

# **The effect of a novel plant activator on photosynthesis in wheat**

by

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“Scientific work must not be considered from the point of view of the direct usefulness of it. It must be done for itself, for the beauty of science, and then there is always the chance that a scientific discovery may become like the radium, a benefit.”

*Marie Curie*

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# **Abbreviations**



## A

ABA	abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
ACS	1-aminocyclopropane-1-carboxylic acid synthetase
ACO	1-aminocyclopropane-1-carboxylic acid oxidase
ADP	adenosine-5'-diphosphate
APAF 1	apoptotic protease activating factor-1
ASA1	anthranilate synthase
ATP	adenosine-5'-triphosphate
<i>Avr</i>	avirulence

## B

BSA	bovine serum albumin
BTH	benzo (1, 2, 3) thiadiazole-7-carboxylic acid S-methyl ester

## C

CC	ComCat <sup>®</sup>
CER	CO <sub>2</sub> exchange rate
<i>CIEP</i>	chloroplast inner envelope protein
CK II	casein kinase II
Cq	quantification cycle
CTR1	Raf-like serine/threonine (Ser/Thr) kinase
CWDP	cell wall degrading protein

## D

D	plants grown in the light and moved to the dark for 24 hours
d <sub>2</sub> MeJA	deuterated methyl jasmonic acid
dCTP	[ $\alpha$ - <sup>32</sup> P]-deoxycytidine triphosphate
DMPC	dimethyl dicarbonate
dNTPs	deoxynucleotide triphosphates
DTT	dithiothreitol
DyNAzyme	DNA polymerase

## E

E	reaction efficiency
EBR	24-epibrassinolide
EDTA	ethylenediaminetetraacetic acid
EIN4	ethylene insensitive 4
ER	endoplasmic reticulum
ERS1	ethylene response sensor 1
EST	expressed sequence tags
EtBr	ethidium bromide
ET	ethylene
ETI	effector-triggered immunity
ETR1	ethylene response 1
ETS	effector-triggered susceptibility

## F

F <sub>0</sub>	ground state fluorescence
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$F_m$	maximum fluorescence
$F_m'$	maximum steady state fluorescence
$F_s$	minimum steady state fluorescence
$F_t$	steady state fluorescence
$F_v/F_m$	maximum quantum efficiency of PSII
FLS2	flagellin sensing 2
FMS-2	fluorescence monitoring system 2
$\Phi$ PSII	quantum efficiency of PSII

## G

<i>GAPDH</i>	glyceraldehyde-3-phosphate dehydrogenase
<i>GST</i>	glutathione S-transferase

## H

$H_2O_2$	hydrogen peroxide
Hpt	hours post treatment
HR	hypersensitive response/ reaction
HSTs	host selective toxins

## I

IMM	inner mitochondrial membrane
INA	2, 6-dichloroisonicotinic acid
IPCC	intergovernmental panel on climate change
ISR	induced systemic resistance

## **J**

JA                   jasmonic acid

## **L**

L                    plants grown in the light

*Lr*-gene           leaf rust resistance gene

LRR               leucine rich repeat

## **M**

MALDI           matrix-assisted laser desorption/ionisation

MAMP           microbe associated molecular pattern

MAP              mitogen activated protein

MAPK            mitogen activated protein kinase

MAPKK          mitogen activated protein kinase kinase

MAPKKK        mitogen activated protein kinase kinase kinase

MeJA            methyl jasmonate

MeSA            methyl salicylate

MET             methionine

MIQE            minimum information for publication of quantitative real-time PCR experiments

M-MuLV RT     Moloney Murine Leukaemia virus RT

MOPS            3-(N-morpholino)-propanesulfonic acid

## N

NB-ARC	nucleotide binding Apaf-1, R proteins, and CED4 homology
NBS	nucleotide binding site
NBS-LRR	nucleotide binding site-leucine rich repeat
NDP	nucleotide diphosphate kinase 2
NLR	nucleotide-binding oligomerisation domain-like receptors
NO	nitric oxide
NOD	nucleotide-binding oligomerisation domain
NPQ	non-photochemical quenching
NPR1	non-expresser of <i>PR1</i>
NTC	no template control
NTP	nucleoside triphosphate

## O

$O_2^-$	superoxide anion
$ONOO^-$	peroxynitrate
<i>OsBSMT1</i>	<i>Oryza sativa</i> salicylic acid/benzoic acid carboxyl methyltransferase
<i>OsSGT1</i>	<i>Oryza sativa</i> UDP glucose: SA glucosyltransferase

## P

<i>PAL</i>	phenylalanine ammonia-lyase
PAMP	pathogen associated molecular pattern
PCD	programmed cell death
PDO	pectin-derived oligosaccharides
PGAL	phosphoglyceraldehyde

<i>PGK</i>	Phosphoglycerate kinase
<i>PR</i>	pathogen related
<i>PR2</i>	glucanase
<i>PR3</i>	chitinase
PRR	pattern recognition receptor
PSI	photosystem I
PSII	photosystem II
<i>PSI</i> subunit	putative chloroplast photosystem I
PTI	PAMP-triggered immunity
<i>Ptr ToxA BP</i>	<i>Ptr ToxA Binding Protein</i>
PVP	polyvinylpyrrolidone

## Q

QA	primary electron acceptor for PSII
qP	photochemical quenching
qPCR	quantitative polymerase chain reaction

## R

<i>R</i> -gene	resistance gene
<i>RbcL</i>	Rubisco large subunit
<i>RbcS</i>	Rubisco small subunit
RGD	arginyl-glycyl-aspartic
RLK	receptor-like protein kinase
RME	receptor mediated endocytosis
ROI	reactive oxygen intermediates
ROS	reactive oxygen species

RT	reverse transcription
RT-PCR	reverse transcription polymerase chain reaction
RT-qPCR	reverse transcriptase quantitative polymerase chain reaction
Rubisco	ribulose-1, 5-bisphosphate carboxylase/oxygenase
RuBP	ribulose-1, 5-bisphosphate

## S

SA	salicylic acid
SABP2	salicylic acid binding protein 2
SAM	<i>S</i> -adenosyl-methionine
SAR	systemic acquired resistance
SDS	sodium dodecyl sulphate
Ser/Thr	serine/threonine
SS	<i>Lupinus albus</i> L. seed suspension
SSH	suppression subtractive hybridisation
STAND	signal transduction ATPases with numerous domains

## T

TIR	toll/interleukin-1 receptor
TLR	toll like receptor
TMV	tobacco mosaic virus
TRIS	tris-hydroxymethyl aminomethane
TTSS	type-three secretion system
Tween 20	polyoxyethylene sorbitanmonolaurat

## V

VOC                    volatile organic compound



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# **Chapter 1**

## **Introduction**

Wheat is one of the world's most cultivated crops and was known as one of the founder crops that initiated agriculture in the 'Old World' (Zohary, 1999). The first domestication of wheat is believed to have taken place between the second half of the 8<sup>th</sup> and the 7<sup>th</sup> millennium BC and originated from South Western Asia (Zohary, 1999).

Today, the distribution of wheat ranges from the lowlands of northern Mexico to Kazakhstan ([http://www.cimmyt.org/english/wpp/rainf\\_wht/index.cfm](http://www.cimmyt.org/english/wpp/rainf_wht/index.cfm)). It currently provides the world with one-fifth of the calorific input and is grown on more than 200 million hectares worldwide (<http://faostat.fao.org/site/567/DesktopDefault.aspx?PageID=567>). The global demand for wheat is increasing at a faster rate than what is annually produced. This poses the question of how agriculture is going to provide for this growing demand while cultivating the crop on the same area of land. It is thus certain that the increase in agricultural output is of cardinal value.

One of the main concerns regarding agricultural productivity is climate changes that are currently being observed. These changes might be natural, but are mostly due to the influence of man. Agriculture is vulnerable to climate changes. Most countries will be able to adapt to these changes, but not Africa. It is believed that by 2050, crop yield in Africa might decrease by between 10 to 20% due to drought (Jones and Thornton, 2003). This, coupled with the Intergovernmental Panel on Climate Change (IPCC)'s reported increase in temperature of between 0.2 and 0.5°C per decade over the next couple of decades, will have a crucial effect on crop production (IPCC, 2001a; b). If farmers are not able to adjust to these changes, problems regarding crop yield as well as plant health, will be more common.

Researchers have however made progress in the last 20 years regarding the increased productivity of wheat by means of genetic intervention. Two such interventions include the improvement of carbon fixation efficiency in C<sub>3</sub> species (Zhu *et al.*, 2008) and the importance of spike fertility in establishment of yield potential (Fischer, 2007). An increase in the CO<sub>2</sub> levels will increase photosynthetic rates in C<sub>3</sub> plants which will lead to crop yields being 30% higher (Poorter, 1993).

Except for these environmental obstacles, plants are also exposed to a range of pathogens and pests (Zipfel and Felix, 2005). Plants, however, have an effective innate defence response to

overcome infection and infestation by pathogens and pests respectively (Marathe and Dinesh-Kumar, 2003). Included in this response are physical and chemical barriers, as well as the activation of an inducible defence response (Hammond-Kosack and Jones, 1996). Through breeding, wheat cultivars carrying resistance genes against pests and pathogens have been developed (Lagudah *et al.*, 2006; Leonard *et al.*, 2008). Even though effective, pathogens are able to overcome these resistance genes with the development of new virulent races (Jin *et al.*, 2008). Once the plant defence response is breached, diseases threaten crop production with farmers suffering great financial losses due to uncontrolled spreading of these diseases.

To overcome these challenges, farmers across the globe are increasingly turning to chemical treatments to aid food production. The most common chemicals used in current farming communities include fungicides and pesticides. The constant use thereof might negatively affect crop production in the long run with productivity being compromised. Pesticides and fungicides also negatively affect the soil and surrounding environments (Allison *et al.*, 2007). Another downside is that not all small scale farmers can afford fungicides or pesticides to protect their crops.

A new approach in agriculture to improve crop production is the use of plant activators. These activators are proposed to increase yield, growth and plant health amongst others (Melkamu *et al.*, 2008). Natural plant activators include salicylic acid (SA) (Van Wees and Glazebrook, 2003), jasmonic acid (JA) (Choh *et al.*, 2004), harpin (Krause and Durner, 2004), abscisic acid (Zhang *et al.*, 2006) and ComCat<sup>®</sup> (Meaza *et al.*, 2007). A number of synthetic plant activators such as BION<sup>®</sup> and Messenger (Türküsay *et al.*, 2009) also exist. Plant activators act independently of the environment to improve yield and activate the plant's defence responses. The use of plant activators is becoming more important and is making an important difference in farming communities.

