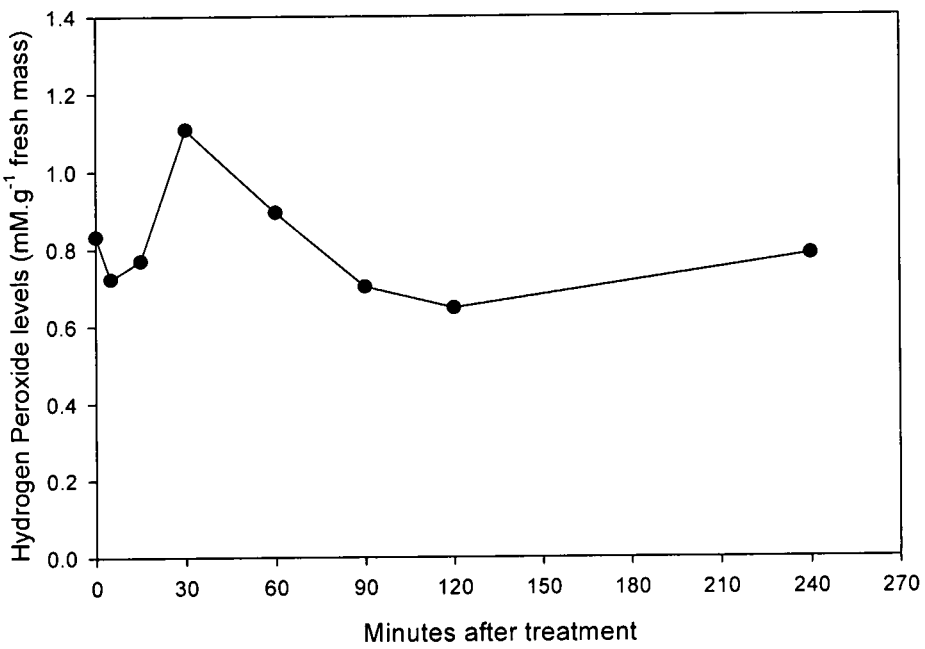


Fig. 4.16. The effect of 0.12 $mg.ml^{-1}$ BTH on the accumulation of H_2O_2 in *A. thaliana* plants.

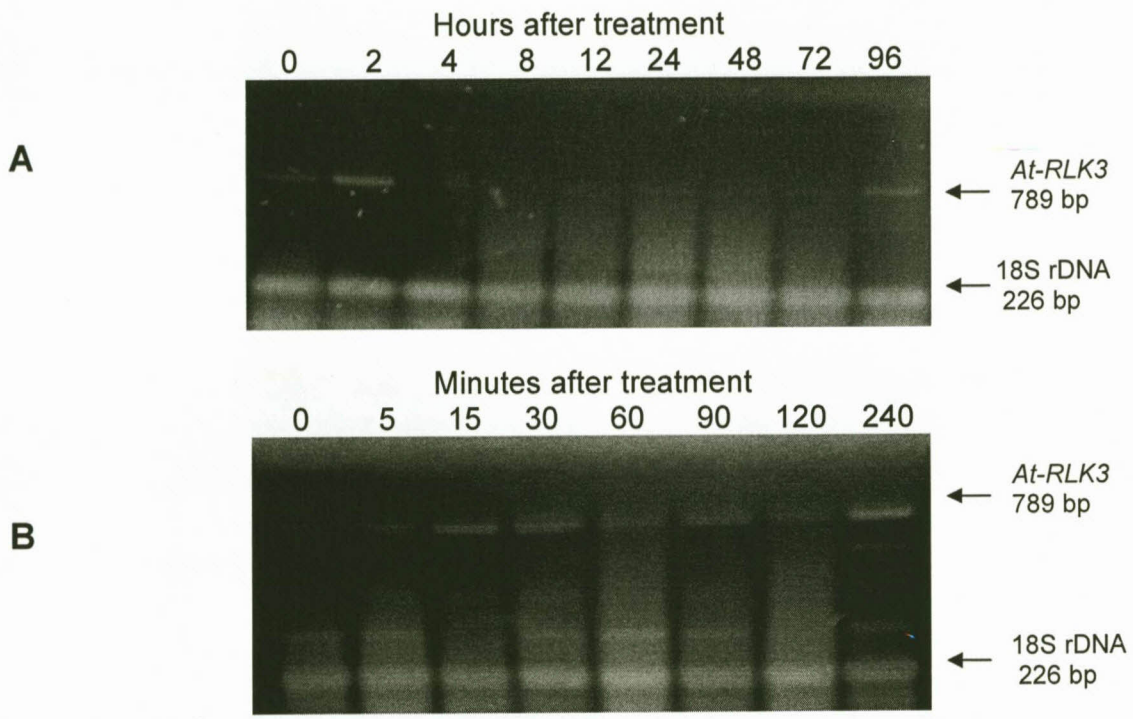


Fig. 4.17. Analysis of *At-RLK3* expression in *A. thaliana* plants treated with 0.2 mg.ml^{-1} BTH over a longer (A) and shorter (B) time interval. The 18S rDNA was included to verify that equal quantities of RNA were used for the RT-PCR reaction.

