Analysis of gene expression in wheat upon treatment with a novel plant activator

by

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List of Abbreviations:

ARR	Age-related resistance
Avr	Avirulence
BABA	DL-3-aminobutyric acid
BAK1	BRI1 associated protein kinase 1
BIT	Benzisothiazole
BL	Brassinolide
BR	Brassinosteroid
BSA	Bovine serum albumin
BTH	Benzo (1, 2, 3) thiadiazole-7-corbothiotic acid S-methyl ester
CC	ComCat®
CMPA	3-Chloro-1-methyl-1*H*-pyrazole-5-carboxylic acid
CS	Castasterone
dCTP	Deoxycytidine triphosphate
DDCC	2,2-Dichloro-3, 3-dimethylcyclopropane carboxylic acid
DMPC	Dimethyl pyrocarbonate
DMSO	Dimethylsulfoxide
dNTPs	Deoxyribonucleotide triphosphates
EDTA	Ethylenedinitrilo tetraacetic acid
EST	Expressed sequence tag
Hpt	Hours after treatment
HR	Hypersensitive response
INA	2,6-dichloroisonicotinic acid
IPTG	Isopropyl-β-D-thiogalactoside

ISR	Induced systemic resistance
JA	Jasmonic acid
LRR	Leucine-rich repeat
MAPK	Mitogen activated protein kinase
MeJA	Methyl jasmonate
MeSA	Methyl-salicylate
MSB	Menadione sodium bisulphite
NBS	Nucleotide binding site
NCI	*N*-Cyanomethyl-2-chloroisonicotinamide
NO	Nitric oxide
NOS	Nitric oxide synthase
PAL	Phenylalanine ammonia-lyase
PBZ	Probenazole
PCD	Programmed cell death
PGPR	Plant growth-promoting rhizobacteria
PR	Pathogenesis related
PVP	Polyvinylpyrrolidone
R	Resistance
RLK	Receptor-like protein kinase
ROS	Reactive oxygen species
RT-PCR	Reverse transcription PCR
Rubisco	Rubilose-1,6-bisphosphate oxidase/carboxylase
SA	Salicylic acid
SAR	Systemic acquired resistance
SBP	Steroid binding protein
SDS	Sodium dodecyl sulphate
SIPK	Salicylic acid induced protein kinase

SS	Plant activator (methanol:water fraction of ComCat®)
SSH	Suppression subtractive hybridization
TIR	Toll/Interleuken1 receptor-like
Tris	Tris-hydroxymethyl aminomethane
Tween20	Polyoxyethylene sorbitanmonolaurat
WAF1	(11E,13E)-labda-11,13-diene-8α,15-diol
WIPK	Wound-induced protein kinase
WRKY	W-Box DNA binding domain
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

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Chapter 1
Introduction

1. Introduction

The agriculture industry today remains just as important as it ever was. Due to the rapidly growing world population, the need to increase yields and output of important crops remains an important objective. Yet with the focus in agriculture moving towards sustainable development and more environmentally favourable farming techniques, new approaches are necessary. Harmful pesticides and fertilizers are still being used to control the problems of plant pathogens and low yields respectively. The use of said chemicals is not very cost effective and is also harmful to the environment.

A solution for these problems is the use of molecules called plant activators that have the ability to switch on the plant's defensive arsenal against potential pathogens, without acting on the environment (Kessmann *et al.*, 1994). An added bonus for the use of plant activators is that they don't have to be applied as often as conventional pest control methods which would make for a more economically viable alternative. Some plant activators also have the added ability to improve growth and yield.

Today a number of well known plant activators have been identified like the naturally occurring products like salicylic acid (Mauch-Mani and Metraux, 1998), jasmonic acid (Turner *et al*., 2002), menadione sodium bisulphite (Borges *et al*., 2003) and harpin (Takakura *et al*., 2004) to name a few. A number of synthetic molecules that can act as plant activators have also been synthesized with some even being the active ingredient of commercial plant activator products like for instance benzo (1,2,3) thiadiazole-7-corbothiotic acid S-methyl ester or BTH. Yet with all the above being said, the knowledge of how specific plant activators mediate their end results on an intracellular level in the target crops remains limited. Wheat was specifically used during this study due to the fact that its one of the crops that has been most extensively studied with plant activators (Kogel and Langen, 2005).

Recently it has also been shown that plant activators can fall into distinct categories according to their intracellular effects. Some activators like ComCat® have the ability to induce plant defence related *PR* gene expression and also increase photosynthesis thereby increasing yield (Berger *et al*., 2004). On the other hand, the commercial product BION® containing a BTH active ingredient has been shown to be detrimental to yield whilst still inducing defence against pathogens (Heil *et al*., 2000). Thus it is important to determine the complete effect of a putative plant activator in its target crop looking specifically at plant defence against pathogens, growth, yield and stress tolerance.

The main aims of this study was to confirm field results of a novel plant activator on a molecular level thereby elucidating the effect the activator has on an intracellular level in the plant, and to identify possible genes involved in the action of the plant activator that could further explain its effect on cellular processes like photosynthesis, respiration, growth and defence against plant pathogens. By identifying genes activated or repressed after treatment, the possible mechanisms of action by the activator can be better explained and positive field trial results confirmed. The characterization of novel expressed genes could also subsequently be used in further studies

The significance of this study in the current realm of plant activator research is to contribute to the existing library of plant activator information, which can allow for comparisons with well known activators like BTH. By the use of molecular biology techniques, a complete picture of the specific plant activator could be painted along with previous results.

Chapter 2

Literature Review

2. Literature review

2.1. Introduction

In the past, pathogen infection of plants, especially economically important crops, has been a huge problem. Even today viruses, fungi, bacteria, nematodes and herbivorous insects cause astounding financial losses each year in the agricultural industry. The need to use conventional pesticides and fungicides containing environmentally harmful agents to control the problem remains higher than ever and with the focus in agricultural science moving towards sustainable development, a solution for this problem is essential.

A recent exciting development in the field of plant pathogen control has been the identification of chemical compounds that switch on the plant's own natural defense mechanism against pathogens. The inducible defense mechanisms differ from the plant's constitutive defense systems, which include mostly physical barriers and preformed anti-pathogenic compounds. These chemicals compounds are called plant activators. To be considered a plant activator, a compound has to have the following general characteristics: (1) the activator must induce resistance against the same spectrum of pathogens compared to the biological model; (2) neither the activator nor its significant metabolites should have direct antimicrobial activity and (3) chemical treatment should induce expression of the same biochemical markers as the biological model (Kessmann *et al*., 1994).

Another field related to plant activators, is known as the biocontrol of pathogens. Biocontrol can be defined as the suppression of pathogen growth with other organisms or their products (McSpadden Gardener and Fravel, 2002). Biocontrol agents include a large variety of microorganisms and their products that can activate the plant's own defense against a pathogen or act directly on the pathogen. This particular field of research is growing very rapidly and new products are developed regularly.

The challenge is to successfully incorporate these plant activators and biocontrol agents into commercial products that have a minimal negative effect on the environment. To develop activators that can achieve levels of resistance that are economically viable and that are not harmful to the crops it is applied to, is essential. A number of large pesticide companies already sell products that fall under the plant activator/biocontrol category, although the number of available products is still very small when compared to the traditional pesticides that act directly on the pathogen.

Before the different plant activators are discussed, it is necessary to first describe the basic principles of plant–pathogen interactions.

2.2. Overview of plant defense interactions

Plant defense responses can be divided into two categories, namely passive (constitutive) and active (induced) defense (Johal *et al*., 1994). Constitutive defense mechanisms are always present in the plant and plants use it to deter potential pathogens. When a pathogen overcomes these constitutive defensive barriers and compounds, active resistance can be induced against the pathogen infection.

Induced resistance can be triggered in the plant in three different ways: (1) by a necrotizing pathogen infection, (2) by treatment of the plant with plant activators and (3) by colonizing the rhizosphere with selected plant growth-promoting rhizobacteria (PGPR). The induced resistance response present in the first two examples is called Systemic acquired resistance (SAR), while the third trigger leads to Induced systemic resistance (ISR) (Pieterse *et al.*, 1996). SAR and ISR can be induced by chemicals and pathogens or biocontrol organisms (Pieterse *et al.,* 1996; Kessmann *et al*., 1994), but treatment with plant activators does not usually damage the plant.

Another more direct and localized manner of pathogen resistance by plants is based on elicitor-receptor interactions. An elicitor is defined as any compound

that can activate the defense system of a plant (Montesano *et al.*, 2003). There are two classes of elicitors, namely race specific and general elicitors. They have the ability to trigger the defense response in both host and non-host plants (Montesano *et al.*, 2003). General elicitors induce a resistance response similar to resistance (*R)*-gene mediated defense by interacting with a variety of different receptors located on the plant cell surface. With race-specific elicitors, a *R* gene product from the plant interacts either directly or indirectly with a specific corresponding avirulence (*Avr*) gene product from the pathogen (Ellis *et al.,* 2000). This interaction leads to the activation of a signal transduction cascade that most of the time results in a hypersensitive response (HR). The HR is characterized by the death of the infected cell through a phenomenon called programmed cell death (PCD), but also leads to SAR in uninfected parts of the plant (Greenberg, 1997; Nimchuk *et al.*, 2003).

2.2.1. Constitutive plant defense mechanisms

Plants posses an arsenal of preformed defense mechanisms against potential pathogens. These passive resistance mechanisms can be divided into two groups, namely physical barriers and preformed chemical molecules (Johal *et al*., 1995; Keen, 1999). Physical barriers include the cell wall and cuticle that prevent certain pathogens like bacteria to enter the host (Johal *et al.*, 1995). Actin microfilaments in the plant cytoskeleton has been shown to play a major role in combating fungal penetration of the plant cell (Mysore and Ryu, 2003).

The chemical compounds that constitutively exist in plants are mostly secondary metabolites like the alkaloids and terpenoids (Wittstock and Gershenzon, 2002). These molecules are almost exclusively toxins that have negative effects on the pathogen or herbivorous invader. Because of the experimental difficulty in observing the constitutive chemical defense, the exact mechanisms of most constitutive plant toxins are largely unknown.

There are however some molecules that have been relatively well studied and their modes of action deciphered. Included are (a) saponins that act on the

cellular membranes of pathogens and herbivores (Osbourn, 1996), (b) tomatine (a steroid glycoalkaloid) that has intrinsic antifungal activity as well as a detrimental effect on certain insects (Costa and Gaugler, 1988), (c) cardenolides that inhibit ion channel functioning (Wittstock and Gershenzon, 2002) and (d) hydrogen cyanide that has a detrimental effect on the respiration of pathogens and herbivores (Zagrobelny *et al*., 2004).

It has been observed that some of these constitutively produced toxins and certain preformed peptides may also have the ability to act in a synergistic fashion (Wittstock and Gershenzon, 2002). An example of this was found when the combined toxic effect of two essential oil constituents of *Thymus vulgaris, trans*-anethole and thymol, on larval growth exceeded the effects of the oil constituent's additive effect thus indicating a synergistic relationship (Hummelbrunner and Isman, 2001).

These are only a few molecules of the vast array that has been discovered to date. As this review and project deals exclusively with induced plant defense mechanisms, more detailed information will be supplied on these mechanisms.

2.2.2. Induced local defense responses

R-gene mediated defense is probably the best-studied plant defense system against pathogens (Nimchuk *et al*., 2003). General elicitor mediated defense is a rare phenomenon and is not well characterized, but the only difference with the former is that the receptors that recognize the general elicitors, are different (Montesano *et al*., 2003). In the case of general elicitors no gene-for-gene interaction takes place and an *R*-gene corresponding to the pathogenic *Avr*-gene is not needed (Montesano *et al*., 2003).

During the pathogenic infection of a plant, an *Avr*-gene product from the pathogen is recognized by a specific corresponding R protein (Nimchuk *et al*., 2003). This recognition is mediated either through the direct or indirect interaction of the two proteins (Ellis *et al*., 2000). While the protein encoded by

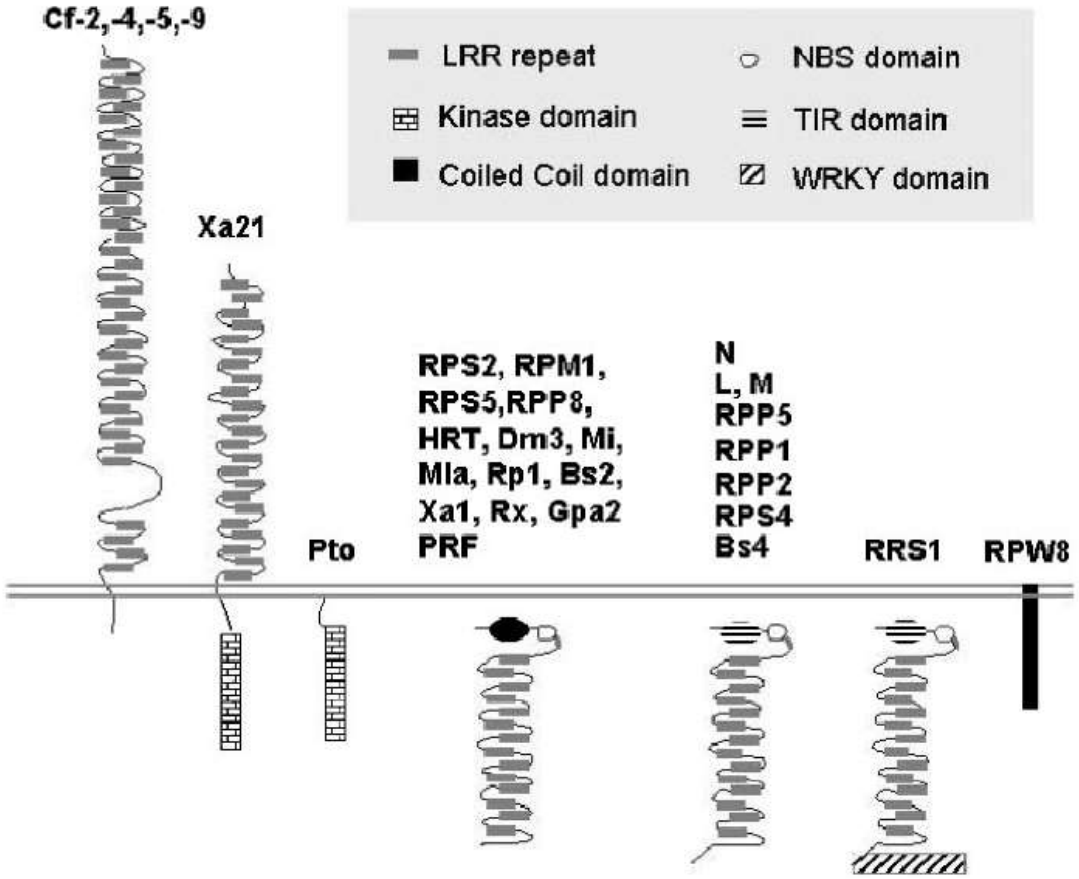
the *Avr*-gene does not necessarily have to be involved in the virulence of the pathogen, many *Arv*-gene products have no clear function in the pathogen itself (Keen, 1999). Certain *Avr*-gene products can also increase the production of secondary plant molecules that can act as additional elicitors of the defense response (Ellis *et al*., 2000). R proteins on the other hand are usually receptor proteins that are located extracellularly or intracellularly (Nimchuk *et al*., 2003). The recognition of the pathogen Avr-product is a crucial step, since without the *R*-gene the plant is not resistant against the pathogen.