Recently, a new plant activator called *Lupinus albus* L. seed suspension (SS) was developed. SS has been shown to improve growth and yield in agricultural and horticultural crops (Van der Watt, 2005). The main aim of this study was to investigate the effect of SS application on wheat. This was firstly done by determining the effect of SS application on photosynthesis by measuring chlorophyll *a* fluorescence. Secondly, its influence on gene expression was studied. The latter was done to determine whether the expression of certain genes is either

induced or repressed after treatment. Thereby, proposed roles of the encoded proteins in the improvement of wheat after SS application will be stated. Finally, a hypothesis on how SS improves photosynthesis in wheat will be made.



## **Chapter 2**

### **Literature review**

## **2. Introduction**

The activation of a protective defence response in plants is a natural process, occurring more often than not. Plants react to all changes they encounter, whether it is an increase in light intensity (Nakano *et al.*, 2009) or a foreign invader (Truman *et al.*, 2007). The plant defence response is thus a normal occurrence.

With an ever increasing world population, and changing climatic conditions, it is important to cultivate specific crop cultivars that are able to withstand the challenges brought about each day. Whether it is chemical or environmental, these cultivars must be able to adjust to changes and still be able to produce high yields to provide in the demand for that specific crop. During the past two decades scientists have been developing products that chemically enhance the plant defence response and thus prepare the plant against possible infection or environmental change. These products are known as plant activators and besides the defence response, also affect growth, rate of photosynthesis, flowering, fruiting, plant metabolism and eventually yield (Van der Watt, 2005). The use of plant activators practically ensures the possibility of producing sufficient food for all nations.

### **2.1 Plant defence as an example of adaptation**

In order to study the effects of a novel plant activator, it is important to first understand the defence response and the mechanisms involved in activating such a response in plants. The response involves a broad spectrum of pathways and signalling mechanisms. The following section will give an overview of the defence response and signalling mechanisms that occur in plants in response to biotic and abiotic stress conditions.

#### **2.1.1 Types of fungal / plant interactions**

Plant pathogens can be characterised either according to their phylogeny or their mechanism of infection. Based upon these characterisations, pathogens are grouped into three classes. The first is known as biotrophs which feed off living tissue and can either have an obligate or non-obligate relationship with host plants. Rust causing fungal pathogens (*Puccinia* spp.),

for instance, are obligate biotrophic pathogens. This means that in order to grow and multiply it needs living plant cells (Jarosz and Davelos, 1995).

After landing on the surface of a leaf a biotrophic pathogen gains entry into the leaf by means of a germinating spore, an appressorium and the formation of a penetration peg. Infection hyphae grow on the surface of the leaf and penetrate the leaf either through the stomata or through open wounds. In this manner they gain access to the host's water and nutrient supply (Underwood and Somerville, 2008). According to Hammond-Kosack and Parker (2003) the plant defence response against biotrophic pathogens is regulated through salicylic acid (SA) dependent defence pathways.

Necrotrophs are able to grow on wounded or weak plants and eventually colonise and kill the host plant (Glazebrook, 2005). They are able to grow as saprophytes outside the host (Agrios, 1988; Slater *et al.*, 2003). Parasitic and saprophytic fitness are two important aspects of necrotrophic fungi. Necrotrophs must be able to colonise, grow and reproduce on both living and dead organic matter to ensure the highest levels of fitness. Leonard (1977) found that saprophytic fitness is however reduced by genes associated with pathogenicity, but no avirulence (*avr*) genes have as yet been linked to the pathogen fitness of *Cladosporium fulvum* and *Magnaporthe grisea* (Leach *et al.*, 2001). The defence response against necrotrophs is regulated through jasmonic acid and ethylene signalling pathways (Hammond-Kosack and Parker, 2003).

### **2.1.2 Pathogen detection**

In order for the plant to detect an invading pathogen, plants use a branched detection strategy (Jones and Dangl, 2006). The first phase is a non-specific detection step where the plant responds to a range of different molecules containing different conserved motifs called microbe- or pathogen associated molecular patterns (MAMPs/PAMPs). After detection, a general defence response is activated to prevent spreading of the invading pathogen. Virulent pathogens can however overcome this general defence response. The second phase of the response is then activated when the resistance (*R*) gene product in the host plant, which is already awaiting the pathogen invasion, interacts with a specific *Avr* gene product from the pathogen. This interaction leads to a stronger defence response which will possibly lead to

the hypersensitive reaction (HR) and systemic acquired resistance (SAR) (Jones and Dangl, 2006).

#### 2.1.2.1 Pathogen detection through PAMPs

Pattern recognition receptors (PRRs) situated in the extracellular matrix of plants (Block *et al.*, 2008), recognise and bind pathogenic PAMPs or MAMPs (Jones and Dangl, 2006). These molecules are conserved structures or motifs that occur on the surface of pathogen membranes and include flagellin, peptidoglycans and lipopolysaccharides (Chisholm *et al.*, 2006).

In mammals the recognition of PAMPs relies on toll like receptors (TLRs) (Zipfel, 2008). These TLRs form the first line of defence where they recognise microbial products and initiate a defence response. Another important mammalian PRR is the nucleotide-binding oligomerisation domain (NOD)-like receptors (NLR) (Akira *et al.*, 2006). NLRs are also called NOD-leucine-rich repeats (LRRs) or CATEPILLAR proteins. This class of proteins consist of 23 cytosolic proteins that all have a conserved NOD region (Inohara *et al.*, 2005). The structure of NOD-LRRs includes a terminal effector binding region for amino acids. The binding region is made up of protein-protein interacting domains. These domains contain the structures necessary for the detection of certain PAMPs (Inohara *et al.*, 2005). NLR proteins are similar to *R*-gene encoded proteins in plants and it is believed that they play an important role in the detection of a pathogen and the generation of a suitable immune response (Chisholm *et al.*, 2006).

Plant PRRs situated in the extracellular matrix of plants (Block *et al.*, 2008), recognise PAMPs and activate the first level of general defence called the PAMP-triggered immunity (PTI) (Jones and Dangl, 2006). Defence responses that are associated with PTI include the activation of MAP kinases (section 2.3.2), cell wall modifications (section 2.4.1) and production of reactive oxygen species (ROS) (2.4.2.1) (Zipfel, 2008). However, PTI does not lead to a clear defence response or an HR. The PTI can be overcome by virulent pathogens that, once provoked, interfere with PTI which cause an effector-triggered susceptibility (ETS) (Jones and Dangl, 2006).

### 2.1.2.2 Pathogen detection through the *R-Avr* interaction

Interference of the plant PTI occurs via pathogenic effectors that are secreted into the plant cells where they either suppress or inhibit PTI (Nomura *et al.*, 2005). The second recognition response then occurs via receptor proteins containing nucleotide binding site - leucine rich repeat (NBS-LRR) motifs which are associated with the gene-for-gene model (Block *et al.*, 2008; Wan *et al.*, 2008). Thus an effector-triggered immunity (ETI) is activated which could lead to the HR and SAR and an effective defence response (Jones and Dangl, 2006). Most R proteins fall into this NBS-LRR containing protein class (Schulze-Lefert, 2004).

One of the most important discoveries in plant pathology was made by Flor (1971). He showed that during an incompatible plant-pathogen interaction, for every gene that cause resistance in a particular plant a complementary *avr* gene is present in the pathogen (Flor, 1971). The gene-for-gene model also proposes that a disease resistance response is activated if the specific *avr* gene product is recognised by an *R* gene product in the plant (Dangl and McDowell, 2006).

The interaction between *R* gene alleles of the host and those of the encoding *avr* gene in the pathogen is very specific (Dangl and McDowell, 2006). Plant R proteins can function in one of two ways. Firstly, it can directly bind to the matching Avr protein from the pathogen which is known as the receptor-ligand model (Jia *et al.*, 2000). This model is supported by different studies where the direct binding of R-Avr proteins is illustrated. One such study was done by Deslandes *et al.* (2006) where they indicated that PopP2, an *avr* protein in *Ralstonia solanacearum*, directly binds to a corresponding *R*-gene, *RRS1-R* in yeast.

The second R protein function model is known as the guard hypothesis. According to this model the pathogenic effector is monitored by the R protein where changes caused by the effector activate the R protein (Jones and Dangl, 2006). The type III effector-induced changes are thus indirectly recognised by the R proteins. There are three proven views concerning the guard hypothesis. The first is that a host contains a specific target for every given effector protein and this target(s) is independent of the corresponding R protein. The second states that by manipulating these target(s) a perturbation is produced by the effector which is then recognised by the corresponding R protein. The third tenet is that these

perturbations are caused as a result of the virulence promoting activity of type III effector proteins (Kim *et al.*, 2005).

## 2.2 Signal transduction pathways

### 2.2.1 Recognition through receptor-like protein kinases

Plant signal transduction pathways are complex with different components like kinases and phosphatases playing different roles in the transduction of a defence signal. Receptor-like protein kinases (RLKs) are key components of both PRR and *R*-gene mediated defence signalling. In *Arabidopsis* alone there are more than 400 RLK genes (Shiu and Bleecker, 2001; Goff and Ramonell, 2007). An RLK consists of three different domains, namely an extracellular ligand binding domain, a transmembrane domain and an intracellular serine/threonine (Ser/Thr) kinase domain. Most of the RLKs in plants belong to the latter receptor kinase subfamily (Hu and Wise, 2008).

One example of a RLK acting as a PRR is the flagellin sensing 2 (FLS2) receptor protein from *Arabidopsis thaliana* (Felix *et al.*, 1999; Chinchilla *et al.*, 2006). FLS2 has all the characteristics of a RLK including a signal peptide, a LRR containing extracellular domain, a transmembrane domain as well as an intracellular Ser/Thr protein kinase domain (Chinchilla *et al.*, 2006). The ligand bound by FLS2 in plants and mammals is flg22, a conserved PAMP of flagellin (Smith *et al.*, 2003). In mammals, this flagellin epitope is formed by the N- and C-terminal of the peptide chain (Felix *et al.*, 1999) that functions as the address-message concept (Schwyzer, 1980). The N-terminal binds to the receptor (address), while the C-terminal of flg22 activates the receptor (message) (Bauer *et al.*, 2001). Chinchilla *et al.* (2006) however indicated that both the address and the message step occurs in the FLS2 protein alone due to the fact that they are perceived in tomato cells expressing the *FLS2* gene in an *Arabidopsis* type manner.