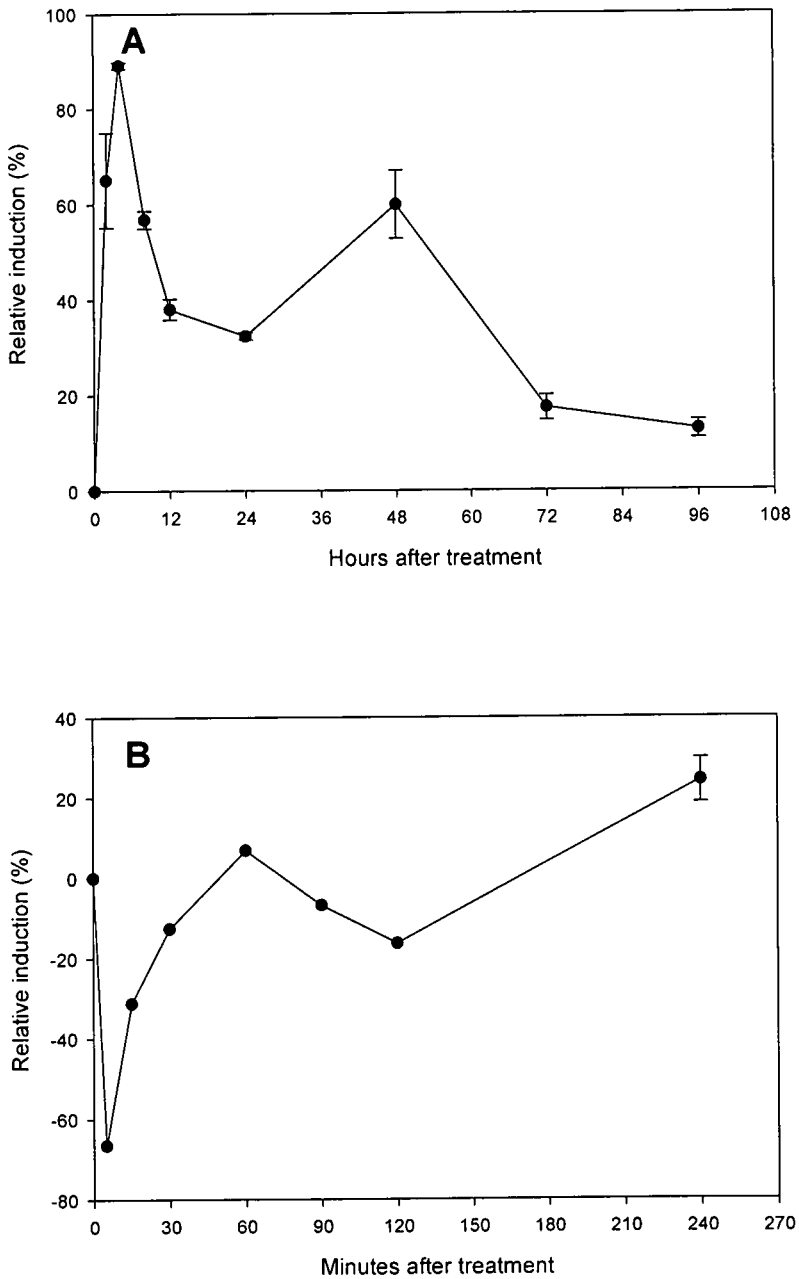


Fig. 4.18. The effect of 0.1 mM INA treatment of *A. thaliana* plants on β-1,3-glucanase activity at longer (A) and shorter (B) time intervals after treatment. Error bars indicate standard deviation, n=3.

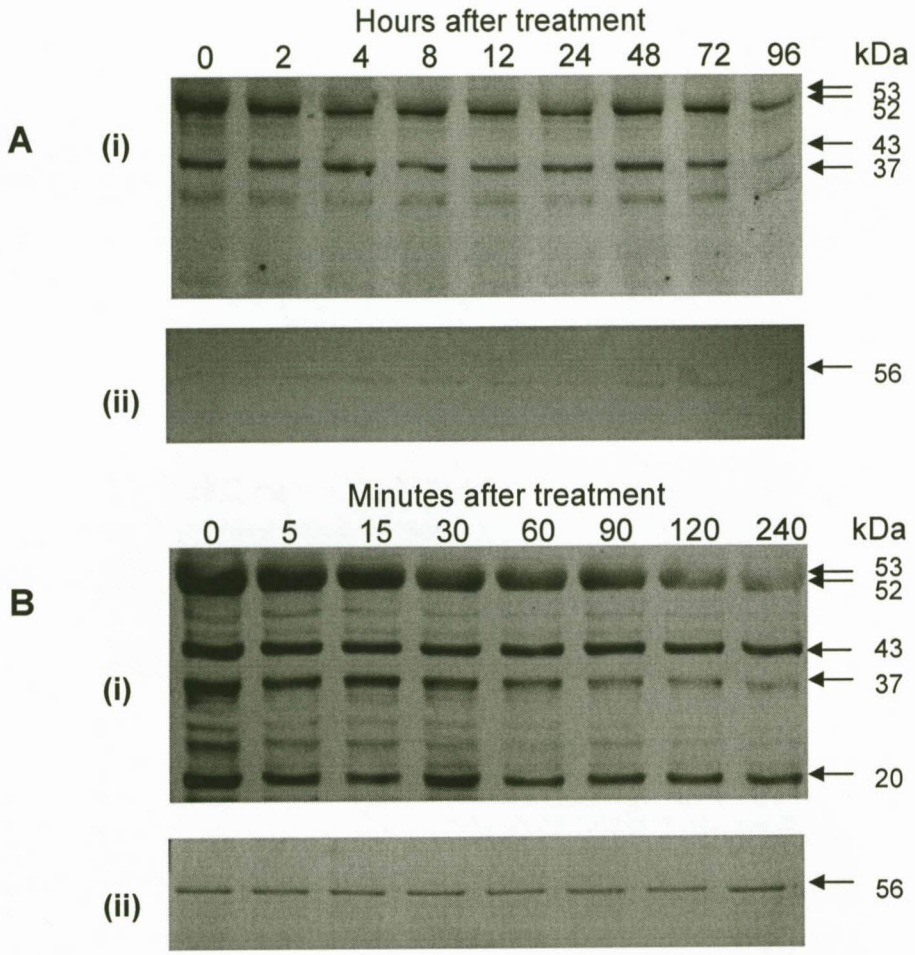


Fig. 4.19. Immunoblot analysis of β-1,3-glucanase polypeptides (i) from *A. thaliana* seedlings treated with 0.1 mM INA. (A) represents the longer time intervals and (B) the shorter time intervals. Coomassie stained gels (ii) were included to verify that equal quantities of proteins were loaded.

was observed during the short time intervals (Fig. 4.19 (B)).. The expression of all the polypeptides was constitutively expressed at equal levels.

In comparison to previous treatments, the H_2O_2 levels in the INA treated plants was almost 5 times lower than that of the BTH treated plants (Fig. 4.20). An early peak induction of the H_2O_2 level occurred 30 min after INA treatment. Another but slower increase is visible 4 h after treatment.

It was found that *At-RLK3* mRNA in the INA treated plants accumulated to a maximum level at 4 h (Fig. 4.21 (A)). In addition, an increase 5 min after treatment was seen in the shorter time period (Fig. 4.21 (B)). Thereafter the levels dropped. In contrast to all the other treatments, a second amplified fragment was also visible with a similar induction profile.

4.2.3 The generation of transgenic *A. thaliana* plants overexpressing *At-RLK3*

4.2.3.1 Transformation of *A. thaliana* and recovery of F_2 plants

Seven independent hygromycin-resistant plants were selected by screening F_1 seeds on media containing hygromycin. Only one of the seven initial plants survived and was replanted in soil for seed collection. The overexpressing plant did not develop a primary influorescence, but had several secondary bolts. In the wild-type plants, the primary bolts are usually clipped to encourage the proliferation of many secondary bolts, but in the transformed plant this was unnecessary. The plant also produced more leaves, forming an almost 'bush' like form while the lengths of the roots were much shorter than those of the wild-type plants. The plant grew much longer in comparison with the wild-type plant before seed could be collected.