There are a number of different classes of resistance proteins (Fig. 2.1). Leucine-rich repeat (LRR) motifs are present in most *R*-genes and are highly variable both in number and organization. The LRR regions serve the important function of helping the receptor protein to recognize and bind ligands originating from the continually evolving pathogen (Romeis, 2001; Nimchuck *et al.*, 2003;). Other important domains that have been identified include the Toll/Interleuken1 receptor-like (TIR) domain, nucleotide binding site (NBS) and W-box DNA binding domains (Nimchuck *et al*., 2003).

The important similarity between all the different classes of these receptors is that they are all responsible for the initial recognition of the pathogen. When *R*-gene encoded protein kinase receptors are bound to the cell membrane, they are called receptor-like protein kinases (RLKs) (Shiu *et al.*, 2004). Plant RLK's are most likely to function through perception of signals by their extracellular domains, whilst the intracellular kinase domain amplifies the signal inside the cell (Shiu *et al.*, 2004). A large number of RLK's have thus far been identified in various plants and are thought to play key roles in diverse actions such as plant-pathogen interactions, hormone signaling and plant growth and metabolism (Shiu *et al.*, 2004).

Once the elicitor has been recognized by the receptor, a number of processes take place. One of the earliest reactions after recognition is the production of reactive oxygen species (ROS) (Torres *et al*., 2006). These ROS include H_2O_2, O_2^- and nitric oxide (NO).

Extracellular



Intracellular

Figure 2.1 – Classes and conserved domains of plant R proteins. The different domains present in R proteins are indicated schematically. Above each class a number of examples of such proteins are indicated (Nimchuck *et al*., 2003).

ROS may have direct anti-microbial properties, but also initiates the cross-linking of proline-rich cell wall proteins (Baker and Orlandi, 1995). This cross-linking strengthens the cell wall and makes it difficult for the pathogen to penetrate the cell and prevent the digestive enzymes of the pathogen to have their full effect.

The chief source of H_2O_2 and O_2^- during plant-pathogen interactions is the membrane bound NAPDH oxidase complex, although pH dependent cell wall bound peroxidases, amine oxidases and oxalate oxidases are also proposed to generate H_2O_2 (Desikan *et al*., 2001; Vranová *et al*., 2002). NO produced during plant defense processes is principally synthesized by nitric oxide synthase (NOS) (Neill *et al*., 2002).

Over the last years the important signaling properties of ROS in plant defense have been demonstrated during various experiments (Desikan *et al*., 2001; Orozco-Cárdenas *et al*., 2001; Neill *et al*., 2002; Torres *et al*., 2006). ROS involved in these signal transduction pathways are principally H_2O_2 and NO. O_2^- is not recognized as an effective signaling molecule due to its short life span (Neill *et al*., 2002; Vranová *et al*., 2002). Signaling mediated by H_2O_2 is extremely complex, with abundant cross regulation occurring. Microarray analysis of *Arabidopsis* cell suspension cultures treated with H_2O_2, revealed that H_2O_2 induces the expression of 113 genes and repressed the expression of a further 62 (Desikan *et al.*, 2001). Included were a number of genes encoding signaling components as well as other proteins involved in a variety of cellular functions including HR, PCD, defense (*PR1* and glutathione-S-transferase), cell wall cross-linking, stomatal closure, DNA and protein degradation and lipid peroxidation (Desikan *et al.*, 2001; Neill *et al*., 2002; Vranová *et al*., 2002).

One of the interesting genes induced by H_2O_2 is calmodulin (Desikan *et al*., 2001; Neill *et al*., 2002). Calmodulin regulates intracellular Ca^{2+} levels, while NADPH oxidase is regulated by Ca^{2+}. This suggests that H_2O_2 signaling regulates its own production. H_2O_2 also induced the expression of various heat shock proteins, indicating it's wide roll in signalling processes (Desikan *et*

al., 2001). The important signaling role of H_2O_2 was reiterated in this study when H_2O_2 induced the expression of various genes encoding signaling related proteins. Included were genes encoding protein tyrosine phosphatases, copper-binding proteins, transcription factors, hormone inducible genes and various mitogen activated protein kinases (MAPK's) (Desikan *et al*., 2001). H_2O_2 is proposed to be a secondary messenger in wounding processes and plant activator treatment as was indicated by systemin and methyl jasmonate treatment of tomato plants (Orozco-Cárdenas *et al*., 2001).

NO plays an important role in early signaling events during plant defense against pathogens. NO synthesis is activated very quickly in plants under pathogen attack and is often synthesized in conjunction with H_2O_2 under these conditions (Neill *et al.*, 2002). NO signaling is proposed to be mediated through cGMP synthesis that eventually results in the induction of defense related genes like *PAL1*, *PR-1*, MAPK and glutathione-S-transferase genes (Neill *et al.*, 2002). It is also suggested that NO and H_2O_2 act synergistically during the oxidative burst due to pathogen infection and plant activator treatment (Neill *et al.*, 2002; Vranová *et al*., 2002).

Another early event in the plant defense response mediated in part by the action of ROS, is the change in ion levels. This include Ca^{2+} and H^+ influx and Cl^- and K^+ efflux (Dixon *et al.*, 1994). This changes the pH of the cellular interior and probably plays a role in signal transduction by activating protein kinases and phosphatases which can in turn activate ROS generating enzymes like peroxidases bound to the cell wall (Vranová *et al*., 2002).

Other reactions that happen shortly after elicitation are protein phosphorylation and dephosphorylation mostly initiated by the *R*-genes. This, together with ROS signaling, leads to the activation of MAPK cascades that play important roles in downstream signal transduction (Zhang and Klessig, 2001). More events that also happen shortly after recognition is the accumulation of salicylic acid (SA) and jasmonic acid (JA) and the activation

of calcium dependant protein kinases and calmodulin by higher intracellular calcium levels that are all involved in signaling processes (Romeis, 2001).

Once all the signaling events have taken place, the end result is changes in the expression of defense related genes (Nimchuck *et al.,* 2003). The individually expressed genes include pathogenesis related (*PR*) genes, as well as genes that help with the synthesis of antimicrobial compounds such as phytoalexins (Melchers and Stuiver, 2000). The end effect of the expressed genes is the HR. Some of the expressed proteins like the PR proteins, migrate to the cell wall to act directly on the pathogen (Fritig *et al.,* 1998). Examples of PR proteins are beta-1-3-glucanases and chitinases which act directly on the cell walls of fungal pathogens (Fritig *et al*., 1998). Once the HR has taken place, SAR can also be induced through as yet unclear signaling events (Sticher *et al.,* 1997; Romeis, 2001). The HR eventually leads to the destruction of the infected cell (PCD) that prevents the pathogen from spreading and infecting other parts of the plant (Solomon *et al.,* 1999).

PCD is achieved through the action of various different molecules and enzymes (Solomon *et al.,* 1999). This process is characterized by Ca^{2+} flux, chromatin condensation, DNA fragmentation into smaller fragments and further to nucleosomal ladders by endonucleases, protease activation, membrane damage and cell shrinking (Greenburg, 1997; Solomon *et al.*, 1999; Vranová *et al*., 2002). H_2O_2, and to a lesser extent NO, play a crucial role in the eventual death of the infected cell either directly by degrading DNA and proteins or indirectly through signaling processes (Neill *et al*., 2002; Vranová *et al*., 2002). Blocking H_2O_2 production through various inhibitors or the overexpression of a catalase gene that degrades H_2O_2, has been shown to have detrimental effects on PCD in soybean suspension cultures and *Arabidopsis* plants (Solomon *et al.*, 1999; Vranová *et al*., 2002).

Experimentally it has been proven that in soybean cells where PCD has been triggered by oxidative stress, plant cysteine proteases play an instrumental part in mediating the death of the cell (Solomon *et al.*, 1999). The authors suggested a novel role for plant protease inhibitor genes to modulate PCD. JA

synthesis during herbivore feeding induced higher levels of the inhibitor cystatin which prevented unnecessary cell death by inhibiting cysteine proteases (Solomon *et al.*, 1999). In contrast, application of SA that is involved in normal microbial pathogen-related defense, repressed the production of the proteinase inhibitor and resulted in increased sensitivity to H_2O_2 and death of the cell at lower H_2O_2 concentrations (Solomon *et al.*, 1999).

2.2.3. Systemic Acquired Resistance

Defense mechanisms that are activated upon contact with a necrotizing pathogen or chemical activators are termed induced or acquired resistance. When this resistance is expressed systemically in other parts of the plant distant from the primary infection site, this phenomenon is called SAR (Fig. 2.2).

SAR gives longer lasting resistance to the plant and also acts upon a wider range of pathogens than the normal pathogen-induced local resistance (Sticher *et al*., 1997). Up to date, SAR has been identified in a host of plant species, including dicots and monocots (Sticher *et al*., 1997; Mauch-Mani and Metraux, 1998). Although many areas regarding SAR still remain unclear, there has been a large amount of research done on this subject during the last decade.

The induction of resistance in parts of the plant distant from the primary infection site is postulated to be the result of the translocation of an as yet unknown systemic signal (Sticher *et al.*, 1997). A number of molecules have been proposed that can possibly act as systemic signals or secondary signals in SAR.

One of the best studied possible signaling molecules in SAR, is SA. Various experiments with mutant plants have shown the importance of SA in SAR (Sticher *et al*., 1997; Mauch-Mani and Metraux, 1998). Plants containing the overexpressed *nahG* gene that produces SA-hydroxylase showed lower

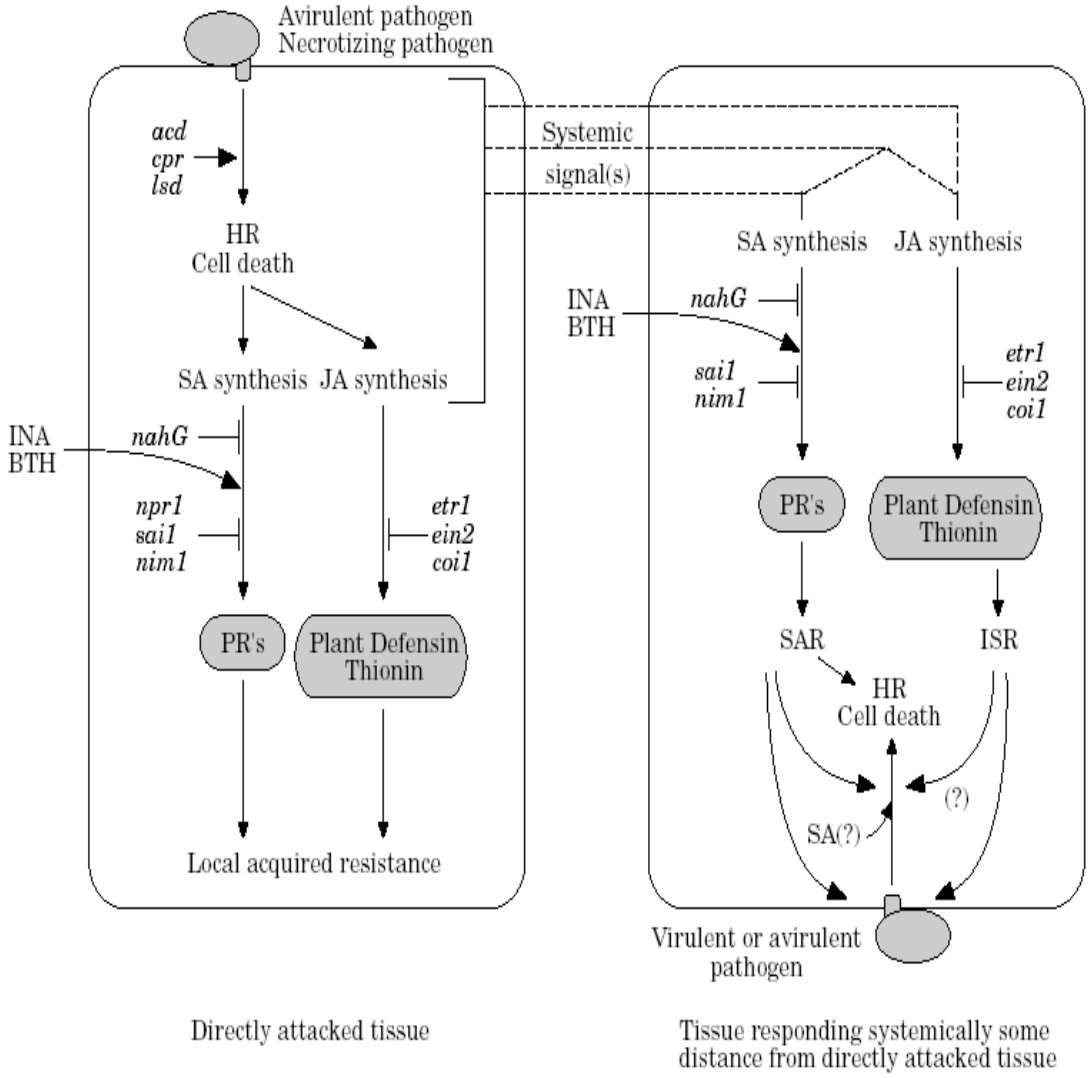


Figure 2.2 – Diagrammatic representation of the induction and expression of SAR and ISR through necrotizing pathogens and chemical induction (Mauch-Mani and Metraux, 1998). The entry point of two synthetic plant activators, Benzo (1, 2, 3) thiadiazole-7-corbothiotic acid S-methyl ester (BTH) and 2,6-dichloroisonicotinic acid (INA) is indicated.

levels of SAR since the enzyme catalyses the formation of catechol from SA, which decreases intracellular SA levels (Mauch-Mani and Metraux, 1998). It has also been shown through genetic studies that *nahG* modifies SA-independant defence pathways in *Arabidopsis* (Heck *et al.,* 2003). SA also accumulates in parts of the plant showing SAR and SA can induce the expression of the same PR proteins as in biologically induced SAR (Mauch-Mani and Metraux, 1998). An experiment in cucumber using radioactively labeled SA have indicated that SA is transported systemically (Mölders *et al.,* 1996).

It has however been shown in grafting experiments between *nahG* and wild-type tobacco plants that SA may not be the mobile translocated signal in SAR (Vernooij *et al.*, 1994). There seems to be a redundancy in the signaling of SAR, with both SA and other signals being involved. SA is believed to be the primary endogenous signal involved in SAR but is probably not the systemically transported signal (Sticher *et al.*, 1997).