The leaf rust resistance (*Lr*) genes of wheat have been extensively phenotypically characterised (Knott, 1989). In a study done by Feuillet *et al.* (1997) they mapped the *Lrk10* gene to the *Lr10* resistance locus in wheat. *Lrk10* is a typical RLK that acts as a resistance gene against leaf rust pathogens in wheat.

### 2.2.2 Downstream signalling

Signalling events following PRR or R-protein mediated recognition of pathogens involve mitogen activated protein (MAP) kinases that form one of the largest protein kinase groups (Nakagami *et al.*, 2005). The MAP kinase (MAPK) cascade is a diverse internal signalling regulator that is important in plant immunity (Nürnberg *et al.*, 2004). It involves three different ser/thr protein kinases (Shan *et al.*, 2007; Schweighofer and Meskiene, 2008). The first, MAPK kinase kinase (MAPKKK) functions as the starting point of the MAPK cascade. Physical interaction or phosphorylation of the specific receptor itself, intermediate bridging factors and interlinking MAPKKKs cause a receptor-mediated activation of MAPKKK. The latter is activated by means of phosphorylation in a S/T-X<sub>3,5</sub>-S/T motif on two ser/thr residues (Nakagami *et al.*, 2005). The second kinase is MAPK kinase (MAPKK) which is known to be dual-specificity kinase. They phosphorylate MAPKs in a T-X-Y motif on threonine and tyrosine residues (Nakagami *et al.*, 2005; Qi and Elion, 2005). The third is MAPK which is able to phosphorylate a range of substrates like transcription factors and protein kinases. They are promiscuous Ser/Thr kinases (Qi and Elion, 2005).

Whereas phosphorylation activates a MAPK signalling cascade, the inactivation of the same MAPK cascade forms another important aspect of defence signalling. Zhang *et al.* (2007) indicated that HopA11, which is able to remove the phosphate group from phosphothreonine by acting as a phosphothreonine lyase, inhibits the MAPK cascade. *Pseudomonas syringae* that produces the protein is therefore able to suppress the plant innate immune response and promote pathogen infection. However, when plants were treated with flg22, HopA11 might also have been able to suppress the activation of these kinases (Zhang *et al.*, 2007). AvrPtoB, challenged with *P. syringae* pv. *tomato*, has also been found to suppress the activation of a MAPK. The expression of *avrPtoB* in transgenic *Arabidopsis* inhibits flg22 signalling (De Torres *et al.*, 2006) and it thus prevents the activation of MAPK.

The different described MAP kinases can be linked to a wide range of plant responses. In a study done by Bögre *et al.* (1999) it was illustrated that the MAP kinase MMK3 was active in the division of aphidicolin synchronised cell cultures. This protein kinase activity was also temporarily activated during mitosis. The activity of some MAPKs has also been shown to be affected by certain hormones. Examples include the induction of MAPK after treatment

with abscisic acid (ABA) (Mori and Muto, 1997) and the association between Raf-like MAPKKK (CTR1) and ETR1 (section 2.1.3) (Clark *et al.*, 1998). Cold and heat stress also activates the *Arabidopsis* MAPK kinase, MKK2 (Teige *et al.*, 2004).

Once the appropriate signalling network has been activated, the plant initiates its defence response.

## **2.3 Plant defence**

Plant defence can be divided into two different responses. After an infection, the passive defence response is activated. This response is always present in the plant and anticipates pathogen infection (Peumans and Van Damme, 1995). Passive defence can also be seen as the primary innate immune response. After the primary response has been breached, the plant relies on its secondary defence response. This response is also known as the inducible defence response and consists of a branched defence pathway.

### **2.3.1 Passive defence response**

PAMPs are key components of the plant defence response. As already stated, plants depend on their innate immune response to defend themselves against invading pathogens (Zipfel and Felix, 2005). This defence can be seen as a two-layered innate system and the interaction between the *R* and *Avr*-genes forms the basis of this innate immunity in plants (Marathe and Dinesh-Kumar, 2003). This interaction is also recognised as the race specific elicitor response (Ellis *et al.*, 2000). Elicitors are pathogen-derived-molecules which causes a cell-death like effect in plants that is similar to the hypersensitive response (see section 2.4.2.3) (Greenberg and Yao, 2004). In a study done by Nürnberger *et al.* (2004) they indicated that general elicitors are theoretically the same as PAMPs (Zipfel and Felix, 2005). One of the passive responses that can be linked to PTI is cell wall modifications (Zipfel, 2008). These modifications, together with the formation of surface wax and other antimicrobial enzymes, all contribute to the passive defence responses.

To initiate a defence response, the host plant alters the cell wall by means of cell wall degrading proteins (CWDP). This, together with pathogen produced enzymes, affect the



polysaccharide cell wall to actually increase susceptibility of the plant against the pathogen (Cantu *et al.*, 2008a; b). Since biotrophic organisms need to keep the infected host cells healthy in order to survive, biotrophs cause less cell wall lysis compared to necrotrophs. It was found that *Ustilago maydis*, a biotrophic smut fungus, has less CWDP-encoding genes in comparison with the necrotrophic fungi *Fusarium graminearum* (Kämper *et al.*, 2006). CWDP produced by both pathogen and plant cell then generates pectin-derived oligosaccharides (PDOs) that, in turn, act as signals for the activation of a defence response. In a study done by An *et al.* (2005), different PDOs were purified from three regions of *Botrytis cinerea*-infected tomato to characterise the structure and degree of polymerisation by using matrix-assisted laser desorption/ionisation (MALDI). Cell wall modifications like methyl esterification occurred and this together with the fact that healthy tissue had more PDOs present, indicated that PDOs act as signal molecules during pathogen infection (An *et al.*, 2005; Osorio *et al.*, 2008). These cell wall modifications together with the fast deposition of callose and phenolic compounds all play a role in plant defence (González *et al.*, 2006; Kang *et al.*, 2008).

The production of leaf hairs is another defence strategy to protect plants against pathogen infection. Kortekamp and Zyprian (1999) studied the effect of four *Vitis* species after treatment with Tween 20. This detergent caused water droplets to adhere to leaf hairs. After exposure to *Plasmopara viticola*, plants treated with Tween 20 were more resistant to pathogen infection due to hydrophobic hairs on the leave surface compared to plants that were not treated with this detergent.

Once the passive defence response has been breached, plants need to find another, stronger response in order to prevent disease. This response depends on the innate immune system.

### **2.3.2 Inducible defence response**

The secondary defence response, also known as inducible defence response, is known to be more specific and intense than the passive defence response and can include the HR or SAR.

### 2.3.2.1 Hypersensitive response

Programmed cell death (PCD) is a common occurrence in animals during pathogen infection (Yao and Greenberg, 2006). PCD causes proliferation on a cellular level and involves different processes, namely apoptosis and a newly described form called autophagy (Yu *et al.*, 2006). In plants, PCD occurring alongside disease resistance, is called the HR (Heath, 2000). This response causes the death of cells surrounding the primary site of infection (Hammond-Kosack and Jones, 1996). The HR is induced through the genetic interaction between the pathogenic *avr* gene and the plant *R* gene (Dodds *et al.*, 2006). During the hypersensitive response (Fig. 2.1), antimicrobial components are released from the primary infected host cells. These components signal the surrounding plant cells to commit suicide by activating their defence mechanisms, thereby stopping the growth of the virus or biotrophic fungus. Although the infected cells die, the plant is saved because the pathogen is contained (Bent, 2003).

The HR is not an obligatory component of disease resistance. For example, in tomato plants exposed to high humidity, the *Cf* genes are able to cause a resistance response against the fungus *Cladosporium fulvum* without visibly inducing a HR (Hammond-Kosack and Jones, 1996). Another example is the potato *Rx* gene that suppresses the replication of a virus in the absence of a HR (Bendahmane *et al.*, 1999). These studies indicated that a certain threshold must be reached in order to activate a HR (Jones and Dangl, 2006). When this threshold is reached, one of the key plant organelles that play a role in the HR is the mitochondrion (Van der Heiden *et al.*, 2000). This is supported by a study done by Chivase and Carr (1998) where they illustrated that an inner mitochondrial membrane (IMM) enzyme, called AOX, helps to suppress a HR after treatment with cyanide and thus decreases the formation of lesions and necrosis.

Fig 2.1 (a) and (b) illustrates *Arabidopsis thaliana* mesophyll tissue that is infected with downy mildew (*Peronospora parasitica*) (Bent, 2003). In Fig. 2.1 (a) the resistant plant shows a hypersensitive response. A resistance gene recognised the pathogen and blocked its growth. The blue cells at the initial site of infection died due to the hypersensitive response.

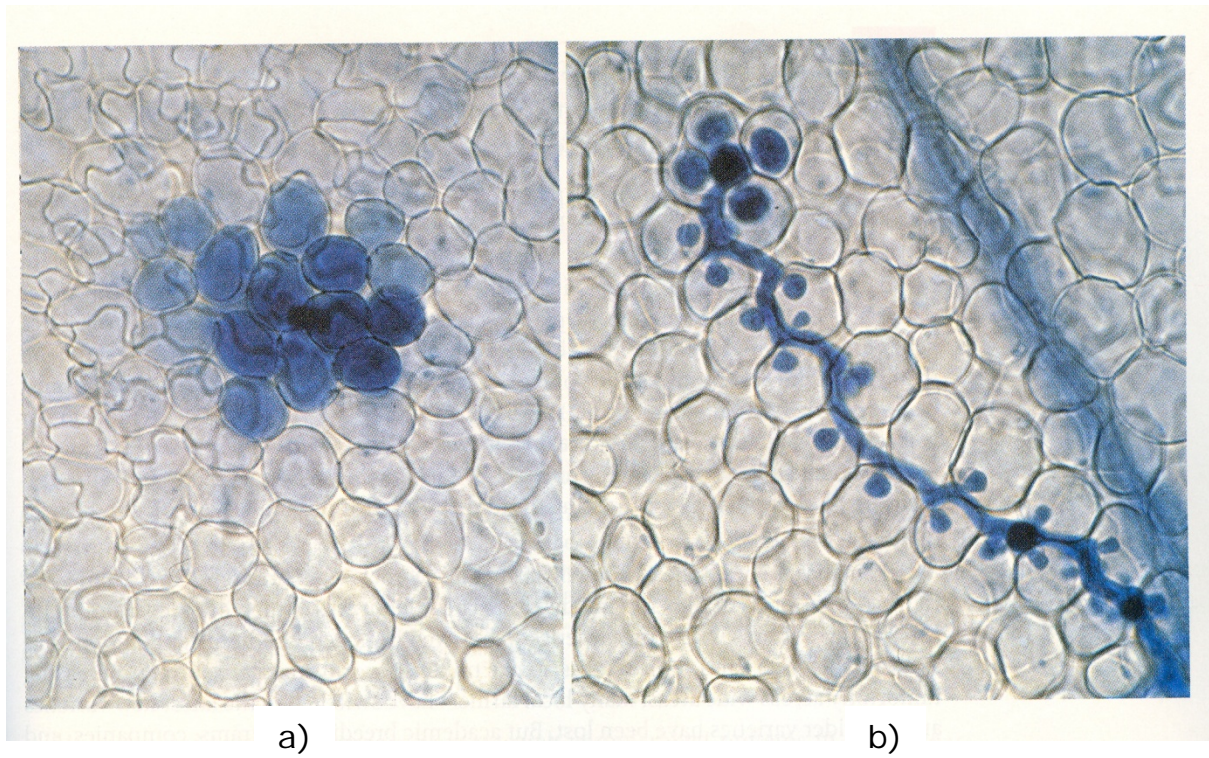


Figure 2.1 The hypersensitive response in *Arabidopsis* plant cells after infection with downy mildew (Bent, 2003). In (a) the resistant plant indicated a HR but the susceptible plant (b) was not able to limit the pathogen to the site of infection.