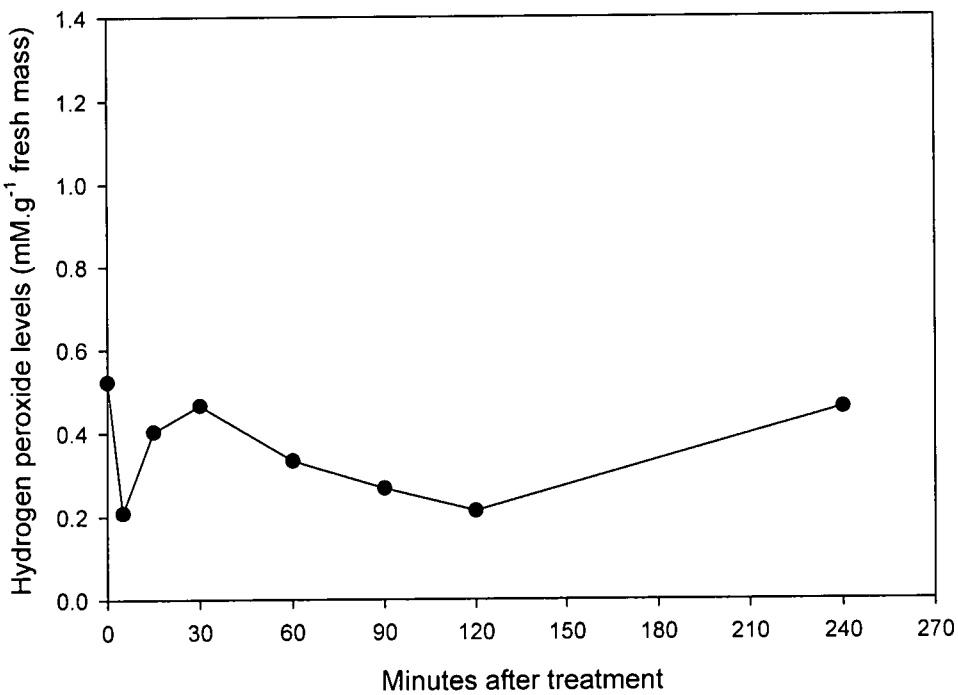


Fig. 4.20. The effect of 0.1 mM INA on the accumulation of H_2O_2 in *A. thaliana* plants.

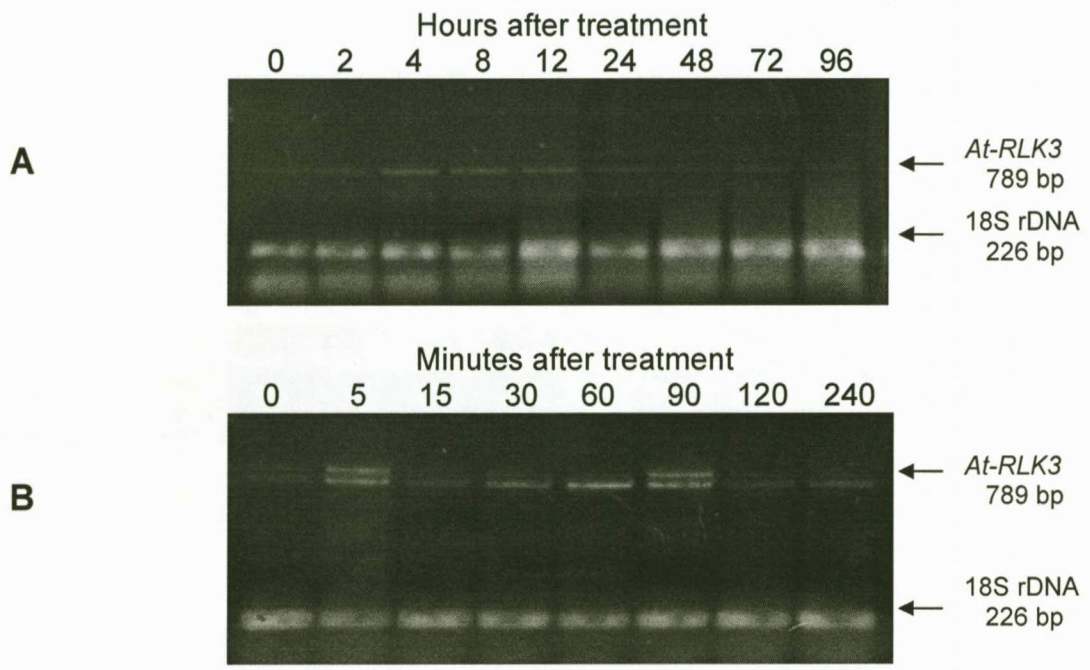


Fig. 4.21. Analysis of *At-RLK3* expression in *A. thaliana* plants treated with 0.1 mM INA over a longer (A) and shorter (B) time interval. The 18S rDNA was included to verify that equal quantities of RNA were used for the RT-PCR reaction.

4.2.3.2 F_2 phenotypes

Seed obtained from this F_1 overexpressing plant was collected and replanted on MS medium containing hygromycin for further analysis. The F_2 plants showed several phenotypic differences from the wild-type (Fig. 4.22).

All the plants in figure 4.22 (B) were collected from a single transgenic F_1 plant, but after replanting it on the medium there were both smaller and larger plants present. The smaller plants had smaller leaves that were harder in structure with a darker green color. Others had longer leaves and stems, with a softer greener color.

Figure 4.23 shows the differences in root lengths between the transgenic (B) and wild-type (A) plants. The root length of the wild-type plants was almost 13 times longer than that of the transgenic plant. It is important to realize that these phenotypic differences were in plants of which the expression of the transgenic *At-RLK3* gene was not induced. It was however reported that the expression of genes in the plasmid could be leaky, allowing low levels of constitutive expression.

4.2.4 The analysis of *At-RLK3* expression after DEX treatment

The inducible expression of *At-RLK3* in the transgenic plants depended on the treatment of the plants with a synthetic glucocorticoid hormone, DEX. A RT-PCR reaction was done to analyze the effect of the DEX treatment on the mRNA expression level of *At-RLK3* in both wild type and transgenic plants (Fig. 4.24). At time zero the *At-RLK3* mRNA level of the transgenic plant was lower (0.8x) in comparison to that of the wild-type plant.