NPR1 is a crucial factor involved in the signal transduction pathways of many defense related processes. Mutant studies, using the *npr1-1* mutant, have shown that SAR cannot be induced in plants that do not have a functional NPR1 protein (Spoel *et al.*, 2003). SA activates the NPR1 protein in the cytosol, which then migrates to the nucleus of the cell where it interacts with transcription factors that induce defense gene expression (Spoel *et al.*, 2003). Activated NPR1 in the nucleus binds to transcription factors of the TGA transcription factor family which then bind to promoter regions of defense related genes like *PR-1* which is a marker gene for SAR (Johnson *et al*., 2003). This again indicates the crucial role for SA during SAR.

Another group of molecules proposed to be involved in the activation of SAR is jasmonates, principally JA and its methyl ester (MeJA) (Turner *et al.,* 2002). These molecules are derived from linolenic acid and move easily in both the gaseous and liquid phases and play important signaling roles in processes such as fruit ripening, pollen production, root growth, tendril coiling, abiotic stress, wounding and defense processes (Devoto and Turner, 2003).

Jasmonates play important roles in the response of plants to stress and herbivore attack. JA has the ability to induce a number of defense related molecules such as defensins and is involved in some SA-independent defense pathways (Pieterse *et al.*, 1996). JA and MeJA induce increased systemic resistance in *Arabidopsis* against caterpillars (van Poecke and Dicke, 2003). It has been proposed that these jasmonates act as secondary messengers in SAR.

SA and JA are the better-characterized proposed signaling molecules in SAR but there are also others that have been identified in plants. The first is systemin, a small 18-amino acid peptide that is released by tomato plants after insect attack (Ryan, 1990; Sticher *et al.*, 1997). It is a systemic signal that increases the synthesis of proteinase inhibitors that inhibit the activity of the digestive proteases of insects (Ryan, 1990). Electrical signals have also been shown to possibly act as systemic signals in potato (Herde *et al*., 1995).

Ethylene, a volatile plant hormone, is involved in numerous physiological reactions within the plant. It is produced after wounding as well as after infection by pathogens (Romeis, 2001). Ethylene has been shown to activate the expression of *PR* genes such as *PR-1* (Clarke *et al.,* 2000) as well as □-1-3-glucanase (*PR-2*) and chitinase (*PR-3*). Ethylene also enhances the fortification of the cell wall to increase resistance to a pathogen and can interact with SA and JA mediated defense pathways (Wang *et al.*, 2002). This might indicate that ethylene acts as a signal during SAR but several experiments have indicated that ethylene is not likely to be a signaling molecule but rather a modulator of SAR (Sticher *et al.*, 1997).

Once the signal transduction event has occurred, SAR is activated systemically throughout the plant. This resistance includes a number of processes that also occur in a HR.

One of the most obvious events is the induced expression of *PR* genes, since this is a major characteristic of SAR (Sticher *et al.,* 1997; Mauch-Mani and Metraux, 1998). These PR proteins accumulate in the extracellular spaces as well as in the vacuole (Fritig *et al*., 1998). Examples of well-known PR proteins involved in SAR are PR1, PR2, PR3 and osmotin (Fritig *et al*., 1998). All of these proteins show direct antimicrobial activity. □-1-3-glucanases and chitinases degrade the cell walls of fungi (Fritig *et al*., 1998). These PR proteins accumulate in large amounts at the primary infection site, but also in tissues showing SAR.

The other major mechanism of SAR is strengthening of the cell wall through lignification. Lignin is formed from precursors in the phenylpropanoid pathway (Sticher *et al.*, 1997). The first step in this pathway is catalyzed by phenylalanine ammonia-lyase (PAL). The activity of PAL is increased in tissue showing SAR and an increased lignin concentration is the result. This lignin is incorporated into the cell wall and strengthens it mechanically against pathogens and their degradating enzymes. It also stops free nutrient movement from the plant cell to the pathogen. It has been proposed that lignin itself may be toxic to the pathogens (Sticher *et al.*, 1997).

2.2.4. Induced systemic resistance

ISR is another form of induced resistance found in plants. ISR differs from SAR in that resistance is achieved without SA accumulation and *PR*-gene expression (Pieterse *et al*., 1996). ISR can be activated in *Arabidopsis* by PGPR that colonize the rhizosphere. These bacteria are non-pathogenic to the plant and are mainly from the fluorescent *Pseudomonas* species. Strain WCS417r of *Pseudomonas fluorescens* has been identified as an inducing organism of ISR in tomato, radish, carnation and *Arabidopsis* (Pieterse *et al.*, 1998).

ISR, like SAR, induces resistance in plants against various organisms. There are a number of similarities between SAR and ISR signaling as well as a number of significant differences (Pieterse *et al*., 1996, 1998). Through mutant studies it was shown that ISR is dependent on elements of the JA, as well as the ethylene response (Pieterse *et al*., 1998). Plants with the *nahG* overexpression mutation did not show a decrease in ISR, which confirms that ISR is an SA independent process. ISR induces defensin synthesis like thionin, which are anti-pathogenic proteins that act on the pathogen to mediate the defense process (Mauch-Mani and Metraux, 1998).

Jasmonates are the principal signals involved in ISR and induced resistance against herbivores and insects (Pieterse *et al*., 1998; van Poecke and Dicke, 2003). The two most important factors in the JA signal transduction pathways are JAR1 and COI1 (Devoto and Turner, 2003). Another important protein in JA mediated responses like ISR is an E3 ubiquitin ligase that has been identified as a regulator of JA responses in *Arabidopsis* (Turner *et al.*, 2002; Devoto and Turner, 2003). JA mediated signaling has been shown to be also dependent on a functional □□ subunit of the G protein in *Arabidopsis*, and deficiency of the subunit obliterated the action of MeJA in the plants (Trusov *et al.,* 2006). Significant cross-talk regularly occurs between the SA-mediated pathway involved in SAR and JA-mediated responses during ISR (Spoel *et al*., 2003).

As has been mentioned, NPR1 is a key component in the SA-mediated signaling pathway that leads to SAR induction. Interestingly the *npr1* mutation also affects ISR indicating that NPR1 is necessary for ISR to occur (Pieterse *et al.,* 1998). This may indicate that although the signaling processes in ISR and SAR differ, that they still overlap. NPR1 in the cytosol has been identified as a key point of cross-talk between SA and JA mediated defense pathways in that SA activated NPR1 in the cytosol negatively regulates the JA mediated signaling pathways (Spoel *et al.*, 2003).

Another point of convergence besides NPR1 between SA- and JA-mediated signaling pathways is the WRKY70 transcription factor (Li *et al.*, 2004). This transcription factor is involved in both pathways and acts in much the same way as NPR1. SA activated NPR1 increases levels of activated WRKY70, which in turn activates the expression of SA-responsive genes in the nucleus while it represses the expression of some JA-responsive genes (Li *et al*., 2004).

COI1 is a F-box protein activated by JA that is proposed to have the ability to inactivate active WRKY70 (Turner *et al*., 2002; Li *et al*., 2004). COI1 was also seen as the factor that disrupts SA's activation of certain SA-response genes when the JA-mediated defense pathways were activated.

2.2.5. Age-Related Resistance

Age related resistance (ARR) is a distinctly different defense response that develops in plants as they mature, and has been shown to be a unique defense response by the use of mutant plants (Kus *et al*., 2002). Allthough ARR is a distinct defense response, it was found to be dependent on SA accumulation, suggesting that SA may also act as a signal during this form of defense (Kus *et al*., 2002). ARR has been observed in a number of plant species but at present the mechanisms involved in the process, is poorly understood.

2.3. Plant activators

To date a variety of chemicals have been identified as potential plant activators. Most of these induce resistance in the plant by means of SAR. In this section the focus will be on different chemicals that have been identified, how they work and in which plants they work.

2.3.1. Natural organic compounds

Activators that fall in this class are organic compounds that occur naturally in the plant (Sticher *et al*., 1997). A variety of different chemicals fall under this category, and some not only have the ability to induce defense processes in plants, they are also involved in other processes such as signal transduction and the promotion of growth.

The best studied example of a plant activator is SA. As well as being involved in the signal transduction of SAR, SA also induces SAR in a number of plant species. Evidence for SA acting as an activator of SAR is extensive and has been well documented in various species including wheat, tobacco and *Arabidopsis* (Kessmann *et al.,* 1994; Bertini *et al.*, 2003). Exogenous application of SA induces the same *PR*-genes within the correct time frame as the induction of SAR by pathogens. Neither SA nor its significant metabolites like methyl-salicylate (MeSA) has any significant direct anti-microbial activity (Kessmann *et al.,* 1994). A drawback of using SA as a plant activator is that low concentrations of SA must be used, because when the SA concentration exceeds a certain (low) limit, it becomes toxic to the plants (Görlach *et al.*, 1996). SA also gives unsatisfactory low levels of resistance in wheat compared to other activators (Görlach *et al.*, 1996).

Another class of molecules that are classified as plant activators, is the jasmonates. Exogenous application of JA and MeJA mostly induces defense responses against herbivores and insects, but can also lead to ISR which protects the plant against various microbial pathogens. Foliar application of JA to *Phaseolus vulgaris* leaves induces increased levels of enzymes involved in defense in a systemic fashion (Alba-Meraz and Choe, 2002). The genes that are induced include lipoxygenase, peroxidase and polyphenol oxides which produce ROS. It has also been observed that a volatile catabolite of JA, *cis*-jasmone or (Z)-jasmone can also induce defense against insects in plants (Bruce *et al*., 2003).

Menadione sodium bisulphite (MSB) is a vitamin K_3 water-soluble compound (Borges *et al.*, 2003). MSB is a novel plant defense activator in oilseed rape, which enhances local and systemic resistance to infection by *Leptosphaeria maculans,* the causal agent of stem canker (Borges *et al.*, 2003). Application of MSB to the plants does not increase *PR-1* gene expression, but it does however increase expression of an ascorbate peroxidase gene. The results suggested that MSB induces resistance by increased production of ROS. MSB is a known ROS generator, producing both H_2O_2 and O_2^- (Vranová *et al*., 2002; Borges *et al.*, 2003). It is postulated that MSB acts systemically in oilseed rape either by translocating to other parts of the plant and increasing ROS there or by increasing H_2O_2 production which induces a systemic signal or acts directly as the systemic signal (Borges *et al.*, 2003). MSB can be classified as a plant activator in oilseed rape because even at very high concentrations it still remains non-phytotoxic to the plant and does not act upon the pathogen. MSB also acts as a plant activator in banana, where it was tested against *Fusarium oxysporum*, which is the causal agent of Panama disease (Borges-Perez and Fernandez-Falcon, 1996).

Ethylene is a volatile plant hormone that is derived from the amino acid methionine and is involved in various important physiological processes within the plant (Wang *et al.,* 2002). Ethylene is produced by the plant in response to wounding and also during infection by pathogens and treatment with elicitors (Wang *et al.,* 2002). Ethylene together with JA and MeJA play an important role in SA-independent defense pathways (Pieterse *et al.,* 1998; Wang *et al*., 2002).

Harpins are a group of bacterial proteins that can elicit a number of defense responses in plants (Keen, 1999). The harpin proteins are typically glycine-rich, protease sensitive, heat stable, acidic proteins produced by some Gram-negative bacteria but was originally isolated from *Erwinia amylovora* (Peng *et al.*, 2003). Allthough the precise role of harpins in plant defense remains unclear, they are believed to be involved in the recognition of bacteria both as foreign substances in non-host plants and as virulence factors in host plants (Keen, 1999).

Harpin treatment can induce the HR and cell death in various plants and suspension cultures including *Arabidopsis* and tobacco (Peng *et al*., 2003; Takakura *et al*., 2004). Interestingly harpin treatment of tobacco BY-2 suspension cultures induced cell death in cells treated with the H_2O_2 inhibitor DPI and catalase, which suggests that in this case H_2O_2 generation is not required for cell death (Ichinose *et al.*, 2001). When the harpins bind to receptors, they can induce signaling through a number of pathways. Through the SA mediated defense pathway, harpins can induce *PR*-gene expression that leads to resistance through SAR activation. It can also act through the JA mediated defense pathway, which induces the expression of defensins and lead to resistance to insects and herbivores. Finally harpins also induce genes involved in plant growth, increasing the rate of photosynthesis and nutrient uptake by the plant (http://www.edenbio.com).

Some fatty acids have the ability to induce SAR. Arachidonic, linolenic, linoleic and oleic acid have been shown to induce SAR in potato against *Phytophthora infestans* (Cohen *et al.,* 1991). Some of these may act as normal elicitors that are released by the pathogen after it has infected the plant, which then through HR, induces SAR (Sticher *et al*., 1997).

A diterpene, (11E,13E)-labda-11,13-diene-8□,15-diol (WAF-1), was recently recognized as a defense inducer in tobacco against tobacco mosaic virus and other pathogens (Seo *et al.*, 2003). WAF-1 was identified as an endogenous signal in tobacco that has the ability to activate the MAP kinase, WIPK (wound induced protein kinase), that is involved in wounding and defense responses (Seo *et al.*, 2003). When WAF-1 was applied exogenously to tobacco, it induced WIPK as well as SIPK (SA induced protein kinase) gene expression and led to increased defense and *PR*-gene expression.

Systemin is another signaling molecule that is involved in both SA- and JA-mediated pathways, which can also act as a plant activator (Holley *et al.,* 2003). Systemin is produced in plants that are under herbivore and insect attack. It binds to the SR160 receptor that initiates the defense response (Holley *et al.*, 2003). Treatment of tomato plants with systemin generates the

systemic expression of proteinase inhibitor genes that deter insects (Orozco-Cárdenas *et al*., 2001; Holley *et al.*, 2003). Systemin induced resistance was also observed in *Lycopersicon peruvianum* cultured cells (Holley *et al*., 2003).

The Brassinosteroids (BR) are a group of cholesterol-like steroid molecules that occur naturally in plants in very low concentrations. Amongst the plant hormone families, brassinosteroids are a relatively recently discovered (Grove *et al.,* 1979) group of plant hormones that have a variety of functions (Bishop and Koncz, 2002).

Exogenous application of BR's has a number of effects on the plant. These include the promotion of cell elongation and division (growth), increased responses to gravitropism and stress tolerance, increased rates of ethylene biosynthesis, enhancement of the differentiation of the tracheary element, proton-pump-mediated polarization of the cell membrane, retardation of abscission, and the induction of defense against pathogens (Shimada *et al*., 2003; Kim *et al.,* 2004).

The important role of brassinosteroids was proven in several different mutant plants that are BR-deficient. Most mutant plants displayed a dwarf phenotype as well as characteristic dark green leaves (Bishop and Koncz, 2002). This suggests that BR's are essential for normal plant growth and development.

The first BR that was isolated was brassinolide (BL) in 1979 (Grove *et al.,* 1979). Since then approximately 40 different BR's have been identified and isolated from a variety of plants. BL is however the most common of all the brassinosteroids and is also the most active. The biosynthetic pathway of BL can be seen in figure 2.3. This pathway is currently the most complete synthesis pathway to describe the synthesis of Brassinosteroids (Bishop and Koncz, 2002).