No hypersensitive reaction occurred in the susceptible plant (Fig. 2.1 b) and the pathogen was able to spread through the leaf tissue. Each cell it reached was penetrated and disease followed (Bent, 2003).

One of the first induced responses after infection by a fungal pathogen is the production of reactive oxygen intermediates (ROI)/ reactive oxygen species (ROS) (Yoda *et al.*, 2006). The oxidative burst might be the trigger that activates the HR (Hammond-Kosack and Parker., 2003). According to Grant and Loake (2000), the production of ROI occurs in two phases. Phase I is a rapid and transient phase while phase II is longer and more sustained. The ROI levels are also higher during phase II (Draper, 1997). The highest rate of oxidative burst occurs between 15 min and 2 - 3 h after infection with a pathogen (Minibaeva and Gordon, 2003).

The production of ROI, including superoxide, hydroxyl radicals and hydrogen peroxide (Bestwick *et al.*, 1997), leads to the oxidative burst. Sources of ROI include NADPH oxidase (Keller *et al.*, 1998) and polyamine oxidases (Yoda *et al.*, 2003). During the polyamine oxidase dependent response, MAPK signalling plays an important role (Yoda *et al.*, 2006). In *Arabidopsis* Kovtun *et al.* (2000) indicated that hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was able to activate MPK3 and MPK6 while it was also able to increase the expression of nucleotide diphosphate (NDP) kinase 2 (Moon *et al.*, 2003). This suggests that ROS production activates MAPK signalling cascades.

ROS are also produced in the chloroplasts where they cause a range of different modifications. This includes alterations in thylakoid and chloroplast proteins as well as degradation of Rubisco (Desimone *et al.*, 1996; Ishida *et al.*, 1997).

The role of nitric oxide (NO) in plant defence signalling is becoming more important. One of the most well known effects involving NO is oxidative damage that occurs when NO interacts with superoxide anion (O<sub>2</sub><sup>-</sup>) (Tewari *et al.*, 2009). This leads to the formation of the oxidant, peroxynitrate (O<sub>2</sub><sup>-</sup>+NO→ONOO<sup>-</sup>) (Mur *et al.*, 2006). In a study by Delledonne *et al.* (2001), the authors illustrated that the production and dismutation of O<sub>2</sub><sup>-</sup> is crucial in the NO/H<sub>2</sub>O<sub>2</sub> trigger during the oxidative burst. The role of NO can, however, not be properly explained without mentioning apoptosis which can be caused due to uncontrolled production

of NO (Bagci *et al.*, 2008). Apoptosis is the process of mammalian PCD that causes the nucleus and cytoplasm to shrivel and the cell is ultimately phagocytised by surrounding cells (Creagh *et al.*, 2003). An important molecule in the process of apoptosis is caspase which is a member of the cysteine protease family (Green and Kroemer, 1998). Caspase is activated by the release of proteins from disrupted mitochondria. One of these proteins is cytochrome *c* which is also released in response to heat (Balk *et al.*, 1999) and oxidative stress (Tiwari *et al.*, 2002).

#### 2.3.2.2 Systematic acquired resistance

SAR is a secondary defence response that provides a plant with the mechanism to protect itself against any subsequent invaders (Ryals *et al.*, 1996). If a plant is infected with a pathogen in only one leaf, the SAR can activate a defence response throughout the whole plant (Métraux *et al.*, 1990). This response is effective even if the plant was not initially resistant to the specific pathogen. In the primary infection the leaf may be seriously damaged with clear necrotic lesions. Necrosis will cause a signal to move through the plant and activate the expression of the SAR genes (Ryals *et al.*, 1996). The resistance response in these tissues is stronger than that in the tissue at the original site of infection. This is due to the fact that the plant has a much stronger defence system this time around (Bent, 2003).

According to Hunt *et al.* (1996) the development of SAR is strongly associated with the biosynthesis of SA and the expression of the pathogenesis related (*PR*) defence genes. The activation of SAR correlates with the induced expression of especially *PR1a* and *PR2* (Uknes *et al.*, 1992).

*PR*-proteins are antimicrobial components (Sels *et al.*, 2008) and the *PR* genes are usually expressed in plant materials after infection by foreign invaders. The *PR*-proteins include the following classes: *PR1a*,  $\alpha$ -1,3-glucanase (*PR2*), chitinase (*PR3*, -4), thaumatin-like (*PR5*), proteinase inhibitors (*PR6*), proteinase (*PR7*), additional chitinases (*PR8*, -11), peroxidase (*PR9*) and ribonuclease-like (*PR10*) (Van Loon and Van Strien, 1999). Van Loon *et al.* (2006) also described other *PR*-protein classes, which included *PR12* – 17.

### 2.3.3 Induced systemic resistance

Induced systemic resistance (ISR) is part of a broad spectrum of resistance (Bostock, 2005). SAR and ISR are phenotypically similar, but ISR is initiated without the expression of *PR*-genes or the presence of SA. It does however respond to jasmonic acid and ethylene (ET) (Bostock, 2005). ISR develops after certain rhizobacteria colonise plant roots (Hammerschmidt, 1999) and is able to protect the plants against fungal, bacterial and viral pathogens (Bostock, 2005).

The non-expresser of *PRI* (*NPRI*) gene plays a role in the activation of the SA signal pathway as well as SAR (Kinkema *et al.*, 2000). Although *NPRI* is a positive regulator of SA signalling, the gene is still necessary for the activation of ISR after infection with a non-pathogenic rhizobacteria (Pieterse *et al.*, 1998). Spoel *et al.* (2003) also stated that *NPRI* acts in the cytosol during ISR and this gene is important in the cross-talk between chemical components in the plant.

## 2.4 Plant activators

Plant activators are compounds that are able to activate the defence response of a plant (Von Rad *et al.*, 2005), improve the growth of the plant ([www.biconet.com/soil/pgaplus.html](http://www.biconet.com/soil/pgaplus.html)) or improve the rate of photosynthesis (Cavalcanti *et al.*, 2006). They are able to affect the ripening of fruit and increase the total yield of crops. To be classified as a plant defence activator, a compound must be able to induce an identical resistance response in the plant compared to a spectrum of different pathogens. When compared to the biological model, neither the activator nor its metabolites should have any direct antimicrobial activity (Kessmann *et al.*, 1994).

Plant activators include a wide spectrum of compounds such as SA, benzo (1, 2, 3) thiadiazole-7-carbotioic acid S-methyl ester (BTH), 2, 6-dichloroisonicotinic acid (INA), JA, ET and ABA. Plant activators can be divided into two groups, namely natural or synthetic plant activators. Several plant activators included in both classes will now be discussed.

## 2.4.1 Natural plant activators

### 2.4.1.1 Salicylic acid

The first study done to show that SA acted as an activator by inducing a defence response in plants, was done three decades ago (White, 1979). He illustrated that, when SA was applied exogenously to tobacco, SA was able to inhibit the disease symptoms caused by Tobacco Mosaic Virus (TMV) by inducing a defence response. This indicated that SA is an important signalling molecule during the plant defence response.

Rairdan and Delaney (2002) found that when transgenic *Arabidopsis* and tobacco plants over-expressed the *NahG* gene, they became more susceptible to virulent pathogens. This was due to the fact that the bacterial *NahG* gene encodes the SA metabolising enzyme, salicylate hydroxylase. Salicylate hydroxylase converts SA to catechol (Yamamoto *et al.*, 1965) thus preventing a plant from accumulating SA and ultimately prevents SA signalling in the presence of a pathogen. Van Wees and Glazebrook (2003) and Anand *et al.* (2008) both indicated that *NahG* containing plants express salicylate hydroxylase and thus cannot produce SA when infected with *Pseudomonas putida* and *Agrobacterium* respectively. These studies confirmed that SA is crucial in the establishment of a defence response and a good example of a plant activator.

A key signalling molecule derived from SA is methyl salicylate (MeSA). SA carboxyl methyltransferase synthesises MeSA which is usually not present in plants (Huang *et al.*, 2003a). The presence of MeSA, a volatile ester, is however induced once pathogen infection occurs (Huang *et al.*, 2003a). Forouhar *et al.* (2005) indicated that salicylic acid binding protein 2 (SABP2) catalyse the conversion of MeSA from SA in tobacco. Once SABP2-silenced tobacco plants were inoculated with TMV, these plants had much lower levels of local resistance and were not able to induce SAR (Kumar and Klessig, 2003). This data suggested that MeSA alone is not capable of inducing a defence response, but that it is an important air-borne signalling component of SAR. This was supported by a study done by Koo *et al.* (2007) where it was apparent that when salicylic acid/benzoic acid carboxyl methyltransferase gene (*OsBSMT1*) over-expressing transgenic *Arabidopsis* plants and wild-type plants were incubated together, the neighbouring wild-type plants induced *PR1* gene

expression. After treatment with SA, these plants indicated little induction of *PRI* and were even more susceptible to pathogen infection. This proved that MeSA is unable to induce a defence response without SA but that its volatile characteristics are clearly prominent between plants.

#### 2.4.1.2 Jasmonic acid

JA, together with its methyl ester (MeJA), plays important signalling roles in the plant defence mechanism (Turner *et al.*, 2002). JA act as a plant hormone but when methylated, it plays an important role in expressing genes involved in plant defence (Pickett *et al.*, 2005). JA is synthesised via the octadecanoid pathway from linolenic acid (Schaller *et al.*, 2005). It occurs in ester and amino acid conjugates in a metabolised form like MeJA, JA-Ile and JA-Leu (Staswick and Tiryaki, 2004).