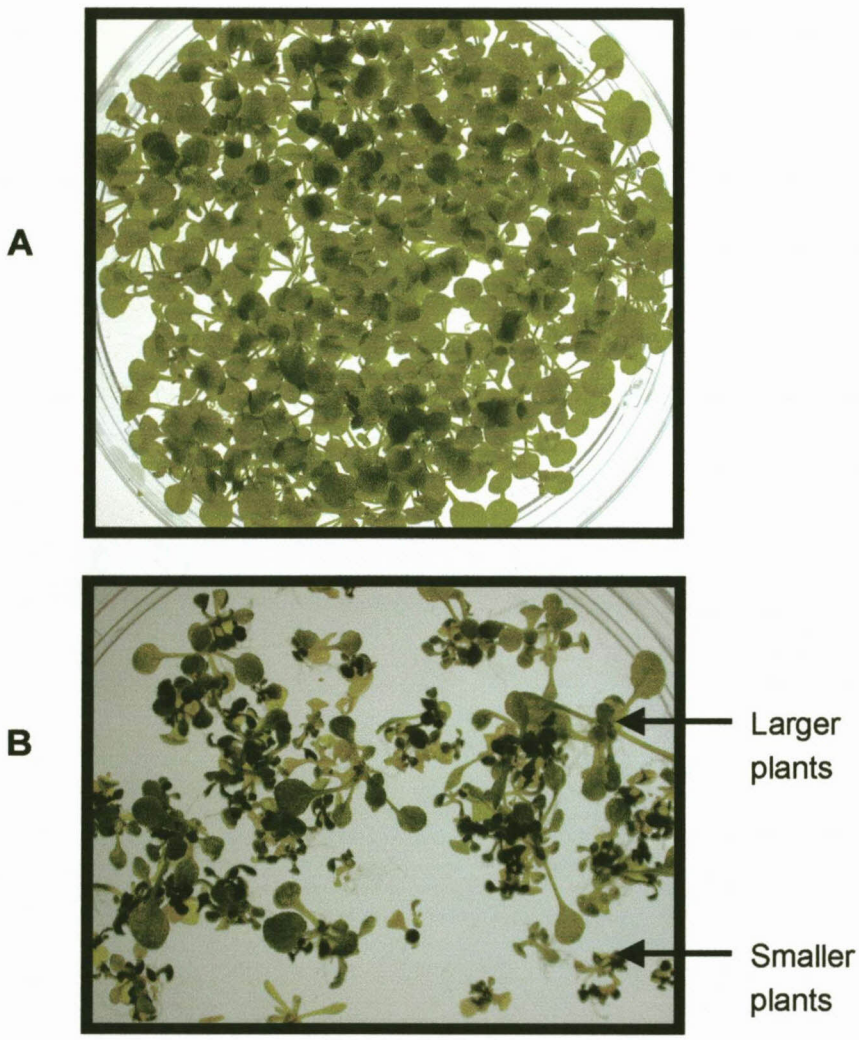


Fig. 4.22. Phenotypic differences between 2-week-old seedlings of (A) wild-type and (B) transgenic *A. thaliana* plants.

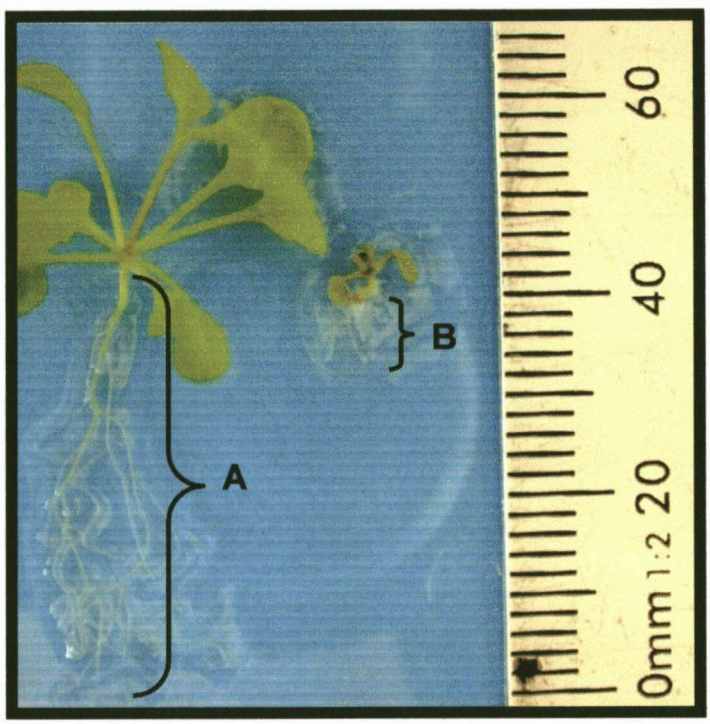


Fig. 4.23. Root length of 2-week-old seedlings of (A) wild-type and (B) transgenic *A. thaliana* plants.

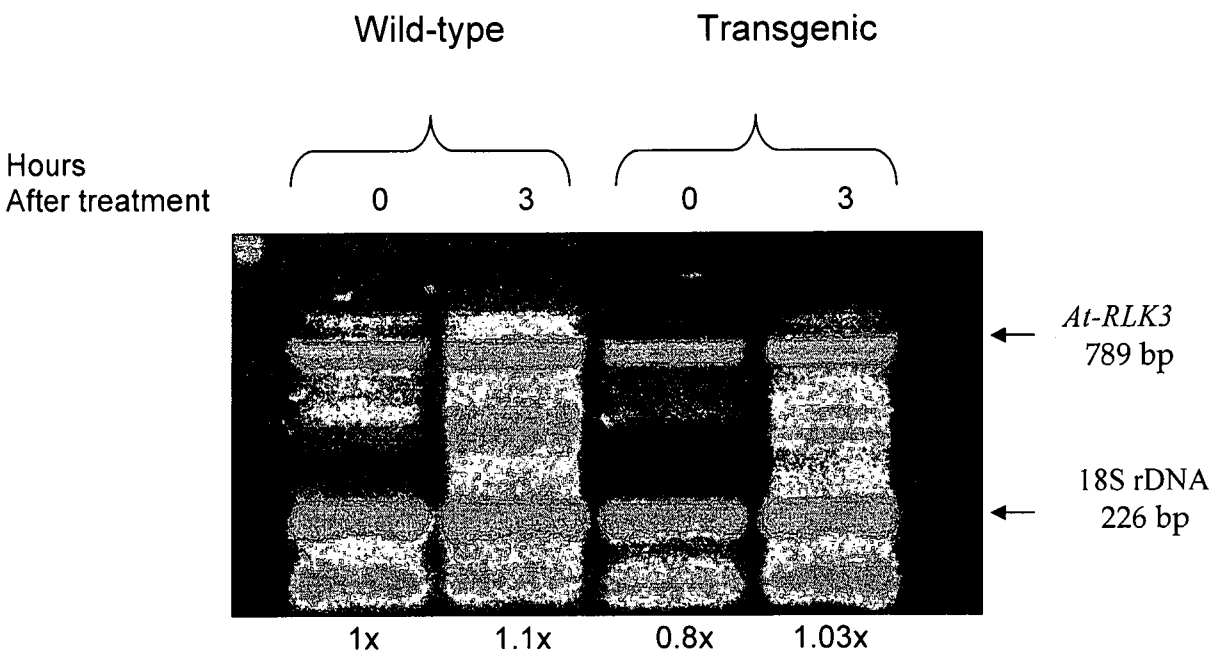


Fig. 4.24. Analysis of *At-RLK3* expression in *A. thaliana* wild-type and transgenic plants treated with DEX. The 18S rDNA was included to verify that equal quantities of RNA were used for the RT-PCR reaction. The expression level of *At-RLK3* at the different times relative to zero hours is indicated at the bottom of the figure.

It therefore seems that the transformation of the plant with the *At-RLK3* construct repressed the normal expression of the native *At-RLK3* gene. Treatment of wild-type plants with DEX had no influence on the expression of the native RLK gene. In the transgenic plant the *At-RLK3* mRNA levels increased (1.03x) after 3 hours, indicating that the DEX treatment induced the expression of the mRNA levels in the transgenic plant.

As was the case with the INA treatment, two DNA fragments amplified during the RT-PCR reaction (Fig. 4.21 (B)).