There are a number of crucial steps in this pathway. The oxidative conversion of castasterone (CS) to BL has been viewed as an important activation step and it is catalyzed by the membrane-associated enzyme castasterone-6-

oxidase/Brassinolide synthase (Kim *et al*., 2004). Recent evidence have shown that this crucial enzyme is in fact a cytochrome P450 enzyme (Kim *et al*., 2004). The other major activation steps of the biosynthetic pathway have been shown to be catalyzed by various other cytochrome P450's (Bishop and Koncz, 2002). These steps are the synthesis of castasterone from typhasterol and also from

Chapter 3
Materials and Methods

3. Materials and Methods

3.1. Materials

3.1.1. Plant material and growth

Two wheat cultivars, namely a leaf rust susceptible (Thatcher) and resistant (Thatcher+*Lr34*) cultivar, were used in this study. The wheat was planted in soil trays in a glasshouse. Conditions in the glasshouse were approximately 25°C with a 16 h light/8 h dark cycle. All plants were watered daily and were treated three times a week with 0.25% (w/v) Multifeed water soluble fertilizer.

3.1.2. Other

The Tripure RNA isolation reagent and mRNA Capture kit were bought from Roche Molecular Biochemicals, the BD PCR-Select™ cDNA subtraction kit from BD Biosciences, the pGEM®T-Easy vector system, competent *Escherichia coli* (*E. coli*) JM109 cells and Im-Prom-II™ reverse transcriptase from Promega, KAPA *Taq* DNA polymerase from KAPA Biosystems, Favorprep™ Gel/PCR Purification kit from Favorgen Biotech Corporation, Hybond™-XL nylon membranes from GE Healthcare and the BigDye® Terminator v3.1 cycle sequencing kit from Applied Biosystems. All other reagents were of the highest quality and purity.

3.2. Methods

3.2.1. Preparation of the plant activator

The plant activator used during this project was purified from the commercially available product ComCat® (CC). CC was first extracted with 100% (v/v) methanol, then with a 100% (v/v) methanol:ethyl acetate (50:50) solution and finally with a 50% (v/v) ethyl acetate solution. The fractions were separated and the ethyl acetate fraction further fractionated with a 90% (v/v)

hexane:methanol (50:50) solution. The two fractions were again separated and the methanol:water fraction (SS) was damped off and used as the plant activator during this study.

3.2.2. Treatment of plants

Wheat seedlings were treated with SS after reaching the three-leaf stage. The plants were sprayed with 0.5 $mg.l^{-1}$ SS dissolved in sterile water containing 0.0001% (v/v) polyoxyethylene sorbitanmonolaurat (Tween 20) to allow for effective absorption by the plants. Control plants were sprayed with water containing Tween 20. All plants were sprayed until microdroplets were visible on the leaves.

Plant material was harvested by cutting off the entire plant just above the soil, where after it was snap frozen in liquid nitrogen and stored at –80°C. Tissue was harvested at 0, 0.5, 1, 2, 4, 8, 12 and 24 h after treatment (hpt).

The frozen plant material was subsequently ground to a fine powder in liquid nitrogen using a pestle and mortar. The pestle and mortar was first sequentially washed with dish washing liquid, 10% (w/v) sodium dodecyl sulphate (SDS), rinsed with dimethyl pyrocarbonate (DMPC) treated water, wrapped in foil and autoclaved.

3.2.3. RNA extraction

All solutions used in experiments involving RNA samples were prepared using RNase-free DMPC treated water. The DMPC water was prepared by adding 0.1% (v/v) DMPC to distilled water, leaving it overnight and finally autoclaving it before use.

Approximately 0.1 g ground plant material was used for total RNA extraction. RNA extraction was done using the Tripure RNA isolation reagent (Roche Molecular Biochemicals) according to the manufacturers instructions. After the final step, the RNA pellet was dissolved in DMPC treated water. The

concentration of the total RNA was determined according to Sambrook *et al*. (1989) and expressed as ng.μl^{-1}.

The quality of the extracted RNA was confirmed by separating 500 ng of each sample on a 1% (w/v) agarose gel containing 0.05 $\mu g.ml^{-1}$ ethidium bromide prepared in 0.5x TAE buffer [20 mM Tris-hydroxymethyl aminomethane (Tris) pH 8; 0.28% (v/v) acetic acid, 0.5 mM ethylenedinitrilotetraacetic acid (EDTA)] (Sambrook *et al.,* 1989). The RNA samples were diluted in DMPC treated water and RNA loading buffer was added to a final concentration of 0.25% (w/v) bromophenol blue, 0.375 M ficoll. Separation was done at 10 $V.cm^{-1}$ for 45 min using 0.5x TAE as running buffer. After separation, the gel was photographed using a Bio-Rad gel documentation system.

3.2.4. Suppression subtractive hybridization (SSH)

Before SSH was performed, poly-A mRNA was captured from the extracted total RNA samples using an mRNA Capture Kit (Roche Molecular Biochemicals) according to the manufacturers instructions.

SSH was performed using a BD PCR-Select™ cDNA subtraction kit supplied by BD Biosciences (Fig 3.1). Two corresponding sets of pooled mRNA were used during the procedure. The tester sample consisted of mRNA samples purified from Thatcher+*Lr34* tissue harvested 0.5, 1 and 2 h after treatment (hpt) after treatment with the activator. The driver mRNA was prepared from Thatcher+*Lr34* tissue treated with water harvested at the same time intervals.

The SSH steps included first strand cDNA synthesis, second strand cDNA synthesis, *Rsa*I digestion, adaptor ligation, two hybridization steps followed by two final PCR reactions (Fig 3.1). The procedure was completed despite the fact that at certain stages where according to the manual, PCR amplified product should have been visible, no products were found.

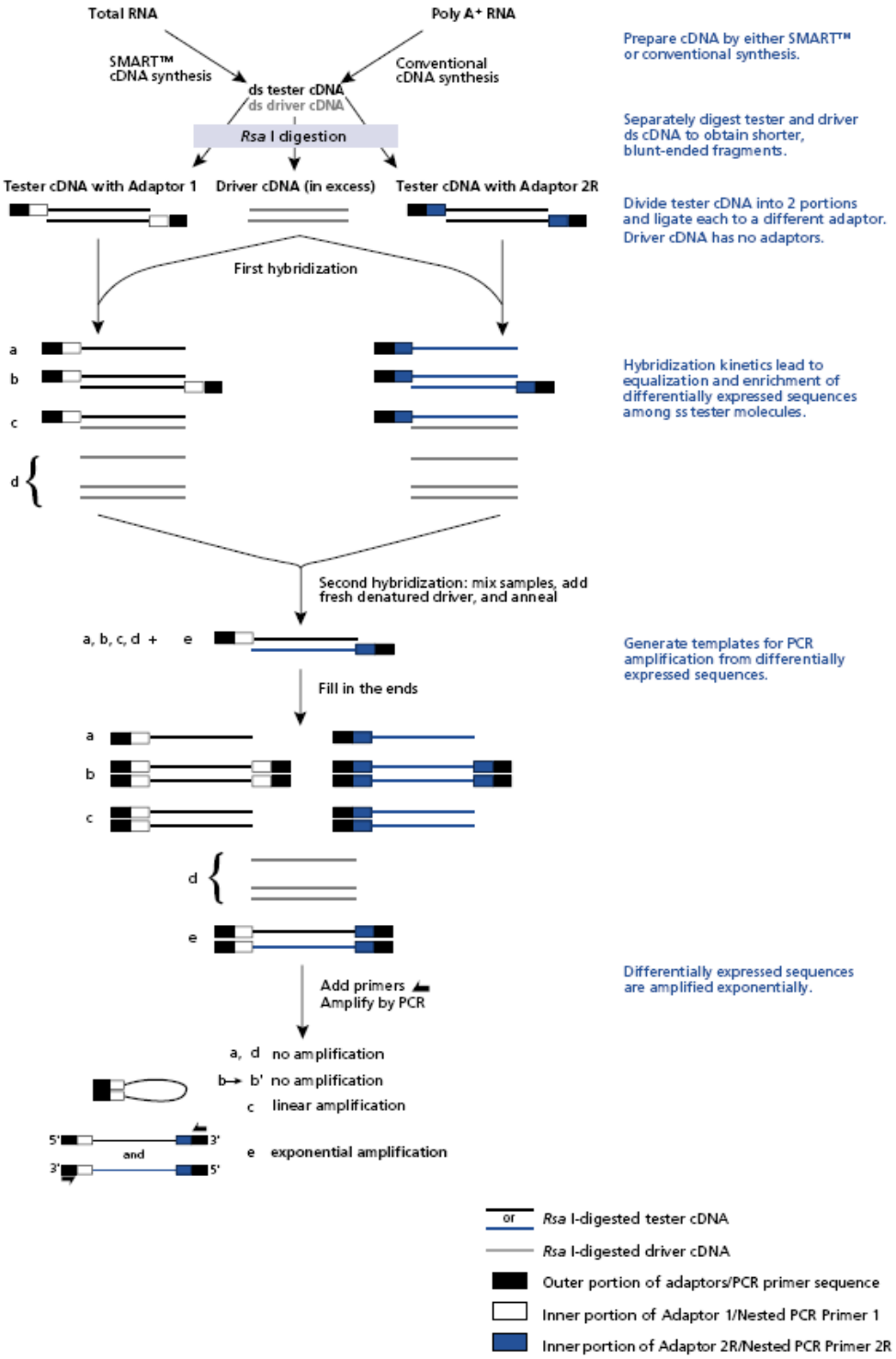


Figure 3.1 – SSH of SS treated wheat seedlings (BD Biosciences, BD PCR-Select^{TM} cDNA subtraction kit manual). The driver sample consisted of pooled, purified mRNA from 0.5, 1 and 2 hpt in SS treated Thatcher+*Lr34* plants whilst the driver was made up by the corresponding water treated Thatcher+*Lr34* samples.

3.2.5. Cloning of SSH products

The end products of the SSH procedure were enriched double strand cDNA fragments that were putatively differentially expressed after SS treatment. These fragments were cloned into the pGEM®T-Easy vector (Promega) according to the manufacturers instructions. Competent *E. coli* JM109 cells (Promega) were transformed with the ligation reaction according to the suppliers instructions. Following transformation, the cells were plated on LB plates [1% (w/v) Tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract, 1.5% (w/v) agar] containing 50 $\mu g.ml^{-1}$ ampicillin, 250 $\mu g.ml^{-1}$ 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and 250 $\mu g.ml^{-1}$ isopropyl-β-D-thiogalactoside (IPTG) and incubated at 37°C overnight. White colonies containing recombinant plasmids were selected and transferred onto new LB plates containing ampicillin.

All recombinant colonies were inoculated in 5 ml LB broth [1% (w/v) Tryptone, 1% (w/v) NaCl, 0.5% (w/v) yeast extract] containing 50 $\mu g.ml^{-1}$ ampicillin and grown overnight at 37°C in an orbital shaker. Cells were harvested by centrifugation at 13000 *g* for 5 min and resuspended in a resuspension solution (50 mM Tris-HCl pH 7.5, 10 mM EDTA, 0.1 $\mu g.\square\mu l^{-1}$ RNase A). The cells were lysed by adding equal volumes of lysis buffer (0.2 M NaOH, 1% (w/v) SDS) and finally neutralized by adding KOAc (pH 4.8) to a final concentration of 0.85 M.

After centrifugation at 13000 *g* for 5 min, plasmid DNA was precipitated from the cleared supernatant with 100% (v/v) ethanol. The solution was centrifuged at 13000 *g* for 10 min and the resulting pellets washed with 70% (v/v) ethanol. Dried plasmid DNA samples were subsequently dissolved in water.

The extracted plasmid DNA was further purified according to the method of McPherson and Moller (2000) before PCR commenced. The DNA was purified by first adding sodium acetate (pH 5.2) to a final concentration of 0.3 M, where after 95% (v/v) ethanol was used to precipitate the DNA. After

centrifugation at 13000 *g*, the samples were washed with 70% (v/v) ethanol. All dried samples were redissolved in 10 mM Tris-HCl (pH 7.5).

3.2.6. PCR amplification of SSH products

The cloned cDNA fragments were amplified from the recombinant plasmids using PCR. The SP6 and T7 primers (Table 3.1) were used to amplify the insert of each recombinant plasmid. Each 20 μl reaction contained 1 μl template DNA, 1x enzyme buffer (5 mM Tris-HCl pH 8.3, 25 mM KCl, 0.75 mM $MgCl_2$), 25 pmoles of each primer, 200 μM deoxyribonucleotide triphosphates (dNTPs) and 0.2 units KAPA *Taq* DNA polymerase (KAPA Biosystems).

The amplification regime was as follows: 94°C for 2 min, 30 cycles of 95°C for 30 s, 50°C for 30 s, 70°C for one min, followed by 72°C for 5 min. The amplified DNA fragments were separated on a 1% (w/v) agarose gel to confirm the success of the reaction (3.2.3).

Following PCR, all amplified cDNA inserts were purified using the FavorPrep™ Gel/PCR Purification kit from Favorgen Biotech Corporation according to the manufacturers instructions. The quality and concentration of all purified PCR fragments were confirmed by agarose gel electrophoresis (3.2.3).

3.2.7. Reverse Northern blot

The purified cDNA fragments were diluted in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and denatured by adding dimethylsulfoxide (DMSO) to a final concentration of 50% (v/v). The samples were then incubated at 60°C for 10 min. Hybond-XL membranes were hydrated once in distilled water and once in 6x SSC buffer (0.9 M NaCl, 0.09 M sodium citrate pH 7.0) until the membrane was thoroughly wet. A 96 well slot blot system was used to transfer the PCR products onto the membranes. Each well was washed with

6x SSC buffer before the denatured cDNA fragments were applied. Once the samples were transferred, each well was washed three times with 6x SSC. The DNA was fixed to the membrane by exposing it to UV-light for 30 s. As a control, a cloned actin gene was similarly amplified and transferred to the membranes.

In total six membranes were prepared in duplicate containing a total of 527 cloned cDNA fragments. The membranes were hybridized with two different probes. The control probe was prepared from pooled mRNA (0.5, 1 and 2 hpt) extracted from Thatcher+*Lr34* plants treated with water. The second probe was prepared using pooled mRNA (0.5, 1 and 2 hpt) extracted from Thatcher+*Lr34* plants treated with SS.

A total of 30 μg total RNA was used to purify mRNA using a mRNA Capture Kit (Roche Molecular Biochemicals). After capturing the mRNA, the probes were prepared by adding 25 pmol of an oligo-dT primer (Bovis 32, Table 3.1) to 4 μl of the pooled RNA samples. This mixture was denatured at 70°C for 5 min before placing it on ice. To this was added 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1x Im-Prom-II™ buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl and 10 mM DTT), 1 μl Im-Prom-II™ reverse transcriptase (Promega) and 30 μCi [α-^{32}P]-deoxycytidine triphosphate (dCTP). The reaction mixture was first incubated at 25°C for 5 min followed by 60 min at 42°C. The labelled probes were purified using Sephadex G75 columns (Sambrook *et al*., 1989), the probe fractions collected, denatured at 95°C for 5 min and immediately cooled on ice before hybridization.