JA acts as a signalling molecule for the ripening of fruit and root growth (Devoto and Turner, 2003). A MAP kinase pathway is activated and the nucleus reacts to this signal by increasing the production of JA (Schweighofer and Meskiene, 2008). It is however best known for its role in mechanical and herbivory wounding (Baldwin, 1998). When attacked by an herbivore, plants release volatiles and this volatile response is mediated by JA (Heil and Bueno, 2007). The increase in the emission of volatiles after wounding and application of JA, has been reported in different studies (Halitschke *et al.*, 2000; Schmelz *et al.*, 2003). Engelberth *et al.* (2007) indicated that the wound-induced JA release is however limited to the site of infection and the immediate surrounding cells. Tamogami *et al.* (2008) also indicated the role of JA as a volatile with volatile organic compound (VOC) being released from other surrounding plants and a defence response being triggered with the formation of these endogenous jasmonates after *Achyranthes bidentata* plants were treated with deuterated MeJA (d<sub>2</sub>MeJA).

Plants are usually exposed to MeJA or the plants are sprayed with JA (Redman *et al.*, 2001). Choh *et al.* (2004) illustrated that when JA was added to the soil, a strong enough signal was generated to activate a defence response in the plant. Although the application of JA to leaves could activate a defence response, Filella *et al.* (2006) showed that JA application might lead to a decrease in the rate of photosynthesis as well as stomatal conduction. This



study was done using *Quercus ilex* leaves, but the mechanisms involved are not yet clear. When JA is applied exogenously to plants there is an increase in the expression of defence related genes (Baldwin, 1998; Redman *et al.*, 2001). However, when applied to healthy uninfected plants the fitness of the plant was negatively affected, since this led to the loss of unnecessary energy (Baldwin, 1998). This energy loss ultimately influences the development of the plant and crop yield (Pickett *et al.*, 2005), which indicates that the use of JA is both advantageous and disadvantageous.

#### 2.4.1.3 Ethylene

ET is a gaseous hormone that is often associated with plant pathogen infections (Van Loon *et al.*, 2006; Harrach *et al.*, 2008). Application of ET affects germination, plant fitness, fruit ripening, PCD and a range of other factors (Bleecker and Kende, 2000). Both ET and JA play important roles in biotic and abiotic stresses (Van Loon *et al.*, 2006). ET is synthesised from methionine (Met) which is converted from *S*-adenosyl-methionine (SAM) into 1-aminocyclopropane-1-carboxylic acid (ACC). The latter is controlled by the enzyme ACC synthetase (ACS) where-after ET is synthesised from ACC by means of ACC oxidase (ACO) (Von Dahl *et al.*, 2007; Schweighofer and Meskiene, 2008).

In *Arabidopsis*, five membrane-associated receptors, ethylene response 1 (ETR1), ETR2, ethylene response sensor 1 (ERS1), ERS2 and ethylene insensitive 4 (EIN4) receptor recognise the presence of ET (Hua *et al.*, 1998; Sakai *et al.*, 1998). They bind the hormone by means of a copper co-factor (Guo and Ecker, 2004). A negative regulator of the ET response, Raf-like serine/threonine (Ser/Thr) kinase (CTR1), is active when ethylene is not present. The ET receptors together with CTR1, localises at the endoplasmic reticulum (ER). It is believed that ET defence responses are inhibited by means of an active repressor complex (Huang *et al.*, 2003b). Downstream of CTR1, EIN2, EIN3, EIN5 and EIN6 act as positive regulators for ET (Guo and Ecker, 2004).

ET receptors bind ET and then signal an ET defence response to be initiated at the nucleus. The signalling of a response from the ER to the nucleus is mediated by a specific MAPK kinase cascade, including the CTR1-MKK9-MPK3/MPK6 cascade (Schweighofer and Meskiene, 2008; Yoo *et al.*, 2008).

The presence of ET could either lead to the activation of a defence response (Li and Yen, 2008) or it could aid in promoting the development of disease. In a study done by Biles *et al.* (1990) it was shown that the amount of anthracnose lesions in cucumber seedlings increased with about 20% after pre-treatment with ET. They also indicated that when seedlings were pre-treated with an ET inhibitor and then treated with ET, the germination and growth of *Colletotrichum lagenarium* could not be inhibited. Ton *et al.* (2002) found that when *Arabidopsis* plants were exposed to *Pyrenophora syringae* pv. *tomato*, ET was required for the establishment of a basal defence response. The time of application of ET is, however, important (Van Loon *et al.*, 2006). If ET is applied before pathogen inoculation, it either reduces disease development or it has no effect on the development of a disease. However, if ET is administered after pathogen inoculation disease development is increased (Van Loon *et al.*, 2006). This illustrates that ET could also assist a pathogen and thus act as a virulence factor during infection.

#### 2.4.1.4 Harpin

Harpins form a group of bacterial proteins (Keen, 1999) that was first isolated from *Erwinia amylovora* (Peng *et al.*, 2003). Harpins are plant activators due to the fact that they can induce an oxidative burst and PCD in plants (Krause and Durner, 2004). They are released, by the infecting pathogen, into the intercellular tissues of plant cells (Perino *et al.*, 1999).

Two separate studies illustrated that when tobacco was treated with harpin, the generation of  $O_2^-$  and  $H_2O_2$  was not necessary for harpin induced cell death (Sasabe *et al.*, 2000; Xie and Chen, 2000). Desikan *et al.* (1998) found that  $H_2O_2$  and harpin induce different sets of defence genes in *Arabidopsis*. The exogenous application of  $H_2O_2$  induces plant defence genes including phenylalanine ammonia-lyase (*PAL*) and glutathione S-transferase (*GST*). Harpin, however, signals the increased expression of *PAL* and anthranilate synthase (*ASA1*) that is not dependent on  $H_2O_2$ . This indicated that in *Arabidopsis* the signalling mechanism of harpin is a branched signal path that entails a wide range of defence responses (Krause and Durner, 2004).

The type-three secretion system (TTSS) increases the virulence of a pathogen in the host cells (Perino *et al.*, 1999). They aid in the delivery of effector proteins to the host (Hueck, 1998).

TTSS delivers harpins to the host plant (Perino *et al.*, 1999). In tobacco, a receptor-mediated MAPK-dependant signal pathway activates a harpin-induced defence response (Lee *et al.*, 2001). The binding of harpin to the plasma membrane causes a pH shift and this in turn causes the influx of calcium across the plasma membrane and the activation of a defence response (Blume *et al.*, 2000).

In a recent study it was shown that the mitochondria also play an important role in a harpin-induced defence response (Xie and Chen, 2000; Livaja *et al.*, 2008). After treatment with harpin, a reduced mitochondrial membrane potential and a decrease in the production of adenosine triphosphate (ATP) was observed (Xie and Chen, 2000). The results found in Garmier *et al.* (2007) illustrated that mitochondrial ROS might also play a key role in the activation of a defence response. This interaction between harpin and the mitochondria is vital because indirectly the whole plant is affected by a decrease in ATP as all of the main pathways in plants need ATP as an energy molecule to function.

#### 2.4.1.5 ComCat<sup>®</sup>

ComCat<sup>®</sup> (CC) is a commercially produced plant activator. It is a natural bio-stimulant and the active substances are brassinosteroids. ComCat<sup>®</sup> contains a mixture of twelve different plant extracts which includes auxins, gibberellins, brassinosteroids, kinetins, amino-acids and natural metabolites (Schnabl *et al.*, 2001), which enhances plant growth and induces stress tolerance. ComCat<sup>®</sup> also improves the development of roots as well as the rate of photosynthesis in plants (Van der Watt, 2005).

Melkamu *et al.* (2008) illustrated that the pre-harvest treatment of tomato with ComCat<sup>®</sup> increased the quality of fruit during storage. ComCat<sup>®</sup> is a good substitute for chemical activators that is able to increase the yield of crops (Melkamu *et al.*, 2008) as well as vegetables (Schnabl *et al.*, 2001; Workneh, 2002). This bio-stimulant is also known to induce the activity of PR proteins and is therefore effective in the activation of a pathogen defence response (<http://comcat.info/descript.html>).

## 2.4.2 Synthetic plant activators

### 2.4.2.1 Benzo (1, 2, 3) thiadiazole-7-carbotioic acid S-methyl ester (BTH)

The synthetic plant activator BTH is also known as acibenzolar-*S*-methyl (Fig. 2.2). It is distributed by Syngenta Crop Protection in the USA as Actigard and in Europe as Bion (Vallad and Goodman, 2004). The main focus behind the production of BTH was to control powdery mildew in wheat and barley (Görlach *et al.*, 1996; Vallad and Goodman, 2004). BTH is an important defence response inducer (Willingham *et al.*, 2002), but it does not affect the pathogen metabolism like fungicides. In order to determine whether BTH played a role in SAR, researchers infected the susceptible wheat cultivar, Kanzler, with *Erysiphe graminis* f. sp. *tritici* (Görlach *et al.*, 1996). Plants were sprayed with BTH ten days after inoculation and a HR was successfully initiated (Görlach *et al.*, 1996). This was a clear indication that BTH did indeed activate SAR to induce a defence response (Pasquer *et al.*, 2005). While the defence response was initiated without the production of SA (Yasuda *et al.*, 2003), the BTH-initiated response did, however, trigger a similar downstream signal pathway than SA (Yasuda *et al.*, 2003).

*Arabidopsis* plants treated with BTH did indeed induce the expression of *PR1* (Dao *et al.*, 2009). Van Hulten *et al.* (2006) also indicated that BTH treated *Arabidopsis* plants induced *PR1* defence gene expression which, after *Hyaloperonospora parasitica* inoculation, led to reduced pathogen colonisation. BTH does, however, not regulate all PR-defence genes in the same way. Once *Brassica oleracea* seedlings were sprayed with BTH, an induction of  $\beta$ -1,3 glucanase activity and *PR2* gene expression was obtained, while *PR1*, *PR3*, *PR5* expression and chitinase activity was unaffected (Ziadi *et al.*, 2001).

Another example where BTH induced a defence response was in wheat. A study by Görlach *et al.* (1996) showed increased resistance against powdery mildew (*Blumeria graminis*), leaf rust (*Puccinia triticina*) and *Septoria* leaf spot (*Septoria* spp.). BTH also induced a resistance response in peach fruit (Liu *et al.*, 2005) and Yali pear infected with *Penicillium expansum* (Cao *et al.*, 2005). Even though it does not protect all plants against pathogens, the induced defence response is much stronger than responses triggered by SA or JA (Pasquer *et al.*, 2005).