4.2.5 *At-RLK3* and β-1,3-glucanase (PR-2) expression in transgenic and wild-type plants after treatment with SA

Since it was previously found that all four plant activators activated the plant defenses, and that *At-RLK3* was implicated in the activated plant defenses, it was decided to determine whether the transgenic plant showed enhanced activation of defenses. The transgenic and wild-type plants were first sprayed with DEX to activate expression of the *At-RLK3* gene and then 3 hours later with SA.

The expression of *At-RLK3* in both the wild-type and transgenic plants were followed after treatment with DEX and SA (Fig. 4.25). When the induction profile between the two plants were compared, the levels of *At-RLK3* at the different time intervals compared to time 0, was much higher for the transgenic plant. Whereas the wild-type showed a maximum increase in expression level of 1.7, the maximum level in the transgenic plant was nearly 4 times that of time 0. It indicated that *At-RLK3* accumulation was several fold higher in the transgenic plant. The higher levels were also sustained over a longer continuous time interval in the transgenic plant than in the wild-type plant.

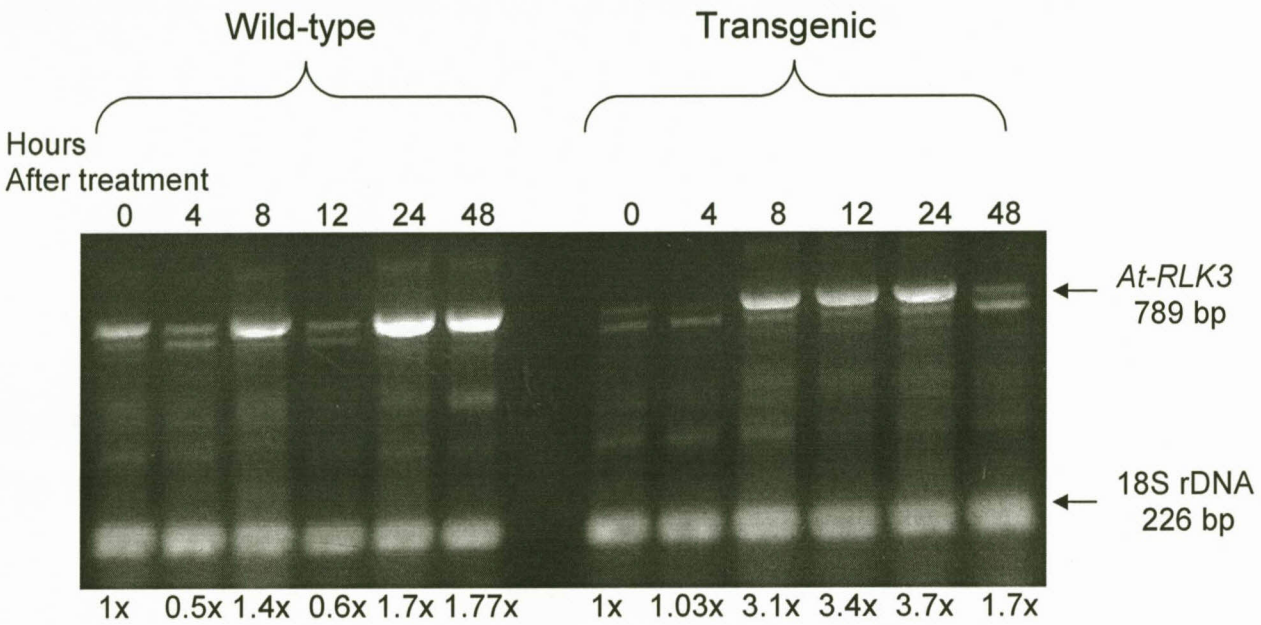


Fig. 4.25. Analysis of *At-RLK3* expression in *A. thaliana* wild-type and transgenic plants treated with DEX and SA. The 18S rDNA was included to verify that equal quantities of RNA used for the RT-PCR reaction. The expression level of *At-RLK3* at the different times relative to zero hours is indicated at the bottom of the figure.

Fig. 4.26 shows the PR-2 gene (β-1,3-glucanases) gene expression in the transgenic and wild-type plants. Similar induction profiles of expression were obtained in both plants, but the PR-2 expression level of the transgenic plants was much higher than that of the wild-type plants. PR-2 expression was induced to much higher levels (4.9x) in transgenic plant 4 h after treatment. At 8 h a slight reduction in the expression of the PR-2 could be seen in both plants and after 12 h the levels of the PR-2 gene expression increased until 48 h. In all cases, the expression of PR-2 in the transgenic plants was at least 2-fold higher than that of the wild-type plant. This clearly indicated that the enhanced levels of *At-RLK3* led to a greater activation of the plant defenses with higher levels of PR-2 expression.