The membranes were pre-hybridized for 2 h at 42°C in a hybridization solution [50% (v/v) formamide, 5x SSPE (0.75 M NaCl, 50 mM NaH_2PO_4, 5 mM EDTA pH 7), 0.5% (w/v) SDS, 5x Denharts solution (1% (w/v) polyvinylpyrrolidone (PVP), 1% (w/v) Ficoll, 1% (w/v) bovine serum albumin (BSA)], 0.1 $\mu g.ml^{-1}$ denatured herring sperm DNA]. This solution was replaced with fresh preheated hybridization solution to which the denatured probes were added. Membranes were hybridized for 24 h at 42°C. After hybridization, the

membranes were washed twice for 15 min each at room temperature using wash buffer 1 [2x SSC (0.3 M NaCl, 0.03 M sodium citrate pH 7.0), 1% (w/v) SDS] and twice for 15 min at 50°C using wash buffer 2 [0.5X SSC (0.075 M NaCl, 7.5 mM sodium citrate, pH 7.0), 1% (w/v) SDS].

The membranes were sealed and exposed to a phosphor screen for a week where after it was developed, scanned and quantified using a Bio-Rad Personal Molecular Imager®. A spot count was done for each sample taking care to exclude the background. Each sample was divided by the spot count of the actin control on the membrane. The mean values of the blots hybridized with the SS probe were then divided by the mean values of blots hybridized with the water probe to estimate the induction level.

3.2.8. Sequencing of clones

cDNA fragments whose induced expression levels were 3 or higher (3.2.7), were sequenced using the BigDye® Terminator technology (Applied Biosystems). The cloned cDNA fragments were amplified with SP6 and T7 primers as described (3.2.6). PCR products were subsequently purified using a FavorPrep™ Gel/PCR Purification kit (3.2.6).

Quarter sequence reactions were preformed by mixing the following: 1x BigDye sequencing buffer, 2 μl Ready Reaction premix, 3.2 pmol of the SP6 primer and a specific volume of DNA template according to the concentration (either 5, 8 or 10 μl).

The amplification regime for all sequencing reactions was as follows: 94°C for 2 min, 30 cycles of 95°C for 30 s, 50°C for 30 s, 70°C for 1 min followed by 72°C for 5 min.

Sequenced products were purified by ethanol/EDTA precipitation (Sambrook *et al*., 1989). This was done by adding 2 μl 125 mM EDTA (pH 8.0) followed by 50 μl 100% (v/v) ethanol. After incubation at room temperature for 15 min,

samples were centrifuged at 13000 *g* for 15 min and the pellet washed with 70% (v/v) ethanol (Sambrook *et al*., 1989). The dried samples were then separated on a 6% (v/v) acrylamide gel and sequenced using an ABI prism™ 377 DNA sequencer. Each sequence was analyzed using Chromas Pro by removing the plasmid DNA sequences. The cDNA sequences were then blasted against the NCBI MegaBlast database and sequences were analyzed accordingly (http://www.ncbi.nlm.nih.gov).

3.2.9. Expression analysis of selected clones

For each cloned cDNA fragment that was analyzed, two gene specific primers were developed (Table 3.1). Primers were designed using the online tool Web primer using the known cDNA sequence (http://seq.yeastgenome.org/cgi-bin/web-primer). All primers used in RT-PCR reactions were first subjected to a temperature gradient PCR to determine the optimum annealing temperature for each primer set. Twelve identical PCR reactions were prepared (3.2.6) for each clone using the original cDNA fragment in the plasmid vectors as template. The PCR reactions were run at a temperature gradient starting at 40°C and ending at 60.3°C. The optimal annealing temperature for each primer set was selected after electrophoresis based on the amplification profile of the particular cDNA fragment.

Reverse transcription PCR (RT-PCR) analysis was done to confirm the differential expression of selected sequenced clones using the RobusT II RT-PCR kit (Finnzymes). Each 10 μl reaction contained 10 ng total RNA, 25 pmoles of each gene specific primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1X optimized kit buffer and 0.2 μl of the enzyme mix (1 U M-MuLV Reverse transcriptase™ and 0.2 U DyNAzyme EXT DNA polymerase™).

Conditions for all RT-PCR reactions were as follows: 48°C for 30 min, 94°C for 2 min and 30 cycles of 94°C for 15 s, 30 s at the specific annealing temperature for each primer pair and 72°C for one min. The amplified cDNA products were separated on a 1% (w/v) agarose gel as described (3.2.3).

The expression of the following cloned cDNA fragments were confirmed: the large and small subunits of Rubilose-1,6-bisphosphate oxidase/carboxylase (Rubisco), Rubisco activase, phosphoglycerate kinase, ribulose 5'-phosphate kinase, Ptr ToxA-binding protein1, β-1,3-glucanase (*PR2*) and ATP synthase CF-1. In order to ensure that the amount of RNA used for all the RT-PCR reactions was consistent, a control amplification of the *18S rRNA* gene was done for each treatment since the gene is constitutively expressed (Table 3.1).

Table 3.1. –Nucleotide sequences of primers used during this study. n=any nucleotide; v=any pyrimidine

Clone name	**Forward Primer**	**Reverse Primer**	**Amplified fragment length**	**Annealing Temperature**
SSH cDNA Clones	SP6: 5'-TATTTAGGTGACACTATAG-3'	T7: 5'-TAATACGACTCACTATAGGG-3'	various	50°C
RNA probes	Bovis 32 5'-GAAGAATTCTCGAGCGGCCGCTTTTTTTTTTTTTTTTVN-3'	n/a	n/a	62°C
Rubisco large subunit (*rbcL*)	Bovis 84: 5'-GAAACTAAAGCAGGTGTTG-3'	Bovis 85: 5'-AGGTGCATTTCCCCAAGGA-3'	1300 bp	60.3°C
Rubisco small subunit (*rbcS*)	Bovis 82: 5'-TGGCTTCCTCTATGCTCTCCTC-3'	Bovis 83: 5'-TGGCTTGTAGGCGATGAAACTG-3'	250 bp	60.3°C
Ribulose-5-phosphate kinase	Bovis 166: 5'-GCCGAGGTACATTGAAATGTG-3'	Bovis 167: 5'-CCGGGCAGGTACCTAGTTT-3'	155 bp	48°C
ATP synthase CF-1	Bovis 168: 5'-CAGGTGCAGGTCGTATCGA-3'	Bovis169: 5'-CCGAGGTGCCTCACAAGT-3'	148 bp	58.9°C
Rubisco activase (*RcaA1*)	Bovis 170: 5'-AGGATACAGGCACATGCT-3'	Bovis 171: 5'-GATGCATATCTTGAAAATG-3'	150 bp	60.3°C
Phosphoglycerate kinase	Bovis 172: 5'-GCCGAGGTACATGAAAGGAAT-3'	Bovis 173: 5'-GGGCAGGTGGCTGATGTTAT-3'	179 bp	59.8°C
Ptr ToxA Binding protein (*TaThf1*)	Bovis 174: 5'-CCGAGGTACTGGACAAGCTTT-3'	Bovis 175: 5'-CCGGGCAGGTACTACACTT-3'	355 bp	60.3°C
PR2	Bovis 37: 5'-TAGGCGATACCTGCCA-3'	Bovis 38: 5'-ACTTCATACTTAGACTGTCG-3'	820 bp	60.3°C
18S rDNA	Bovis 26: 5'-CAACTTTCGATGGTAGGATAG-3'	Bovis 27: 5'-CTCGTTAAGGGATTTAGATTG-3'	226 bp	58°C

Chapter 4

Results

4. Results

4.1. Plant material and growth of plants

The two wheat cultivars used in this study were grown in a glass house under optimum conditions until the three leaf stage was reached three weeks after planting. The plants were photographed in the glasshouse one day before treatment commenced (Fig. 4.1).

4.2. RNA concentration and quality

Before SSH and RT-PCR were done, the quality of the extracted RNA samples was assessed (Fig. 4.2). Shown in figure 4.2 is total RNA extracted from Thatcher+*Lr34* wheat treated with SS. The two bands in each lane correspond to the *18S* and *28S rRNA* fragments. The intactness of these bands indicated that the RNA was of good quality and not broken down. The background smear represents the mRNA. It can also be seen that the particular RNA samples were furthermore of roughly equal concentrations except for 12 hpt which had a lower concentration. The concentration of RNA samples that were not accurate, was adjusted to ensure that equal quantities total RNA were used for all subsequent procedures. RNA isolated from all other treatments were similarly assessed. These samples were therefore fit to be used for SSH.

4.3. Subtractive suppression hybridization

RNA samples from SS and water treated Thatcher+*Lr34* seedlings harvested at 0.5, 1 and 2 hpt were respectively pooled for use as the tester and driver samples during SSH. According to the SSH manual, following PCR analysis at various steps during the procedure, PCR amplified products should have been visible when separated on an agarose gel. Such results were however never obtained during this study. In order to confirm the success of the individual SSH

Figure 4.1 – The cultivation of wheat seedlings. In (a) Thatcher+*Lr34* seedlings are indicated and in (b) Thatcher seedlings.

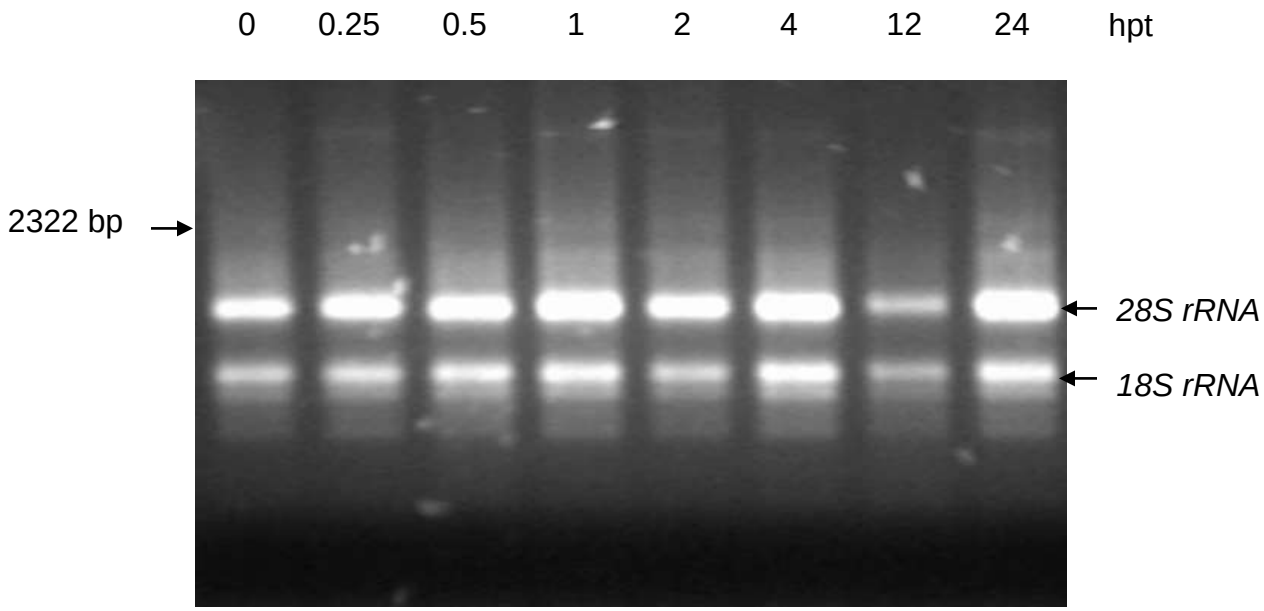


Figure 4.2 - Quality assessment of isolated RNA samples. Each lane contains 500 ng total RNA isolated from SS treated Thatcher+*Lr34* plants harvested at the indicated time intervals. The size of a DNA marker is indicated.

steps, trace amounts of [α-^{32}P]-dATP were added during the respective cDNA synthesis steps. Incorporation of the isotope during DNA synthesis would label the newly synthesized DNA radio-active. The presence of the radio-active DNA was then confirmed using a Geiger counter, confirming the success of the individual SSH steps.

Since the final subtracted cDNA pool was indeed radioactive, it was assumed that the entire subtractive procedure was successful. The final enriched cDNA pool of the SSH procedure was cloned into the pGEM®T-Easy vector system. Recombinant colonies (Fig. 4.3) were selected via α-complementation. The plasmids present within blue colonies contained an intact *lac*Z' gene that resulted in the utilization of the X-gal substrate to yield the blue colour. White colonies on the other hand contained plasmids with cloned inserts that interrupted the *lac*Z' gene resulting in a defective enzyme that could not utilize the substrate yielding white colonies (Fig. 4.3). All white colonies were transferred to new LB-plates using a grid system. In total, 650 white colonies containing putative recombinant plasmids, were obtained.

4.4. PCR amplification of cloned SSH products

The cloned inserts of all the putative recombinant plasmids were amplified from the plasmids to confirm their presence (Fig. 4.4). When a sample showed more than one band or no band whatsoever (Fig. 4.4), that particular sample was tested again. After a third negative result, the clone was discarded. Out of the original 650 clones, a total of 562 were shown to have inserts. The sizes of the inserts varied considerably whilst still falling between 200-500 bp which is the accepted size range for SSH products.

Figure 4.3 – Selection of colonies containing recombinant plasmids using α-complementation. In (a) a white colony containing a recombinant plasmid is indicated, while (b) indicates a blue colony containing a non-recombinant plasmid.