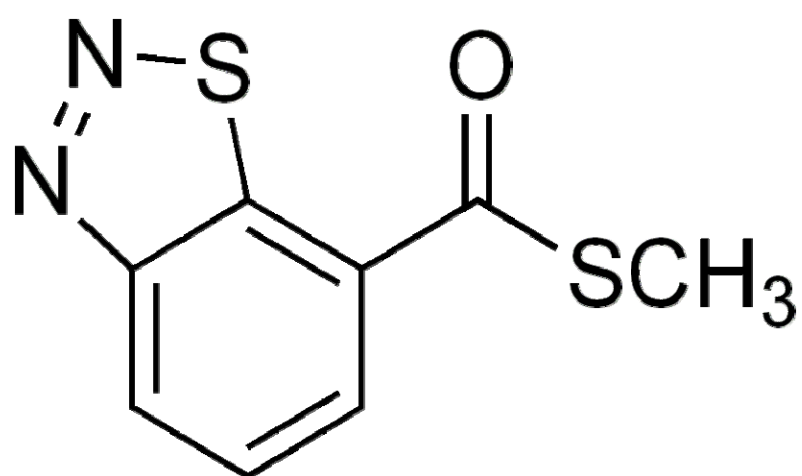


Figure 2.2 The chemical structure of Benzo (1, 2, 3) thiadiazole-7-carbotioic acid S-methyl ester (<http://www.wikipedia.org/wiki/Acibenzolar-S-methyl>).

#### 2.4.2.2 2, 6-Dichloroisonicotinic acid (INA)

INA and BTH are two of the most important chemical activators for the initiation of SAR (Pasquer *et al.*, 2005) and both are able to induce a defence response in the absence of SA (Yasuda, 2007). INA like BTH is believed to signal the induction of SAR via the same signal transduction pathway that SA employs (Vernooij *et al.*, 1995). Another similarity between SA and INA is that both consist of a hexagon-structured ring with a carboxyl group (Fig. 2.3) (Conrath *et al.*, 1995). Basson and Dubery (2007) postulated that the similarity between these two activators might replace central molecules in the ever-present stress-signalling pathway in all plants. They did however find that SA is more effective in the activation of a defence response and that a response is initiated much faster compared to INA.

Research indicated that when a *NahG* expressing plant is sprayed with INA, the defence against *P. infestans* is reduced (Halim *et al.*, 2007). A possible reason for this is that INA is an analogue of SA, and since plants expressing *NahG* is defective in SA dependent signalling (Anand *et al.*, 2008), *NahG* plants treated with INA will also be unable to induce a defence response. It was also found that INA derivatives act as elicitors that can increase secondary metabolism (Qian *et al.*, 2006). In a study done by Umemura *et al.* (2009) it was found that even though rice treated with INA showed a phytotoxic response in leaves, INA was more efficient in the induced expression of the UDP glucose: SA glucosyltransferase (*OsSGT1*) gene.

Even though an INA-induced defence response is not triggered as fast as other plant activators, it is still able to protect a plant against a potential pathogen infection.

#### 2.4.2.3 Messenger<sup>®</sup>

Messenger<sup>®</sup> is a chemical plant activator that is manufactured by Eden Bioscience ([http://www.gardeningthings.com/sm/cimpublic/retrieve.cgi?catalog\\_id=1.11.27](http://www.gardeningthings.com/sm/cimpublic/retrieve.cgi?catalog_id=1.11.27)). It is an excellent product that increases growth and eliminates the use of fungicides and pesticides (<http://gardening.about.com/od/gardenproblems/gr/Messenger.htm>). This activator was

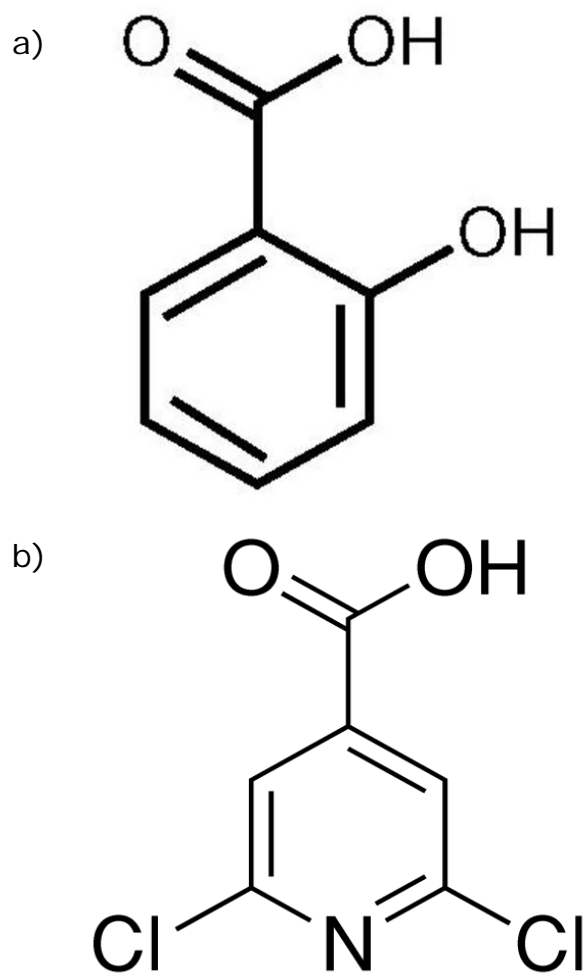


Figure 2.3 The chemical structure of two plant activators. In (a) salicylic acid ([www.drugs.com/pdr/prograniq-ointment.html](http://www.drugs.com/pdr/prograniq-ointment.html)) is shown and in (b) 2, 6-Dichloroisonicotinic acid

([www.sigmaaldrich.com/catalog/ProductDetail.do?N4=456543%7CALDRICH&N5=SEARCH\\_CONCAT\\_PNO%7CBRAND\\_KEY&F=SPEC](http://www.sigmaaldrich.com/catalog/ProductDetail.do?N4=456543%7CALDRICH&N5=SEARCH_CONCAT_PNO%7CBRAND_KEY&F=SPEC)).

developed at Cornell University and the active ingredient is harpin (<http://www.smallfruits.org/SRSFCReserchFunding/Research03/SchermReport03.pdf>).

Messenger<sup>®</sup> activates the plant's defence responses without it being infected with a pathogen. Treatment firstly induces genes involved in immunity, then a growth response is initiated, the rate of photosynthesis increases, more blossoms are produced and the amount of offspring increase (<http://gardening.about.com/od/gardenproblems/gr/Messenger.htm>).

#### 2.4.2.4 *Lupinus albus* L. seed suspension

A *Lupinus albus* seed suspension (SS) was comprehensively investigated for its bio-stimulatory properties as well as its potential to be applied as a natural plant growth regulator in the horticultural and agricultural industries (Van der Watt, 2005). Preliminary bio-tests, including the respiration rate of monoculture yeast cells, seed germination and seedling growth, strongly indicated that the rather crude seed suspension possessed the ability to manipulate these aspects when applied exogenously. Subsequently, the effect of SS on the yields of a variety of vegetable and cash crops was tested under field conditions. Foliar treatment of test crops with SS significantly increased yields. This supplied the rationale for activity directed isolation and purification of the active bio-stimulatory compound which was identified as triglyceride, glycerol trilinoleate. It is postulated that this is a novel plant activator: an aspect that will be elucidated in this study.

## 2.5 Molecular switches involved in plant defence signalling

Plant defence signalling is a crucial part of the defence response and a range of factors affect this response. Some genes and proteins, however, function as molecular switches that are able to activate the signalling response and thus lead to an appropriate defence response in the host.

### 2.5.1 The NB-ARC domain

Most R-proteins contain a nucleotide binding site (NBS) as well as a carboxy-terminal LRR domain (Martin *et al.*, 2003). An NBS-LRR R-protein also carries one of two domains at the



N-terminus, namely a coiled coil domain or a domain that has homology to a Toll/Interleukin-1 receptor (TIR) domain (Pan *et al.*, 2000).

The NBS domain of the R-protein forms part of the nucleotide binding (NB)-ARC domain class (Van der Biezen and Jones, 1998). This is a shared domain between the human apoptotic protease activating factor-1 (APAF-1), its homologue in *Caenorhabditis elegans* called CED-4 and plant R-proteins (Van der Biezen and Jones, 1998). Proteins that contain a NB-ARC domain are related to the mammalian NACHT-LRR (NAIP, CIITA, HET-E, TP1) proteins (NLRs) by means of evolution (Inohara *et al.*, 2005; Leipe *et al.*, 2004). The latter group of proteins as well as the NB-ARC group form part of the signal transduction ATPases with numerous domains (STAND) family of NTPases (Leipe *et al.*, 2004). According to Leipe *et al.* (2004), these proteins regulate signal transduction by functioning as a nucleoside triphosphate (NTP) switch.

The NB-ARC domain is probably also able to act as molecular switch in the plant NBS-LRR proteins (Takken *et al.*, 2006). Takken *et al.* (2006) postulate that in the absence of a pathogen, the NBS-LRR protein is in the resting state. The LRR stabilises the ADP-state by exerting its negative role. However, when a pathogen is present, the LRR is affected by the Avr-elicitor and conformational changes are induced in the NB-ARC domain. ADP is released and a second conformational change is triggered by the binding of ATP to the LRR domain. A signal is released when this change occurs in the N-terminus of the effector protein. A signalling response is attenuated by the ATPase activity of the protein and the protein then returns to the OFF-state (Takken *et al.*, 2006). The exact signalling complexes are however unknown but it is believed that it follows a 'soft-wired signalling complex' mode (Teruel and Meyer, 2000). According to this model the signalling molecules are translocated into a receptor-containing complex where it undergoes reversible binding interactions as well as modifications (Belkadir *et al.*, 2004).

### **2.5.2 The *NPR1* gene**

Different defence related genes contribute to the activation of a defence response in plants. One example is the regulatory protein in *A. thaliana* called NPR1, which is considered a positive regulator for the activation of SAR (Koo *et al.*, 2007). According to Johnson *et al.*

(2008), NPR1 serves as a transcriptional factor and exists as a multimer in the chloroplast cells. After a defence response has been activated and an oxidative burst has taken place, the multimeric NPR1 is reduced to monomers that move into the nucleus of the plant cell. Once inside the nucleus NPR1 interacts with TGA transcription factors to increase their binding to cognate promoter regulatory binding sites and subsequent defence gene expression (Kinkema *et al.*, 2000; Mou *et al.*, 2003). This then activates the SAR and a defence response is initiated throughout the plant. The *NPR1* gene can thus also be seen as a molecular switch that activates defence responses in plants.