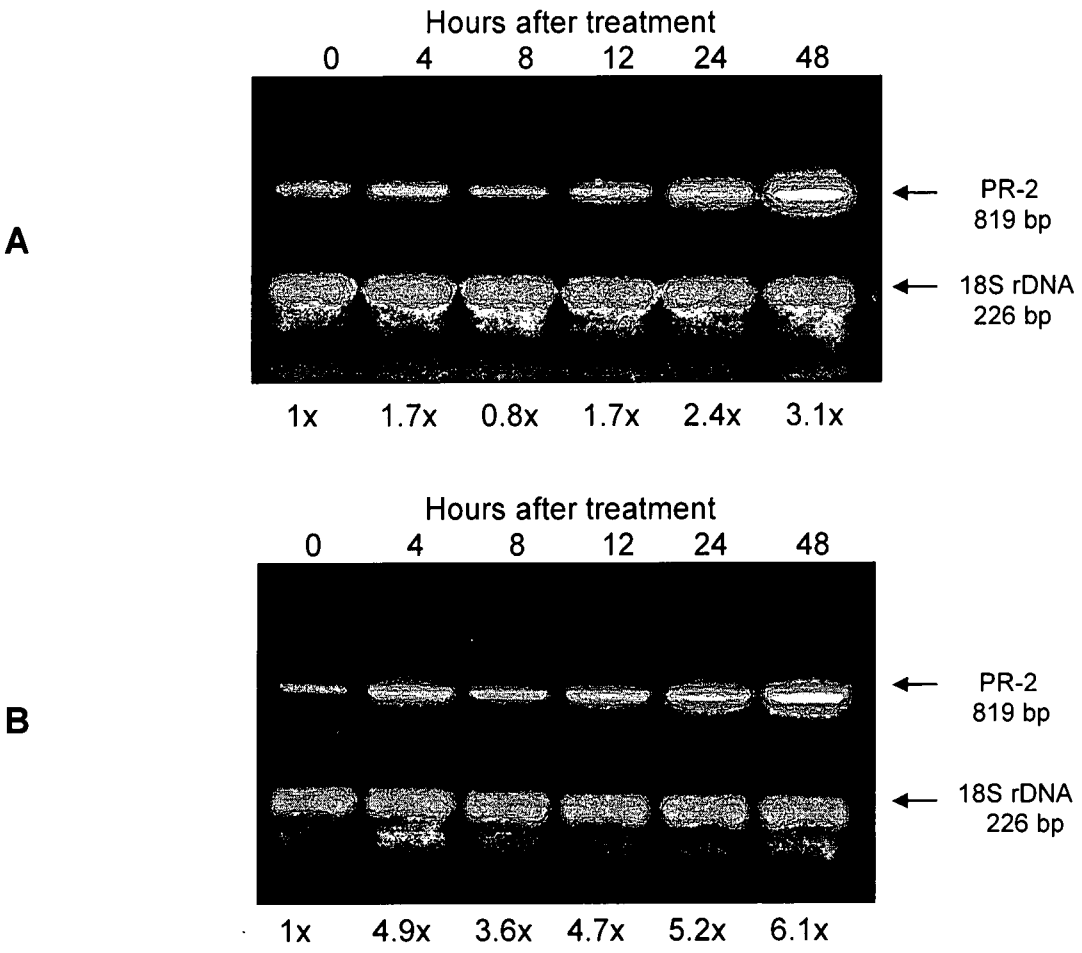


Fig. 4.26 Analysis of PR-2 (β-1,3-glucanase) expression in *A. thaliana* wild-type (A) and transgenic (B) plants treated with DEX and SA. The 18S rDNA was included to verify that equal quantities of RNA used for the RT-PCR reaction. The expression level of *At-RLK3* at the different times relative to zero hours is indicated at the bottom of the figure.

CHAPTER FIVE

Discussion

Discussion

During incompatible plant-pathogen interactions, the HR is activated (Levine *et al.*, 1994), followed by the onset of SAR (Rathjen and Moffett, 2003). This activation only occurs when the plant contains a *R* gene product that recognizes the avr protein from the pathogen. In the absence of a functional *R* gene or avr gene product, no recognition occurs. The result is infection of the host plant.

In tomato the Pto kinase acted as a receptor for the AvrPto bacterial-derived elicitor and requires a LRR-containing protein, Prf, to function (Tang *et al.*, 1999). In other cases, proteins such as Cf9 and Cf4 act as components of the receptor for the recognition of Avr9 and Avr4 fungal peptide elicitors (Hammond-Kosack and Jones, 1996; Blumwald *et al.*, 1998). A number of general peptide elicitors have been characterized, but their receptor counterparts have not yet been identified. Therefore the identification of receptors involved in the recognition of external signals is of great interest.

RLKs is one of the classes of *R* genes that are implicated to be involved in the signal transduction pathways of plants. They perceive the external stimuli, transduce the signal across the plasma membrane to the nucleus through phosphorylation cascades leading to the expression of appropriate target genes (Walker, 1994). *At-RLK3* appears to be a member of a new family of RLKs, possibly involved in plant defense (Czernic *et al.*, 1999). *At-RLK3* shared all the regions characteristic of RLKs described in plants, although the features of its extracellular domain could not fit into any described groups of RLKs. Since the expression of the gene was induced by SA (Czernic *et al.*, 1999), it was also possible that the protein could be involved in the perception of other plant activators.

Plant activators such as SA, BTH, ASA and INA share common structural features between each other, including an aromatic ring with a substituted carboxyl group (Fig. 2.1). Therefore, it is possible that this common feature cause them to be recognized by the same receptor and that their mechanisms of the defense activation might be similar.

This study was performed to determine whether At-RLK3 plays a role in the perception of the four different plant activators. The activation of the defense response was determined by measuring β-1,3-glucanase enzyme activity over a longer time period. Fluctuations in β-1,3-glucanase polypeptide levels were also measured.

The β-1,3-glucanase enzyme activity and the At-RLK3 RNA transcripts between the two time intervals after treatment was expected to give a more or less similar induction level at 2 and 4 h after application, but different values were found in some of the treatments, such as SA (Fig. 4.6, 4.9), ASA (Fig. 4.10, 4.13) and INA (Fig. 4.18, 4.21). These variations could be attributed to differences in treatment, plant age and spraying conditions. The most important aim of the study was however to study the activation pattern of both β-1,3-glucanase activity and gene expression. Therefore the differences found between the two treatments were not considered that critical and in each case the later time intervals were compared with time 0.

The treatment of *A. thaliana* with SA, ASA, BTH and INA led to the activation of β-1,3-glucanases (Figs. 4.6, 4.10, 4.14 and 4.18). The β-1,3-glucanase enzyme activity in the SA treated plants remained high for 24 h and in the ASA for 48 h before it decreased (Figs. 4.6 (A) and .4.10 (A)) The β-1,3-glucanase activity of the BTH treated plants remained high until 48 h after treatment before the activity decreased (Fig. 4.14 (A)).

INA treatment on the other hand led to higher β-1,3-glucanase activity that remained higher than that of time 0 for the whole time course (Figs. 4.18). This clearly indicated that all four activators effectively activated a defense response in the plant. The results for the SA and INA treatments correlated very well with results obtained by Uknes *et al.,* (1992).

They found that PR-2 (β-1,3-glucanases) gene expression was induced within 24 h after treating *A. thaliana* with SA and INA. The gene expression also decreased at 96 h after treatment with SA, but remained high for the INA treatment. Lawton *et al.* (1996) illustrated that treating *A. thaliana* plants with BTH resulted in the accumulation of PR-2 transcripts within 4-8 h after application which reached the highest level at 24 h after treatment before it again decreased.

While SA, BTH and INA showed an increase in β-1,3-glucanase activity immediately after treatment, ASA had a delayed increase after an initial decrease in activity. This delayed increase could be attributed to the hydrolyzation of ASA to the biological active SA in the plant tissue (Klessig and Malany, 1994).

The accumulation of the β-1,3-glucanase polypeptides was studied in order to determine whether defense activation was accompanied by increased gene expression. This would indicate at what stage the activation of the defenses was relayed to transcriptional level.

The number of β-1,3-glucanase polypeptides detected ranged from four to six, with the sizes corresponding very well with that published in the Genbank data base. It was found that the majority of polypeptides were constitutively expressed at equal levels in the SA, BTH, ASA and INA treated plants. SA and BTH treatments did however resulted in the synthesis of new polypeptides 48 h and 96 h after treatment (Figs. 4.7 (A) and 4.15 (A)).

This coincided with a decrease in β-1,3-glucanase enzyme (Figs 4.6 (A) and 4.14 (A)) These newly synthesized polypeptides were probably needed to maintain this activated defense response state, leading to their synthesis.

On the other hand, ASA treatment caused a slight decrease in polypeptide concentration at 72 h, but the enzyme activity at that specific time remained high (Fig. 4.11 (A)).

Czernic *et al.* (1999) found that SA application resulted in a rapid induced expression of *At-RLK3.* It is therefore possible that this enzyme might play a crucial role in the very early signal transduction events after treatment. To determine whether *At-RLK3* play a role during the signal events after application of plant activators, the attention shifted to the earliest activation of β-1,3-glucanases after treatment. During this early event, the signal must be recognized, perceived and transduced to the inside of the cells to activate the defense response.