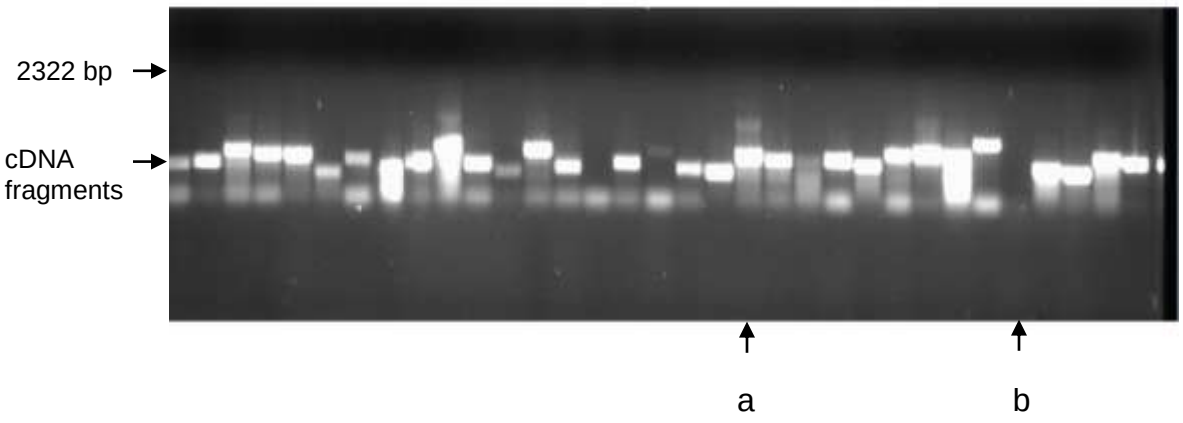


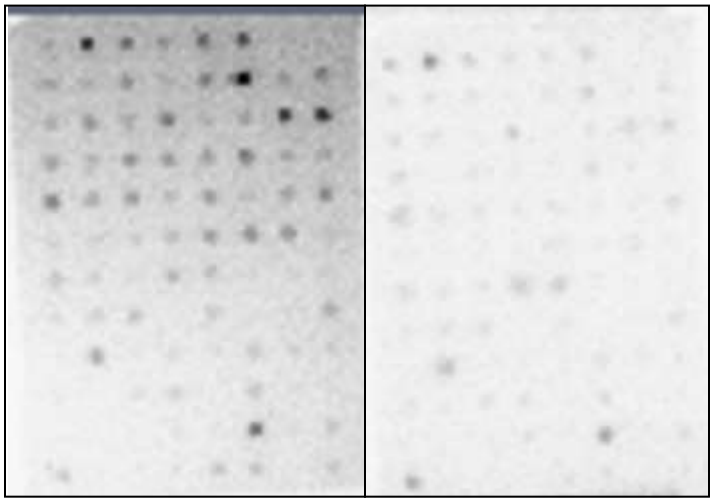
Figure 4.4 – Confirmation of the presence of inserts in recombinant plasmids. Indicated as (b) is a putative recombinant plasmid that did not show an amplified insert while (a) is a recombinant plasmid that had more than one insert. The size of a DNA marker is indicated.

4.5. Reverse northern blot

Once the inserts of recombinant plasmids were confirmed, the differential expression of each gene fragment was tested using a reverse Northern blot. The 562 cDNA clones that were amplified, were transferred to nylon membranes. Two sets of identical membranes were hybridized with radioactively labeled cDNA probes prepared from RNA isolated from Thatcher+*Lr34* seedlings treated with SS and water respectively.

For quantification purposes, each membrane contained two controls (Fig. 4.5). The two controls were an actin gene used for quantification due to its normal constitutive expression and a buffer control acting as a negative control. No hybridization was visible at the buffer control positions, thus excluding any false positive results. Hybridization at actin positions were integrated until visible for all the membranes and were used for quantification of the cDNA fragments. The actin control is not visible in figure 4.5 because integration of the image led to the overexposure of the other clones.

All blots were quantified as mentioned earlier (3.2.7). The expression levels for all the clones were as follows. Of the 562 cloned cDNA fragments, 106 showed repressed expression values between 0.3 and 1, 215 clones showed induced expression values between 1 and 2, 88 between 2 and 4 and 62 clones had induced expression levels higher than 4. The differential expression levels thus varied between 13.6 for clone 460 and 0.3 for clone 560. cDNA clones with an induced expression level of 4 or higher than that of the corresponding water control sample, were sequenced. Clone nr. 560, which showed a 0.3 repression level, was also sequenced.



a b

Figure 4.5 – Reverse northern blot hybridization of isolated cDNA clones. Indicated in (a) is a membrane hybridized with the cDNA probe prepared from RNA isolated from SS treated wheat while in (b) an identical membrane hybridized with the water control probe is shown.

4.6. Sequencing of clones

A total of 51 cloned cDNA fragments with an induction level of 4 or higher were sequenced (Table 4.1). The majority of the cDNA fragments shared homology with chloroplast genes including photosynthetic related genes as well as ribosomal components. In addition, several fragments shared homology with expressed sequence tags (EST) from wheat. These cDNA fragments sharing homology with known genes included: chloroplast RNA (1), Rubisco activase (1), calmodulin (1), Rubisco large subunit (3), ribulose 5'-phosphate kinase (4), the small subunit of Rubisco (2), partial wheat 18S ribosomal RNA (3), wheat 40S ribosomal protein (2), wheat chloroplast DNA and ribosomal protein 12 (4), chloroplast-localized Ptr ToxA-binding protein1 (2), *Triticum aestivum* clone wlk8.pk0011.e10:fis (4), wheat EST's (19), the β and γ subunits of ATP synthase CF-1 (1) and phosphoglycerate kinase (1).

Three potentially novel genes were also found which did not show any significant homology to any known genes. Interestingly, the only cDNA clone that showed repressed expression, clone number 540, showed very high similarity to the genes encoding chloroplast cDNA and ribosomal protein 12 which was shown also to be induced through SS treatment.

4.7. Expression analysis of SSH generated cDNA fragments

Once sequenced a number of clones sharing homology with known genes were chosen for further expression analysis. This was done to confirm the results of both the SSH and the reverse Northern blot, as well as to determine whether SS action differs between the two different wheat cultivars. The chosen cDNA clones were those that showed homology to both subunits of Rubisco, rubilose 5'-phosphate kinase, ATP synthase CF-1, Rubisco activase, phosphoglycerate kinase and Ptr ToxA binding protein1. The gene encoding β-1-3-glucanase (*PR2*) was also included although it was not initially found in the group of sequenced

Table 4.1 – Identification of SSH generated cloned cDNA fragments.

Clone number	Induction	Similarities	E value
462	10.2X	Chloroplast rRNA	$7E^{-51}$
463	10.5X	*Hordeum vulgare* subsp. *vulgare* ribulose 1,5-bisphosphate carboxylase activase (*RcaB*) gene	$7E^{-29}$
485	4.9X	Low homolgy to rice genomic DNA, rice calmodulin clone, EST's	$1E^{-07}$
343	9.3X	No significant similarities/Novel	n/a
465	9.1X	No significant similarities/Novel	n/a
345	7.1X	No significant similarities/Novel	n/a
335	11,2X	Ribulose-1,5-bisphoshate carboxylase/oxygenase, Rubisco (various plant species)	$6E^{-154}$
479	6.2X	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*) gene, partial cds; chloroplast (various)	$4E^{-143}$
363	5.8X	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*rbcL*) gene, partial cds; chloroplast (various)	$4E^{-143}$
460	13,6X	Ribulose 5'-phosphate kinase	$6E^{-35}$
354	7.3X	Ribulose-5-phosphate kinase	$3E^{-60}$
364	5.5X	Ribulose-5-phosphate kinase	$3E^{-60}$
491	4.1X	Ribulose-5-phosphate kinase	$3E^{-60}$
477	7.4X	Rubisco small subunit (*rbcS*)	$2E^{-30}$
486	5.4X	Rubisco small subunit (*rbcS*)	$4E^{-34}$
476	7.9X	*Triticum aestivum* 18S ribosomal RNA gene, partial sequence	$9E^{-10}$
494	4.6X	*Triticum aestivum* 18S ribosomal RNA gene, partial sequence	$6E^{-23}$
473	5.9X	*Triticum aestivum* 18S ribosomal RNA gene, partial sequence	$3E^{-103}$
336	9.1X	*Triticum aestivum* 40S ribosomal protein mRNA, partial cds	$1E^{-66}$
356	4.7X	*Triticum aestivum* 40S ribosomal protein mRNA, partial cds	$1E^{-66}$
466	8.5X	*Triticum aestivum* chloroplast DNA, complete genome, Chloroplast ribosomal protein 12	$6E^{-139}$
467	8.4X	*Triticum aestivum* chloroplast DNA, complete genome, Chloroplast ribosomal protein 12	$9E^{-132}$
338	6.4X	*Triticum aestivum* chloroplast DNA, complete genome, Chloroplast ribosomal protein 12	$6E^{-139}$
540	0.3X	*Triticum aestivum* chloroplast DNA, complete genome, Chloroplast ribosomal protein 12	$2E^{-145}$
464	9.2X	*Triticum aestivum* chloroplast-localized Ptr ToxA-binding protein1(*TaThf1*) mRNA	$1E^{-84}$
470	8.6X	*Triticum aestivum* chloroplast-localized Ptr ToxA-binding protein1(*TaThf1*) mRNA	$2E^{-93}$
468	7.1X	*Triticum aestivum* clone wlk8.pk0011.e10:fis, full insert mRNA	4^{-105}
355	5.9X	*Triticum aestivum* clone wlk8.pk0011.e10:fis, full insert mRNA	$4E^{-105}$

Table 4.1 – Identification of SSH generated cloned cDNA fragments.

340	4.2X	*Triticum aestivum* clone wlk8.pk0011.e10:fis, full insert mRNA	$4E^{-105}$
366	4.2X	*Triticum aestivum* clone wlk8.pk0011.e10:fis, full insert mRNA	$4E^{-105}$
469	8.8X	Various wheat EST's	Various
344	8.4X	Various wheat EST's	Various
361	7X	Various wheat EST's	Various
337	7.6X	Various wheat EST's	Various
471	7.6X	Various wheat EST's	Various
475	6.9X	Various wheat EST's	Various
487	6.1X	Various wheat EST's	Various
484	5X	Various wheat EST's	Various
488	5X	Various wheat EST's	Various
478	5.9X	Various wheat EST's	Various
481	5.7X	Various wheat EST's	Various
381	5.1X	Various wheat EST's	Various
357	4.8X	Various wheat EST's	Various
489	4.6X	Various wheat EST's	Various
496	4.2X	Various wheat EST's	Various
349	4.1X	Various wheat EST's	Various
350	4.1X	Various wheat EST's	Various
353	8.1X	Various wheat EST's, Low homology with calmodulin	Various
346	5.8X	Various wheat EST's, Low homology with calmodulin	Various
370	5.3X	Wheat chloroplast ATP synthase CF-1 gene, beta and epsilon subunits	$2E^{-62}$
474	8X	Wheat mRNA for chloroplast phosphoglycerate kinase (EC 2.7.2.3)	$3E^{-63}$

clones. The gene was included as a marker for the activation of defense responses to examine possible SS action regarding general plant defense.

4.7.1. Optimum temperature determination

A primer pair for each cDNA fragment was designed and synthesized. To determine the optimal annealing temperature, each pair was subjected to temperature gradient PCR amplification (Fig. 4.6). Primer sets for the genes encoding both the large and small subunits of Rubisco were excluded due to the fact that their optimal annealing temperatures were already known. The temperature range used was from 45°C to 60°C using 12 intermediate temperatures. For each primer set, six PCR reactions were prepared and subjected to amplification (Fig. 4.6).

Primers for ribulose 5'-phosphate kinase showed the best amplification at 48°C, showing almost no amplification at higher temperatures (Fig. 4.6a). The best amplification of ATP synthase CF-1 occurred at higher temperatures, peaking at 58.9°C (Fig 4.6b). For Rubisco activase (Fig. 4.6c), Ptr ToxA binding protein1 (Fig. 4.6d) and phosphoglycerate kinase (Fig. 4.6e), the respective primer sets showed maximum amplification throughout the entire temperature range, thus the optimum temperature of 60.3°C was used in these instances.

4.7.2. RT-PCR

RT-PCR reactions were done to confirm the differential expression of the identified cDNA clones in both treated and control resistant and susceptible wheat plants respectively. The *18S rDNA* gene was used as a control because it is constitutively expressed and would therefore confirm that equal amounts of RNA was used in all RT-PCR reactions. When RT-PCR analysis was started, 10 ng total RNA was used in each reaction. However as can be seen, it was very

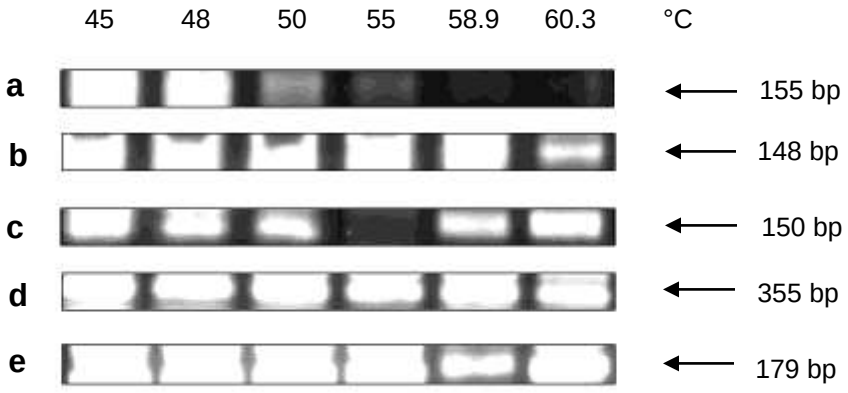


Figure 4.6 – Optimum temperature determination for primer sets used during RT-PCR. The fragment sizes are indicated for amplified fragments of ribulose 5'-phosphate kinase (a), ATP synthase CF-1 (b), Rubisco activase (c), Ptr ToxA binding protein1 (d) and phosphoglycerate kinase (e).

difficult to confirm the differential expression, since the amplified bands had more or less equal intensities (Fig. 4.7b). This could be due to end point saturation, where because of the large amounts of total RNA in each reaction, other ingredients of the PCR reactions are exhausted long before the 30 cycles are completed. This would then give an incorrect result of the RT-PCR.

In order to improve the results, the amount of total RNA used in each RT-PCR reaction was reduced to 1 ng. The obtained results were much clearer in that the induced expression was more evident (Fig. 4.7a). All subsequent RT-PCR reactions were therefore done using 1 ng total RNA as template.

4.7.2.1. Expression analysis of selected cDNA fragments in Thatcher+*Lr34* plants

Once the correct amount of RNA template in each RT-PCR reaction was confirmed, the expression of the chosen genes was determined in SS and water treated wheat respectively. The expression of *18S rDNA* remained constant in Thatcher+*Lr34* plants treated with SS which confirms that equal amounts of RNA were used for all RT-PCR reactions (Fig. 4.8a). SS induced the expression of all the assayed cDNA fragments with a characteristic two step activation being evident for all the cDNA fragments except for the small subunit of Rubisco. The secondary induction resulted in higher levels of expression compared to the primary induction.

The gene encoding the large subunit of Rubisco showed a primary induction of expression at 0.25 hpt that decreased at 1 hpt before the secondary induction started at 2 hpt (Fig. 4.8b). These elevated expression levels were evident up until 24 hpt when a slight decrease was observed. The expression pattern observed for the small subunit of Rubisco differed from that of the large subunit in that a single induction of expression was observed at 12 hpt, much later than the other genes (Fig. 4.8c).