The role of SA and JA in plant defence has already been discussed but the antagonistic effect of these two plant activators (Mur *et al.*, 2006; Koornneef *et al.*, 2008) has not yet been mentioned. Genes involved in the activation of a SA dependent defence response includes *PR1.1* and *PR1.2* (Zheng *et al.*, 2006) while those involved in the establishment of a JA-dependant defence response includes *PDF1.2* (Penninckx *et al.*, 1998; Trusov *et al.*, 2009). Koornneef *et al.* (2008) indicated that when *Arabidopsis* is treated with SA, *PR1* gene expression is activated while *PDF1.2* expression is activated after MeJA treatment. The combined application of SA and MeJA led to the suppression of the JA-responsive *PDF1.2* gene expression which indicated the strong antagonistic signalling effect of these activators.

A study done by Kawamura *et al.* (2009) illustrated that after *NPR1* mutant plants were treated with cell wall protein fractions of *Pythium oligandrum*, the induced expression of two JA responsive genes, *PDF1.2* and *JR2*, was negatively affected. They concluded that the activation of ET and JA signalling pathways, in response to CWDP, both need NPR1 to induce SAR. Spoel *et al.* (2003) indicated that the antagonistic effect of JA and SA signalling in *Arabidopsis NPR1* mutant plants requires the regulatory protein NPR1. They found that the suppression of the JA signalling pathway does not require nuclear NPR1. The expression of *PR1* in wild-type *Arabidopsis* plants was induced after SA treatment, but not in *NPR1-3* mutant plants. When the latter was transformed with *NPR1*, the mutant plants again expressed *PR1* after the treatment with SA (Yu *et al.*, 2001). The induction of these defence genes and the fact that *NPR1* plays a central role in the plant, illustrates that *NPR1* acts as a molecular switch in the signalling of a defence response in plants.

### 2.5.3 *Ptr ToxA BPI*

Another possible molecular switch in plants is a recently described chloroplast gene called *Ptr ToxA binding protein 1 (Ptr ToxA BPI)*.

Tan spot is a well known disease in wheat that is caused by *Pyrenophora tritici-repentis* (Friesen *et al.*, 2003). There are five different races of *Pyrenophora tritici-repentis*. Race 3 and 5 produce chlorosis but not necrosis after infection, while race 4 is avirulent on wheat and produces neither necrosis nor chlorosis (Lamari *et al.*, 1995). Both races 1 and 2 produce the host selective toxin called *Ptr ToxA* (also known as *ToxA*). Race 1 produces necrotic lesions that are usually surrounded by chlorosis, while race 2 only produces necrotic lesions (Friesen *et al.*, 2002). Due to the fact that race 2 does not cause chlorosis, its phenotype is used to describe *ToxA* as a pathogenic factor (Ciuffetti *et al.*, 1997). Race 1 can also produce other pathogenic factors and not just *ToxA*, while race 2 only produces *ToxA* (Friesen *et al.*, 2003). *ToxA*, as a pathogenic factor for tan spot, entails that *ToxA* is able to cause disease (Friesen *et al.*, 2003). These results were supported by Ciuffetti *et al.* (1997) when they transformed a non-pathogenic strain of *P. tritici-repentis* into a pathogenic strain by transferring the *ToxA* gene into this strain. This led to the production of the toxin in the non-pathogenic strain.

*ToxA* is a host selective toxin (Friesen *et al.*, 2003) and a single recessive gene known as *Tsn1* on chromosome 5BL of the wheat genome is possibly involved in toxin sensitivity (Faris *et al.*, 1996; Anderson *et al.*, 1999). It is however important to remember that host signalling and *de novo* gene expression is necessary for necrosis development in toxin induced wheat (Kwon *et al.*, 1998). Therefore toxin insensitivity could possibly be a result of mutations occurring in other host genes (Friesen *et al.*, 2002).

*ToxA* can be seen as a pre-pro-protein (Fig. 2.4), consisting of a pre-domain, a pro-domain and the mature protein (Sarma *et al.*, 2005). The pre-domain consists of a signal sequence which guides the protein to the secretory pathway (Tuori *et al.*, 2000). The pro-domain, also known as the N-domain, is involved in the folding of the protein (Tuori *et al.*, 2000) while the mature protein, known as the C-domain, is the functional toxin (Lamari *et al.*, 1995; Tuori *et al.*, 2000). The tertiary structure of *ToxA* displays an arginyl-glycyl-aspartic (RGD) tri-

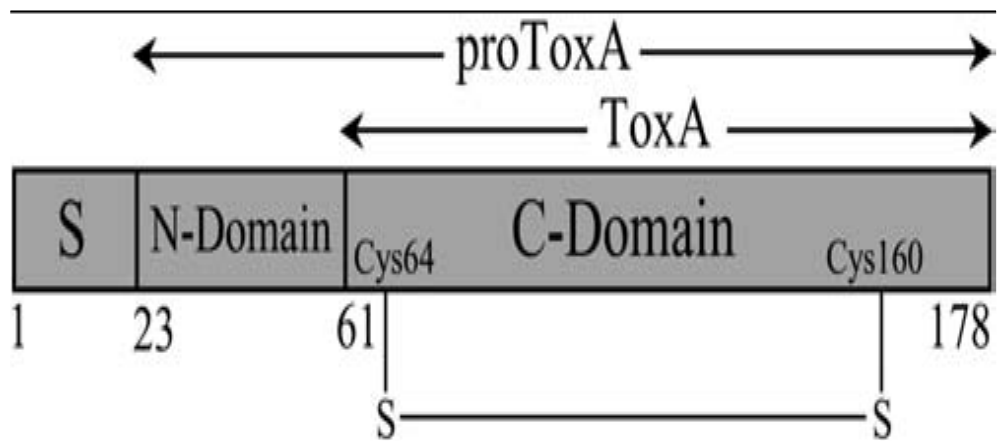


Figure 2.4 The functional structure of Ptr ToxA. The disulfide bond necessary for the formation of the mature ToxA is illustrated and stretches from residue 64 - 160. The signal sequence or pre-domain consists of residues 1 - 22. The N-domain consists of residues 23 - 60 and residues 61 - 178 is part of the C-domain (Sarma *et al.*, 2005).

peptide between residues 140-142. This sequence in ToxA is important in toxin-induced cell death (Manning *et al.*, 2008).

ToxA and a mammalian protein, vitronectin (Hajj *et al.*, 2007), share the RGD peptide sequence (Suzuki *et al.*, 1985; Manning *et al.*, 2004). The RGD sequence in vitronectin interacts with transmembrane proteins, called integrins by means of the cell-attachment motif (Cherny *et al.*, 1993). The cell attachment motif of human vitronectin can be repressed by RGD containing peptides, but this activity can also be increased by phosphorylation of casein kinase II (CK II) (Seger *et al.*, 1998). The binding of the RGD sequence by integrins induces certain cellular processes, like apoptosis, calcium fluxes, protein phosphorylation and dephosphorylation events (Meinhardt *et al.*, 2002). Recent studies (Meinhardt *et al.*, 2002; Sarma *et al.*, 2005; Manning *et al.*, 2008) all indicated that the RGD motif is important for the functioning of the protein.

Vitronectin is present in humans in the cytoplasm, extracellular matrix and blood platelets (Fay *et al.*, 1999). This mammalian protein circulates in the blood in micromolar levels and participates in coagulation, fibrinolysis and in the activation of the complement cascade (Preissner and Jenne, 1991). The role of vitronectin in the immune defence in humans has been illustrated by Singh *et al.* (2005) when they found that the expression levels of vitronectin increased in the lungs of sepsis patients. In a study done by Ekmekci *et al.* (2005) they indicated that the levels of plasma vitronectin increased drastically in patients suffering from coronary artery disease.

The shared RGD motif in vitronectin and ToxA indicates the possibility that these proteins might play similar roles in humans and plants. ToxA is internalised into the mesophyll cells of susceptible wheat cultivars (Manning *et al.*, 2007; 2008) by means of receptor mediated endocytosis (RME). It is however not internalised into ToxA-insensitive mesophyll wheat cultivar cells. Two important aspects of RME are that it is energy and temperature dependent. Temperature dependence of ToxA was proven in a study done by Kwon *et al.* (1998). They found that the effect of the toxin was prevented and host metabolism decreased when plants were incubated at 4°C. They also confirmed that at 30°C ToxA was ineffective. Secondly, the development of ToxA induced cell death was shown to require light (Manning *et al.*, 2007). The dependence on light can be associated with the energy dependence of

RME. After internalisation into the endosomal compartment, the RME receptors have one of two fates. They can be recycled inside the plant cells where they become receptors again or they enter the vacuole or lysosome where they are degraded (Geldner and Jürgens, 2006). ToxA breaks away from the receptor and the endosome, moves into the cytosol and then enters the chloroplast where it matures, alters chloroplast function (Fig 2.5) and ultimately leads to cell death (Manning *et al.*, 2008).

An important aspect of ToxA is how plant signalling mechanisms can protect the host against its toxic actions. Rasmussen *et al.* (2004) studied the effects of host signalling mechanisms on toxins, using the calcium influx as well as protein phosphorylation and dephosphorylation as tools. Using the electrolyte leakage bioassays of Kwon *et al.* (1998), they found that wheat leaves were protected from toxin actions by inhibitors of the plant signalling mechanisms. The inhibitors included okadaic acid and phenylarsine oxide, which inhibits the activity of protein phosphatases. It also included inorganic calcium channel blockers and protein kinase inhibitors. This experiment indicated that for disease development to fully occur, Ptr ToxA need to be internalised (Ciuffetti *et al.*, 1997; Manning *et al.*, 2008) and calcium fluxes as well as the protein phosphorylation cascades are necessary to induce cell death in wheat (Rasmussen *et al.*, 2004).

A recent study found that ToxA and a binding protein called ToxA binding protein 1 (ToxABP1) normally interacts (Manning *et al.*, 2007; 2008). Manning *et al.* (2007) indicated that the threonine 137 of the mammalian vitronectin sequence is necessary for this interaction but the RGD-cell motif is not. This binding protein is found in the membrane and stroma of wheat chloroplasts. ToxA binding protein forms part of a 60 - 70 kDa complex in the membrane and a 45 kDa complex in the stroma. Wang *et al.* (2004) found that the transcript level of ToxABP1 is light regulated which is in agreement with a study done by Manning and Ciuffetti (2005). They found that dark incubation of plants caused a decrease in the ToxA BP transcript level while the movement of plants from dark to light again increased expression (Manning *et al.*, 2007).