SA, BTH and INA showed a very early transient activation of the defense response. The earliest activation caused by SA, BTH and INA treatment (Figs. 4.6 (B), 4.14 (B) and 4.18 (B) was within 15 min while ASA treatment led to activation 30 min after treatment. (Fig. 4.10 (B)). This activation of β-1,3-glucanases had a small effect on the expression of the β-1,3-glucanase polypeptides, since minor differences in polypeptides were visible (Figs. 4.7, 4.11, 4.15 and 4.19) This indicated that upon treatment, the presence of the plant activator is recognized, the signal relayed into the cell where a possible pool of inactive proteins already present, were activated. It is only later on that gene expression was induced.

The expression of *At-RLK3* was then investigated to determine its involvement in the signal transduction within these very short time intervals.

It was expected that the *At-RLK3* levels of both the longer and shorter time intervals at time 0 should be similar. In some treatment it was not the case. It was therefore decided that the induction profiles were again compared relative to time 0 of each time study. It was found that *At-RLK3* mRNA accumulated rapidly within *A. thaliana* treated with SA, BTH, ASA and INA (Figs. 4.9, 4.13, 4.17 and 4.21).

At-RLK3 expression in the SA treated plants showed a rapid increase at 15 min after treatment. It decreased after 30 min, but the transcript level increased again between 60 and 120 min after application (Fig. 4.9 (B)). These results correlated with results found by Czernic *et al.,* (1999) where they found that induced *At-RLK3* gene expression in *A. thaliana* was observed between 30 min and 1 h after treatment and remained high for up to 4 h after treatment.

ASA and BTH induced the expression of *At-RLK3* rapidly within 15 min after application (Figs. 4.13 (B) and 4.17 (B)), while after INA treatment, *At-RLK3* expression increased even earlier, 5 min after application (Fig. 4.21 (B)). Results obtained from experiments where *At-RLK3* expression was investigated over longer time intervals confirmed the early expression of *At-RLK3* i.e. induction within 2 h after treatment with these three plant activators. This rapid accumulation of *At-RLK3* mRNA clearly indicated that *At-RLK3* must play a crucial and very early role in the perception of these plant activators.

The specific signal responsible for the induction of the defense response in *A. thaliana* after treatment with SA, BTH, ASA and INA still has to be identified. The question remained whether At-RLK3 was the primary receptor through which the defense response was activated or whether other receptors co-operated via several interconnected pathways. Tanaka *et al.* (2003) found that flagellin from *Acidovorax avenae* induced the expression of defense genes in rice.

Flagellin was however not the sole factor responsible of inducing the rice defense system, but another signal was also involved. It is known that one of the earliest responses after host plant recognition is the oxidative burst where the ROS levels rapidly increase (Mehdy, 1994).

Other rapid responses include the hypersensitive cell death, crosslinking of cell wall proteins, the activation of protein kinases, the production of various plant protectants and the expression of defense genes (Levine *et al.*, 1994). Therefore, further analysis of the earliest activation of the defense responses was done by investigating the accumulation of H_2O_2. According to Tanaka *et al.* (2003), H_2O_2 generated during the interactions between plants and incompatible pathogens could be involved in the activation of defense responses.

Chen *et al.* (1995) found that an increase in endogenous SA levels correlated with the resistance of tobacco to TMV and the induction of defense related genes. They found that SA and its biological active analogues inhibited catalase which led to elevated levels of cellular H_2O_2. This higher H_2O_2 levels induced PR-1 gene expression. This showed that H_2O_2 perhaps acted as a second messenger in the activation of defense responses.

In this study with SA, ASA and INA treatments an initial decrease in H_2O_2 levels was observed, while with BTH treatment the H_2O_2 level increased immediately (Figs. 4.8, 4.12, 4.16 and 4.20). Despite showing an initial decrease, an oxygen burst was visible in SA, ASA and INA treated plants. This transient increase occurred after 15 min in the SA treated plants, after 60 min in the ASA treated plants and after 30 min in the INA treated plants. Also significance was the fact that these four H_2O_2 level profiles were very similar. The increase in H_2O_2 levels in tobacco plants after BTH treatment correlated with results of Wendehenne *et al.* (1998), were they found that BTH inhibited catalase and ascorbate peroxidase (APX).

There are two pathways involved in the breakdown of H_2O_2 (Chen *et al.*, 1995). H_2O_2 can be converted to H_2O and O_2 by catalase, a process that can be inhibited by SA and its analogues. Secondly H_2O_2 can be broken down by specific peroxidases.

The transient increase in H_2O_2 levels after all four treatments could be the result of the inactivation of catalase. Since Chen *et al.* (1995) found that the elevated levels of H_2O_2 after SA and INA treatment led to the induction of PR-2 gene expression, it was speculated that H_2O_2 also acted as a second messenger in the activation of defense response after treatment with these four plant activators.

The fact that H_2O_2 levels rose after *At-RLK3* expression was already induced, led us to suggest that H_2O_2 did not act as the primary signal for the activation of defense responses. Also, the increased H_2O_2 levels after the different treatments did not seem to influence the expression of *At-RLK3*. It is thus believed that H_2O_2 levels rose because of the involvement of *At-RLK3* in a signaling event, and that it acted downstream of *At-RLK3*.

Since At-RLK3 seems to play a role in the activation of the defense responses after activation treatment its role was further analyzed in *At-RLK3* transgenic *A. thaliana* plants overexpressing *At-RLK3*. Should At-RLK3 play an important role, elevated At-RLK3 levels should allow the plant to react either faster after treatment, or to react in a stronger manner. After transformation, the F_1 plants were self-pollinated to produce the F_2 generation. When planted on hygromycin plates, two different phenotypes were present. The one resembled the wild-type, while with the other phenotype, the plants were very small and stunted, dark green and root growth was severely inhibited. The difference in phenotype could be explained by analyzing the possible genotypes. The transformed plant will be heterozygotic for the transformed gene, for instance RLK3/-. After self pollinating the F_1, a F_2 consisting of RLK3/RLK3, RLK3/- and -/- will be found.

Since the number of normal plants found, were the least in number, one can assume that the genotype will be -/-. The affected plants will therefore be either RLK3/RLK3 or RLK3/-. We therefore concluded that the presence of the transgene in some way led to the altered phenotypes, by possibly disrupting some crucial genes, such as those involved in gibberellic acid synthesis or negatively regulating the expression of At-RLK3.

This was confirmed when the expression levels of *At-RLK3* were determined in wild-type and the transgenic plants. It was found that in the transgenic plants *At-RLK3* expression was lower compared to that of the wild-type plants. So, in some way, the transfer of the *At-RLK3* gene to wild-type plants, inhibited expression of the wild-type gene, which then led to the altered phenotype. A decreased expression of *At-RLK3* therefore seems to influence normal plant development.

These phenotypic differences could be compared with phenotypes found in *bri*1 mutant plants (Li and Chory, 1997). They suggested that brassinosteroids play a key role in *A. thaliana* development. Absence of this RLK in *A. thaliana* led to severe developmental alterations with phenotypes similar to that found in this study. Similarly, the reduction of TIP49a mRNA levels also led to morphological and developmental defects in *A. thaliana*, including small rosettes and defective leaves (Holt III *et al.*, 2002). Since TIP49a is involved in plant defense, these results indicated that the development of a plant and its reaction to pathogens must share common proteins, most probably those involved in signal events. These results indicated another possible role for *At-RLK3*, not only in plant defense, but also in the development of the plant.