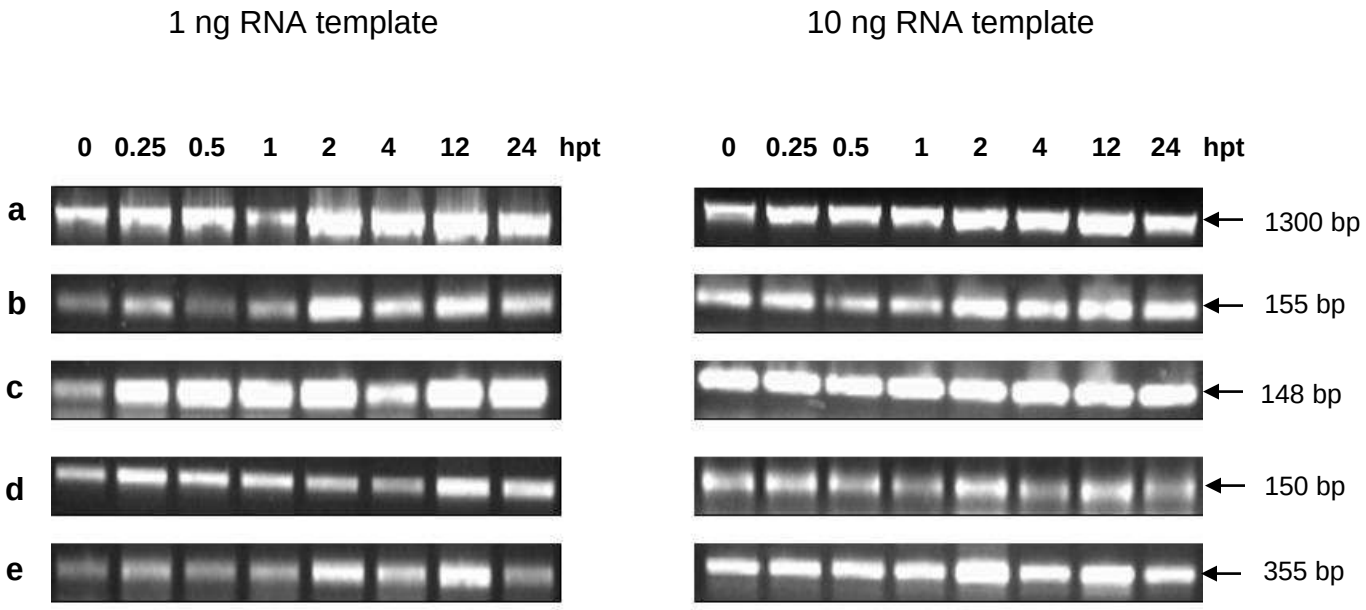


Figure 4.7 – The effect of different amounts of total RNA on RT-PCR reactions. Differences in expression are indicated for genes encoding Rubisco large subunit (a), Ribulose 5'-phosphate kinase (b), ATP synthase CF-1 (c), Rubisco activase (d), and Ptr ToxA binding protein1 (e) at 1 and 10 ng total RNA template. Time intervals are indicated above each figure.

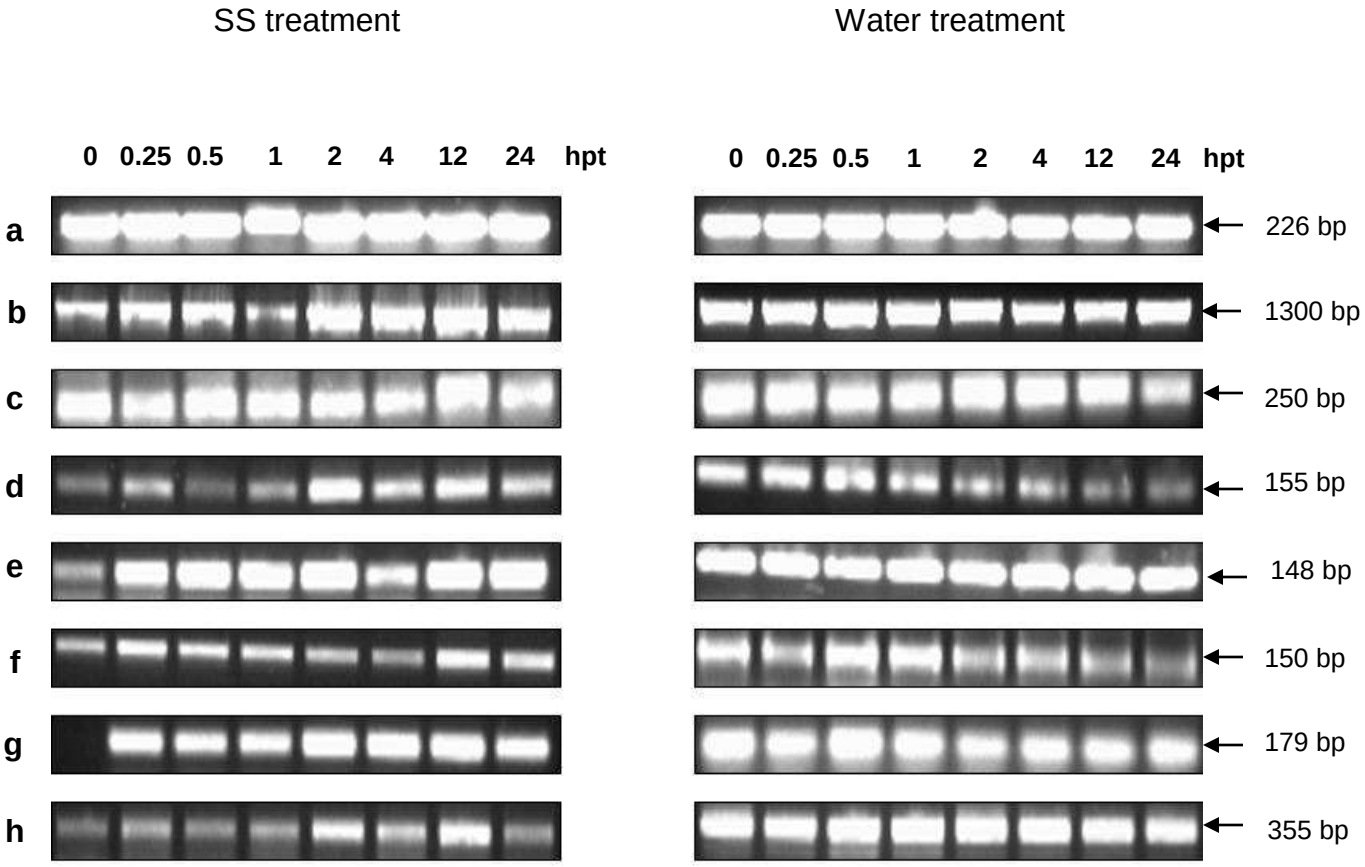


Figure 4.8 – Expression analysis of isolated cDNA clones in Thatcher+*Lr34* plants. Expression patterns and fragment lengths are indicated in SS treated and water treated Thatcher+*Lr34* plants for *18S rRNA* (a) and cDNA fragments encoding Rubisco large subunit (b), Rubisco small subunit (c), ribulose 5'-phosphate kinase (d), ATP synthase CF-1 (e), Rubisco activase (f), phosphoglycerate kinase (g) and Ptr ToxA binding protein1 (h). Time intervals are indicated above each figure.

Similar to what was observed with the large subunit of Rubisco, expression of Ribulose 5'-phosphate kinase (Fig 4.8d), ATP synthase CF-1 (Fig. 4.8e), Rubisco activase (Fig. 4.8f), phosphoglycerate kinase (Fig. 4.8g) and Ptr ToxA binding protein1 (Fig. 4.8h) was induced at 0.25 hpt. All the above mentioned cDNA fragments showed the two step induction of expression that was observed with the large subunit of Rubisco at 2 hpt except for Rubisco activase and ATP synthase CF1 where the secondary induction of expression only occurred at 12 hpt.

The expression of *18S rDNA* remained constant in Thatcher+*Lr34* plants treated with water which confirms that equal amounts of RNA were used (Fig. 4.8a). The expression of all the tested cDNA fragments remained constant throughout all the assayed time intervals of the control plants (Fig. 4.8). A decrease in expression over the 12 and 24 hpt time intervals was observed for ribulose 5'-phosphate kinase (Fig. 4.8d) and Rubisco activase (Fig. 4.8f).

4.7.2.2. Expression analysis of selected cDNA fragments in Thatcher plants

SS treatment was found to also induce the expression of all the assayed genes in the susceptible Thatcher cultivar (Fig. 4.9). The expression patterns observed for the cDNA fragments varied more than in Thatcher+*Lr34* but a two step induced expression pattern was again evident in some samples. The times of the different inductions also differed from those found in the resistant cultivar.

There were small variations in the expression of *18S rDNA* for some of the time intervals in Thatcher plants treated with SS (Fig. 4.9a). This was also reflected in the expression patterns of some genes, like ribulose 5'-phosphate kinase (Fig. 4.9d), Rubisco activase (Fig. 4.9f), the large subunit of Rubisco (Fig. 4.9b) and Ptr ToxA binding protein1 (Fig. 4.9h). Based on this, the expression of these genes seems to be unaffected in the susceptible plants.

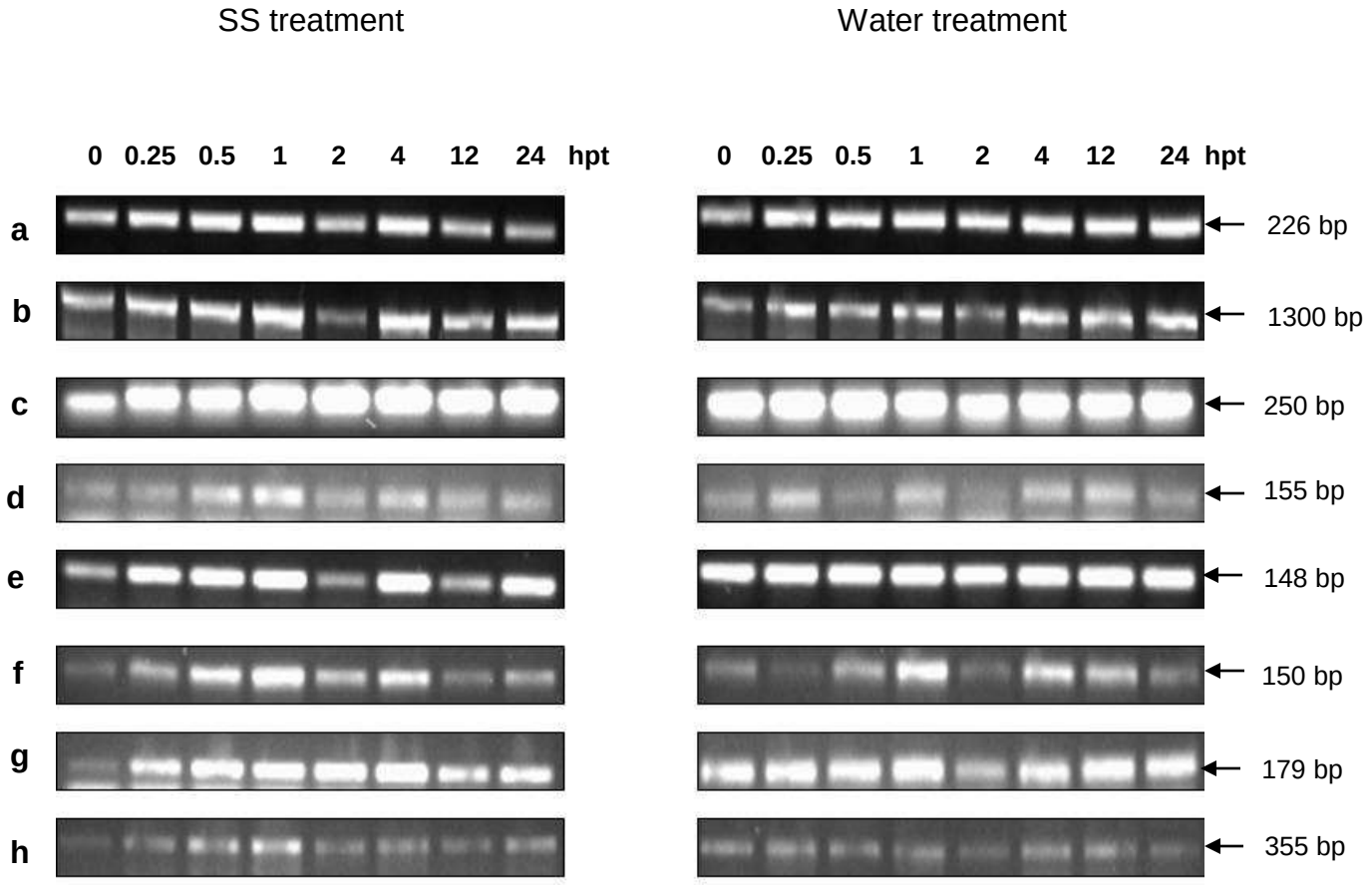


Figure 4.9 – Expression analysis of isolated cDNA clones in Thatcher plants. Expression patterns and fragment lengths are indicated in treated and control Thatcher plants for *18S rRNA* (a) and cDNA fragments encoding Rubisco large subunit (b), Rubisco small subunit (c), ribulose 5'-phosphate kinase (d), ATP synthase CF-1 (e), Rubisco activase (f), phosphoglycerate kinase (g) and Ptr ToxA binding protein1 (h). Time intervals are indicated above each figure.

The expression pattern for the small subunit of Rubisco showed a constant induced expression which started at 0.25 hpt (Fig. 4.9c). Contrasting to the Thatcher+*Lr34* results, there was no decrease in expression present at 24 h after treatment. While the expression pattern of ATP synthase CF-1 resembled that of the 18S control, a clear induction of expression was only evident at 24 hpt (Fig. 4.9e). Phosphoglycerate kinase showed a steady induction of expression starting at 0.25 hpt with no secondary induction being evident as was the case in Thatcher+*Lr34* treated with SS (Fig. 4.9g). Even though the expression at some intervals was similar to that of 18S, a definite induction at 2 hpt was evident.

As was the case with all the other treatments, *18S rDNA* expression remained constant in Thatcher plants treated with water which confirms that equal amounts of RNA were used (Fig. 4.9a). The expression of the large subunit of Rubisco (Fig. 4.9b), the small subunit of Rubisco (Fig. 4.9c), ATP synthase CF-1 (Fig. 4.9e), phosphoglycerate kinase (Fig 4.9g) and Ptr ToxA binding protein1 (Fig. 4.9h) stayed constant throughout all the assayed time intervals with minor variations. Fluctuations in expression levels was however evident for ribulose 5'-phosphate kinase (Fig. 4.9d) and Rubisco activase (Fig. 4.9f).

4.7.2.3. Expression of defense related genes

To evaluate the possible role of SS in the induction of the defense response within wheat, *PR2* was also subjected to RT-PCR. This was done because of the fact that *PR2* is a marker gene for plant defense against pathogens. No induction of expression of the gene was found in any of the tested cultivars after treatment with SS.

Chapter 5
Discussion

5. Discussion

Plant activators are an exciting alternative to current methods used in pest control in agricultural crops. Plant activators generally have two major characteristics, namely being able to induce plant defence against pathogens and increase growth and yield in treated plants (Kessman *et al.*, 1994) Yet, despite all these positive attributes, the molecular mechanisms involved in the action of plant activators on crops are still largely unclear. During this study a plant activator with known positive effects on crops in field trails was evaluated on molecular level. The use of plant molecular biology techniques gave the opportunity to examine possible cellular mechanisms the activator may activate to produce the positive results found in the field trials and to identify which genes are involved in the process.

CC is a commercially available plant activator product that is prepared from a blended extract from 12 different plant species with a suspected brassinosteroid active compound. It has known inductive properties on plant defence against pathogens such as increased expression of *PR* genes (Berger *et al.,* 2004). CC also has a positive effect on photosynthesis and therefore growth as has been shown by its counter regulation of the repression on photosynthesis genes caused by the pathogen *Botrytris cinerea* in tomato (Berger *et al.,* 2004). SS, the plant activator that was used during this study is a purified fraction of CC and was shown to be successful during field trails using different crops at increasing stress tolerance, yield and growth (JC Pretorius, personal communication).

Wheat was selected as the test crop for this study largely because of its importance as an agricultural crop in Africa and due to the lack of plant activator based knowledge currently available. An added bonus for using wheat as a test plant is that although plant activator based work is fairly limited, a number of plant activators have been tested on wheat for possible comparison with SS (Bertini *et al*., 2003; Kogel and Langen, 2005). Two cultivars were treated with

SS, namely the resistant Thatcher+*Lr34* and the susceptible Thatcher cultivar. The resistant Thatcher cultivar differs from the susceptible cultivar in that it has resistance against the fungal pathogen that causes leaf rust which is conferred by the *Lr34* gene (Kerber and Aung, 1999). Thatcher+*Lr34* was used for the gene identification experiments to possibly confirm field results on a molecular level whilst the susceptible line was used in comparison to examine possible differences by SS action in different cultivars. SSH was used as the tool to evaluate gene expression during this study due to the fact that a vast number of potentially differentially induced genes could be identified. Thus, through the use of SSH a more complete picture of SS action in the different cultivars was obtained than would have been the case with other techniques. SSH has been used in plants for large scale gene expression analysis such as for instance the identification of defence related genes in rice and *Arabidopsis* after pathogen infection (Xiong *et al.,* 2001; Dos Santos *et al*., 2003).

The increase in growth and yield by SS in field trials of other crops could possibly be confirmed in wheat on a molecular level due to the increase in photosynthetic capacity mediated by SS. This would appear not to be the case for all potent defence inducing plant activators in wheat. Recently wheat treated with BION®, a commercial plant activator product with a BTH active ingredient, resulted in a decrease in yield when compared to control plants whilst still inducing SAR (Heil *et al.,* 2000). Whilst experiments have shown the potent defence inducing ability of BION®, treated plants achieved lower biomass and produced fewer shoots than control plants possibly due to allocation costs of the induction of SAR that can be detrimental to other metabolic processes (Heil *et al*., 2000). Due to the fact that the induction of growth and yield is not always the case when plant activators are applied to target crops, SS treatment has an obvious advantage especially in wheat.

The fact that the majority of the sequenced cDNA clones showed homology to genes encoding products involved in photosynthesis and metabolism confirms

this statement. A number of genes encoding crucial enzymes involved in photosynthesis showed homology to sequenced cDNA clones. Both subunits of Rubisco was found to be induced by SS treatment. The Rubisco enzyme complex is crucial in photosynthesis where it catalyzes the formation of 3-phosphoglycerate from ribulose-1,5-bisphosphate, CO_2 and water (Mathews *et al.,* 2000). Interestingly, Rubisco is also the main culprit involved in the energy wasting process known as photorespiration which has a detrimental effect on yield (Foyer and Noctor, 2000; Long *et al.*, 2006). Two upstream enzymes involved in photosynthesis have however also been found to be induced by SS namely, Rubsico activase which regulates Rubsico activity (Foyer and Noctor, 2000) and Ribulose-5'-phosphate kinase which phosphorylates ribulose-5'-phosphate to act as substrate for Rubisco. Induced expression of both these genes explains this possible ambiguity. It can therefore be concluded that as a result of the induced expression of these genes, SS application increases photosynthetic capacity within the cell, and hence also yield.

Various genes encoding ribosomal components were also found to be induced by SS treatment again confirming the positive impact that SS treatment has on growth and yield in wheat. A cDNA clone homologous to phosphoglycerate kinase was also found in the sequenced fragments, which indicates that SS might have an effect on respiration of the plants. The enzyme catalyzes the seventh reaction in the glycolytic pathway of cell respiration that oxidates glucose to pyruvate whilst generating ATP (Fernie *et al*., 2004). Another important gene that was found to be homologous to one of the sequenced cDNA fragments was ATP synthase CF-1. ATP synthase is located on the thylakoid membrane of the chloroplast and utilizes the transmembrane proton gradient to form ATP from ADP and phosphate (Groth and Strotmann, 1999). ATP is the most prominent energy source for all living organisms as the hydrolysis of ATP provides the energy needed for energy deficient cellular processes.

SS also induced the expression of a defence related gene. The absence of induction of any significant marker genes for the HR, SAR or ISR suggests that the primary mode of action of SS does not include induction of the defence response against plant pathogens. *PR2* which is a standard marker for plant defence (Sticher *et al.,* 1997) was found not to be induced within the assayed time intervals through RT-PCR testing (Results not shown). It is however possible that the expression of the *PR2* gene is only induced at a later stage due to the fact that during this study, plants were only harvested up to 24 hpt. It must however also be stated that defence related cDNA clones could still be present in the rest of the cDNA clones that still has to be sequenced. *PR2* expression was found during previous experiments to only be induced 48 hpt with CC (Results not shown).

Early induced *PR* gene expression is however commonplace for all the well known plant activators like SA, harpin, BTH, INA and BABA (Friedrich *et al.,* 1996; Jakab *et al*., 2001; Bertini *et al.*, 2003; Peng *et al.*, 2003). No genes encoding products characteristic of other defence processes induced by JA or ROS generating enzymes, were found in this study (Borges *et al*., 2003; Bruce *et al*., 2003). Due to the fact that none of the above mentioned genes were found to be induced by SS application, it can be suggested that if SS induces defence responses it is likely a unique response that differs from the classic model.

Possible evidence for this unique defensive process is the single chloroplast located defence related gene that was induced by the application of SS, namely the gene encoding Ptr ToxA-binding protein1. Ptr ToxA is a host selective proteinaceous toxin produced by *Phyrenophora tritici-repentis* which is the causal agent of tan spot in wheat (Sarma *et al.,* 2005). Ptr ToxA mediates toxicity by internalizing into wheat mesophyll cells where after it localizes into the chloroplasts and mediates chloroplast breakdown and cell death through an as yet unknown mechanism (Sarma *et al*., 2005). It has been experimentally shown recently that Ptr ToxA binding protein1 is the protein which interacts with Ptr

ToxA in the chloroplast and is somehow involved in downstream reactions eventually resulting in cell death (Manning *et al.*, 2007). The precise result of this interaction remains unclear but because of the fact that Ptr ToxA binding protein1 seems to be highly conserved in plants and is expressed in both susceptible and resistant plants, it has tremendous potential for further research (Manning *et al.,* 2007).

Amongst the other sequenced cDNA fragments of interest with slightly lower homologies were a number of fragments that encoded part of the Calmodulin gene. Ca^{2+} is a very important signalling molecule that helps plants respond to a variety of external stimuli including drought, cold, movement, hypoxia, pathogen attack, interactions with symbionts and phytohormones (Snedden and Fromm, 1998). Intracellular changes as a result of Ca^{2+} signalling is dependent on Ca^{2+} binding proteins like Calmodulin. The enzyme has no catalytic activity itself but has the ability to bind Ca^{2+} and subsequently activate target enzymes that continue the signal or mediate cellular responses (Snedden and Fromm, 1998). Finally, large numbers of wheat EST homologies were also found among the sequenced cDNA clones that along with the three potentially novel genes, have potential for further studies.

Expression analysis was carried out using RT-PCR on a number of interesting cDNA clones as well as *PR2* to confirm the results of the reverse Northern blots and to determine a possible mode of action for SS. The RT-PCR's were done in Thatcher+*Lr34* and Thatcher to evaluate the possible differences of SS treatment in susceptible and resistant cultivars. The cDNA fragments that were selected were those that showed homology to the genes encoding the large and small subunits of Rubisco, ribulose 5'-phosphate kinase, ATP synthase CF-1, Rubisco activase, phosphoglycerate kinase and Ptr ToxA binding protein1.

In Thatcher+*Lr34* plants, the expression of all the assayed genes except *PR2* was shown to be induced by SS treatment. A characteristic two step induction

pattern appears to be the mode of action of SS in wheat as was shown by the RT-PCR results with the induced expression starting at 0.25 hpt. The water treatment for Thatcher+*Lr34* stayed constant for the most part, with only a slight decrease in expression that was observed over the latter time intervals for ribulose 5'-phosphate kinase and Rubisco activase.

While some conclusions could be made from the results obtained from the expression analysis in Thatcher plants, some problems were experienced. The *18S rRNA* results in plants treated with SS showed fluctuations in expression levels. All the assayed cDNA fragments did show possible inductions of expression, but when they were compared to the *18S rRNA* results a similar pattern was observed with the results being inconclusive. Only the expression patterns obtained for the small subunit of Rubisco, ATP synthase CF-1 and phosphoglycerate kinase exhibited a different pattern than that of *18S rDNA* and could be seen as induced. SS therefore does seem to be effective in susceptible Thatcher plants based on these results but further RT-PCR analysis would be necessary to confirm this.

The most obvious observation from all the sequencing results is that the majority of sequenced cDNA fragments showed homology to genes encoding chloroplast located products. Since the initial discovery of chloroplast DNA in 1963, vast amounts of genomic work have been done in this area (Sugiura, 2003). Although many of the specific regulatory mechanisms involved in chloroplast DNA remain elusive, what is clear is that the majority of the sequenced genes in the chloroplast genome are involved in photosynthesis and growth in general. Chloroplasts are often also the primary target for pathogens as is the case with ToxA (Manning *et al.,* 2007). Russian wheat aphids have also been shown to inject a phytotoxin that specifically targets chloroplasts and cellular membranes of susceptible plants. It is suggested that maintenance of chloroplast gene expression is essential for resistant plants to overcome stress due to aphid feeding (Botha *et al*., 2006).

This suggests that the primary mode of action by SS in wheat appears to be through the chloroplasts. The overall effect of SS treatment in the plants is likely achieved by induction of expression of genes located in the chloroplast DNA. The microarray analysis of gene expression after BR application in *Arabidopsis* and several plant activators in wheat including BTH and JA, showed no such tendency suggesting that this mode of action may be unique to SS (Goda *et al*., 2002; von Rad *et al*., 2005). BR application induced the expression mainly of genes encoding products involved in BR synthesis and growth although none were similar to what was found in this study (Goda *et al.,* 2002). On the other hand, the SA analogue BTH, induced the expression of genes that are typically involved in SA mediated plant defence responses like *PR* gene expression (von Rad *et al.,* 2005). SS therefore seems to be unique in its action since it targets chloroplast function.

In conclusion, it can be suggested that SS treatment of wheat induced the expression of three sets of genes. These three sets included photosynthetic, glycolytic and defence related genes. The majority of the sequenced cDNA clones thus far showed homology to photosynthetic related genes. Whilst the expression of one plant defence related gene, Ptr ToxA binding protein1 was found to be induced by SS, it is more likely to be involved in the recognition of pathogens than classic SAR and HR responses induced by other plant activators. The main mechanisms of SS action in wheat is likely increased growth due to a higher photosynthetic capacity, higher amounts of intracellular ATP and increased glycolysis as well as a possible detection system for the recognition of potential pathogens.

Future work will mainly concern the sequencing of more of the SS induced cDNA clones as well as further gene expression analysis of these sequenced clones. Due to the fact that there are still many cDNA clones with induction levels between 2 and 4, there are still many potentially important genes that could be discovered. More experimental work can also be conducted in the future on the

obtained results from this study by further elucidating the role SS plays within the chloroplasts, it's role in photosynthesis regulation and it's possible role in plant defence through Ptr ToxA binding protein1.

Chapter 6

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Abstract

Plant activators are an exciting alternative to modern day plant pathogen control procedures. Yet research on plant activators and the mechanisms involved in their action remains limited. The plant activator used during this study, SS, is a purified fraction from the commercial product ComCat® with known positive effects in field trials. The main aims of the study was to confirm the action of SS on a molecular level in wheat, identify genes involved in the process and examine the possible mode of action of the activator.

Leaf rust resistant Thatcher+*Lr34* and susceptible Thatcher wheat cultivars were treated with SS and water. SSH was performed on pooled Thatcher+*Lr34* RNA resulting in 562 putatively induced cDNA clones. Of these, 51 were sequenced. The sequenced clones shared homology with a variety of genes mostly encoding photosynthetic and metabolic products, as well as one plant defence related gene encoding Ptr ToxA binding protein1. Expression analysis confirmed a characteristic two step induction pattern in Thatcher+*Lr34* confirming the induced gene expression

It was concluded that SS application in wheat increases photosynthetic and metabolic capacity on a molecular level thereby confirming field results. The induction of expression of *TaThf1* (Ptr ToxA Binding protein1) by SS suggested a possible unique role in plant defence against pathogens.

Opsomming

Die gebruik van plantaktiveerders bied ‘n opwindende alternatief vir moderne tegnieke om plantpatogene te beheer. Ten spyte hiervan is baie min navorsing op die gebied van plantaktiveerders en hul meganismes tot dusver gedoen. ‘n Gesuiwerde fraksie van die kommersiële produk, ComCat®, bekend as SS, is as plantaktiveerder gebruik gedurende die studie, omdat dit positiewe resultate in veldproewe gelewer het. Die hoof doelwitte van hierdie projek was om die aksie van SS op molekulêre vlak in koring te ondersoek, om gene te identifiseer wat in die proses betrokke is asook om ‘n moontlike meganisme van werking vir SS daar te stel.

Thatcher+*Lr34*, weerstandbiedend teen stamroes, en vatbare Thatcher plante is met SS en water behandel. Saamgepoelde RNA van Thatcher+*Lr34* plante is tydens SSH gebruik. In totaal is 562 moontlik geïnduseerde cDNA klone geïdentifiseer. Die DNA basisvolgorde van 51 van die klone is bepaal. Die meerderheid van die klone het homologie op geen vlak getoon met gene wat vir fotosintetiese en metaboliese produkte kodeer. Een kloon het homologie met die geen wat kodeer vir Ptr ToxA bindingsprotiën1 wat betrokke is by plantverdediging getoon.

Ten slotte is bewys dat behandeling met SS die fotosintetiese en metabolise kapasiteit van koring op molekulêre vlak verhoog. Die induksie van *TaThf1* uitdrukking na SS behandeling dui ook op ‘n moontlike unieke rol van SS in plantverdediging.

Appendix 1:

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