To determine whether *At-RLK3* overexpression leads to higher activation of the defense response in *A. thaliana*, the transgenic plants were analyzed after treatment with SA.

The activation of defense responses was measured through the analysis of PR-2 expression (Uknes *et al.*, 1992). In the wild-type plants treatment with SA resulted in the induction of PR-2 synthesis 4 h after treatment. After a decrease at 8 h, the expression again increased to reach a maximum level at 48 h (Fig. 4.26).

A similar induction profile was obtained within the transgenic plants. What was however clear, was that at all times, the level of PR-2 expression relative to that of 0 h, was at least 2 times higher in the transgenic plants compared to the wild-type plants. This enhanced expression of PR-2 correlated with enhanced expression of *At-RLK3* in the transgenic plants. This clearly indicated that the enhanced levels of *At-RLK3* led to a bigger activation of the plant defense responses after SA treatment.

To conclude, the role of *At-RLK3* in the perception of SA, BTH, ASA and INA is unknown, but in this study evidence was provided that *At-RLK3* plays a key role in the activation of defense responses. It was also demonstrated that the overexpression of *At-RLK3* in *A. thaliana* treated with SA leads to the amplification of the defense response in the transgenic plants. Since the defense activating role of ASA, BTH and INA corresponded to a large degree with SA implicated could also be effective in amplifying the activation of the defenses in the plants overexpressing *At-RLK3*. Taking this possibility into account plants overexpressing At-RLK3 might have an important application in agriculture. This gene might help to improve the resistance in crops after spraying with one of these plant activators, rendering them more resistant to pathogens.

CHAPTER SIX

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SUMMARY

Summary

The involvement of a receptor-like protein kinase, *At-RLK3* in the response of *A. thaliana* to four different plant activators was analyzed in this study. The activators were salicylic acid (SA), benzothiadiazole (BTH), acetylsalicylic acid (ASA) and 2,6-dichloroisonicotinic acid (INA). The activation of the defense response was determined measuring both the activity and expression on protein level of β-1,3-glucanases. SA, ASA, BTH and INA treatments showed an early transient induction of β-1,3-glucanase activity after treatment. On protein level the majority of polypeptides were constitutively expressed in the SA, BTH, ASA and INA treated plants, indicating that a pool of inactive β-1,3-glucanase proteins already present in the cells, were activated upon treatment. It was only in the SA and BTH treated plants that newly synthesized polypeptides were visible during the later stages of the time studies.

Once the activation of β-1,3-glucanases was established, the possible involvement of *At-RLK3* in the activation of plant defense mechanisms by plant activators, was investigated. It was found that *At-RLK3* mRNA accumulated rapidly within plants treated with SA, BTH, ASA and INA. The results indicated a correlation between the onset of SAR in the plant and the expression of the *At-RLK3* gene.

Hydrogen peroxide acts as an early marker for the activation of defenses. The fact that H_2O_2 levels rose after *At-RLK3* expression was already induced, led us to suggest that H_2O_2 did not act as the primary signal that binds to *At-RLK3* but that it acts downstream of *At-RLK3.* All four treatments did however show the characteristic oxygen burst shortly after application. These results however need to be substantiated by repeating each experiment.

In addition to being involved in defense signalling, a role for *At-RLK3* in plant development was also suggested. It was found that the transfer of a second *At-RLK3* gene to wild-type plants inhibited the expression of the native gene. These lower *At-RLK3* levels led to an altered phenotype of the transgenic plant, implicating a possible role for *At-RLK3* in the development of the plant.

Elevated levels of *At-RLK3* in transgenic plants led a higher induction of PR-2 expression after treatment with salicylic acid. A potential application for *At-RLK3* in important crops when treated with plant activators could be envisaged.

Key words:

***Arabidopsis thaliana*, plant activators, induction of defense response, receptor-like protein kinase (*At-RLK3*).**

OPSOMMING

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Tydens die studie is die betrokkenheid van die reseptorproteïenkinase (*At-RLK3*) by die respons van *Arabidopsis thaliana* op bespuiting met verskillende plantaktiveerders bestudeer. Die plantaktiveerders was salisielsuur (SA), bensotiadiasool (BTH), asetielsalisielsuur (ASA) en 2,6-dichloro-isonikotiensuur (INA). Die aktivering van die verdedigingsrespons was bevestig deur beide β-1,3-glukanase-aktiwiteit en proteïenvlakke te ondersoek. SA, ASA, BTH en INA het almal vroeë kortstondige induksies van β-1,3-glukanase-aktiwiteit getoon. Op proteïenvlak is die meeste β-1,3-glukanasepolipeptiede konstitutief uitgedruk. Dit dui daarop dat behandeling met die plantaktiveerders 'n poel van reeds bestaande onaktiewe β-1,3-glukanase-ensieme waarskynlik geaktiveer het. Dit was alleenlik in die SA- en BTH-behandelings dat nuwe β-1,3-glukanasepolipeptiede tydens die latere fases van die tydstudie gesintetiseer is.

Die volgende stap was om te bepaal of *At-RLK3* 'n rol speel by die herkenning van die verskillende plantaktiveerders. Na behandeling, het al vier aktiveerders die uiting van die *At-RLK3*-geen geïnduseer. Resultate dui op 'n verband tussen die uiting van *At-RLK3* en die aktivering van die sistemiese verworwe weestand (SAR).

Waterstofperoksied akkumulasie is 'n vroeë aanduiding van die aktivering van verdediging in plante. Die toediening van al vier aktiveerders het tot 'n kortstondige toename in H_2O_2-vlakke gelei. Die feit dat die vlakke na die induksie van *At-RLK3* geenuiting gestyg het, is 'n aanduiding dat H_2O_2 nie direk aan *At-RLK3* bind nie. Dit funksioneer waarskynlik stroomaf van *At-RLK3*. Hierdie resultate sal bevestig moet word deur die herhaling van elke eksperiment.

Na die oordraging van 'n tweede kopie van *At-RLK3* na *Arabidopsis*, is gevind dat die transgeniese plante minder At-RLK3 gevorm het. Dit het daarop gedui dat die uiting van die oorspronklike geen deur die oorgedraagde geen gerem is. Die gevolg was dat die transgeniese plante verskeie abnormaliteite getoon het. Dit het impliseer dat At-RLK3 ook moontlike 'n rol in plant ontwikkeling speel.

SA-behandeling en die daarop volgende kunsmatige induksie van die oorgedraagde geen, is bevind dat die transgeniese plante 'n hoër uiting van PR-2 getoon het. Die aanwending van At-RLK3 om plantweerstand in belangrike gewasse te verbeter, kan in vooruitsig gestel word.

Sleutelwoorde:

***Arabidopsis thaliana*, plantaktiveerders, induksie van verdedigingsreaksie, reseptorproteïenkinase (*At-RLK3*).**