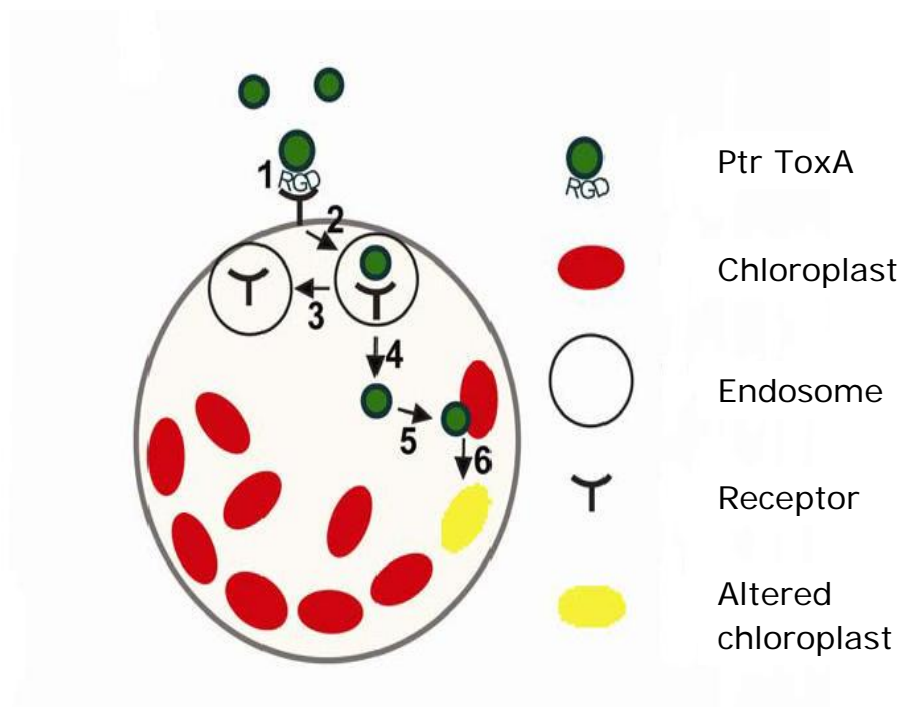


Figure 2.5 Schematic representation of the functioning of ToxA. 1) ToxA binds to the RME receptors by means of the RGD-containing, solvent exposed loop. 2) The RME receptor –ToxA structure is internalised into the endosome. 3) The RME receptor then breaks away and enters the endosome to again become receptors. 4) The ToxA molecule then exits the endosome and enters the cytosol of the plant cell. 5) ToxA then enters the chloroplast and alters chloroplast function. 6) The chloroplast function is irreversibly transformed and this leads to ToxA-induced cell death (Manning *et al.*, 2008).

ToxA also has an effect on the chloroplasts. Keren *et al.* (2005) proposed that the formation of the thylakoid is not prevented by knockout of Thf1 but it does lead to disintegration of the thylakoid membranes. This is caused by means of oxidative stress. This might indicate that the treatment of the ToxA causes the induction of ROS in plants. If this is the case, it is important to try and explain the role of ToxA BP in the chloroplasts and how this toxin affects photosynthesis and ultimately plant defence.



# **Chapter 3**

## **Materials and Methods**

## **3.1 Materials**

### **3.1.1 Plant propagation**

For this study, wheat cultivars that were resistant (Thatcher + *Lr34*) against *Puccinia triticina* were used. Seed was planted in plastic trays in a 1:1 soil and Culterra Acid Compost mixture and kept in the glass house at  $\pm 23^{\circ}\text{C}$ . Plants were watered daily with 50 ml water, while 50 ml 0.2% (w/v) Multifeed fertiliser was applied twice a week. Once the second leaf stage was reached 1% (w/v) 3:2:1 fertiliser was used instead.

### **3.1.2 Preparation of SS**

*Lupinus albus* seeds were firstly ground to a fine powder using a Kenwood coffee grinder and secondly by means of a Freutsch grinder manufactured in Germany. The powdered seeds were transferred to a separating funnel by first extracting with 100% (v/v) methanol. This was followed by a 100% (v/v) methanol:ethyl acetate (50:50) and a 50% (v/v) ethyl acetate extraction, respectively. The fractions were separated and a 90% (v/v) hexane:methanol (50:50) solution was used to further fractionate the ethyl acetate fraction. The two resulting fractions were separated again. The methanol:water fraction was used as SS.

## **3.2 Methods**

### **3.2.1 SS treatment of wheat**

For all plant treatments, SS was dissolved in sterile water to a final concentration of 0.025% (w/v). To improve absorption of SS by the leaves, 0.00025% (v/v) polyoxyethylene sorbitanmonolaurat (Tween 20) was added. Ten day old wheat seedlings were sprayed with the prepared SS solution until micro-droplets were visible on the leaves. Control plants were sprayed with water containing only 0.00025% (v/v) Tween 20. Plant material was harvested at different hours post treatment (hpt). Seedlings were cut just above the soil and immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . A sterilised mortar and pestle was used to grind the leaf material in liquid nitrogen. The mortar and pestle was first washed with dish washing liquid and then with 10% (w/v) sodium dodecyl sulphate (SDS). It was finally

rinsed with 0.1% (v/v) dimethyl dicarbonate (DMPC) treated water, wrapped in foil and autoclaved.

### 3.2.2 Chlorophyll fluorescence measurements

Chlorophyll *a* fluorescence measurement was done using the Hansatech fluorescence monitoring system (FMS-2) according to the manufacturer's instructions. Thatcher + *Lr34* seed was germinated and grown in the glass house at  $\pm 23^{\circ}\text{C}$  for a period of one month. The 0 hpt measurement was taken immediately before SS treatment. Wheat plants were then treated with SS and water as described (3.2.1). Fluorescence measurements of four individual plants were taken for SS and water treatments at every time point for five days each day at noon. Before commencement of each reading, leaves were dark adapted for 5 min using the provided clips. The first reading taken was the ground state fluorescence ( $F_0$ ) followed by the application of a single saturating light pulse for 7 sec. The maximum fluorescence ( $F_m$ ) of a dark adapted leaf was then taken. The actinic light source ( $70 \text{ mol photons.m}^{-2}.\text{s}^{-1}$ ) was then turned on for 20 sec where after the minimum ( $F_s$ ) and maximum ( $F_{m'}$ ) steady state fluorescence were measured. The maximum quantum efficiency of photosystem II (PSII) ( $F_v/F_m$ ), the quantum efficiency of PSII ( $\hat{O}PSII$ ), the proportion of open PSII reaction centres (qP) and non-photochemical quenching (NPQ) were calculated using the following equations (Maxwell and Johnson, 2000):

$$F_v/F_m = (F_m - F_0) / F_m$$

$$\hat{O}PSII = (F_{m'} - F_s) / F_{m'}$$

$$qP = (F_{m'} - F_s) / (F_{m'} - F_0)$$

$$NPQ = (F_m - F_{m'}) / F_{m'}$$

To further evaluate the effect of SS on photosynthetic capacity on water stressed plants, Thatcher + *Lr34* seed was germinated in the glass house (3.1.1) and grown for one month. Chlorophyll *a* fluorescence was measured at 0 hpt where after the plants were treated with SS and water respectively (3.2.1). Photosynthetic measurements for four individual plants were then taken daily at noon for one week after treatment. Plants were then subjected to water stress where they were only watered every third day with 50 ml water. This regime was continued for six days.

The effect of SS treatment on photosynthetic capacity of plants under heat stress was also determined. Thatcher + *Lr34* seedlings were grown in the glass house for 21 days (3.1.1). They were then moved to two separate growth cabinets both at 23°C for one week to acclimatise. Seven days after incubation, photosynthesis was measured (0 hpt). Plants were then treated with SS and water where after the temperature of the one growth cabinet was increased to 37°C. The photosynthetic capacity of both sets of plants was then measured at 24, 48, 72, 96 and 120 hpt.

In the previous experiment plants were treated with SS prior to heat stress. It was however decided to examine the photosynthetic capacity of Thatcher + *Lr34* plants firstly exposed to heat stress and then afterwards treated with SS and water. Thatcher + *Lr34* seedlings were grown in the glass house for 21 days (3.1.1) and then moved to two separate growth cabinets, both at 23°C, to acclimatise. One week later, photosynthesis was measured (0 hpt) and the temperature of one growth cabinet was increased to 37°C. Plants were then incubated for 48 hours at 23°C and 37°C where after photosynthesis was measured for both sets of plants. Both sets of plants were then treated with SS and water and photosynthesis was again measured 24, 48 and 72 hpt.

### **3.2.3 Treatments of wheat seedlings**

#### **3.2.3.1 Light and dark incubation**

Thatcher + *Lr34* seed was germinated, watered and fertilised as described (3.1.1). They were kept in a growth cabinet at ±23°C with a light/dark regime of 12 h light and 12 h dark (1500 micro-einsteins. m<sup>-2</sup>. s<sup>-2</sup>). Seedlings were grown until they reached the third leaf stage. Half of the seedlings were transferred to a completely dark incubator at ±25°C while the other half remained in the light. Leaf material was harvested at 0, 2, 4, 8 and 24 hpt for both the dark and light incubated seedlings as described (3.2.1). The next day half of the dark and light incubated plants were treated with SS and the other half with water respectively (3.2.1). Treated seedlings were then harvested at 2, 4, 8 and 24 hpt and frozen at -80°C. These samples were used for gene expression analysis.

### 3.2.3.2 Dark germination and incubation

Thatcher + *Lr34* seed was germinated and grown in complete darkness at  $\pm 25^{\circ}\text{C}$  (3.1.1). After ten days, half of the seedlings were transferred to a greenhouse at  $\pm 23^{\circ}\text{C}$ . The next day, light and dark incubated plants were harvested at 0 hpt. Both sets of plants were then treated with SS and water respectively, samples harvested at 2, 4, 8 and 24 hpt and used for expression analysis.

## 3.2.4 Gene expression analysis

### 3.2.4.1 Extraction of total RNA

All solutions used for RNA extraction were prepared using water treated with 0.1% (v/v) DMPC to ensure that it was RNase-free. The treated water was left overnight and autoclaved the next morning to destroy the DMPC.

Frozen wheat leaves were ground to a fine powder in liquid nitrogen. Total RNA was extracted from ground leaf tissue using the Trizol<sup>®</sup> Reagent (Invitrogen<sup>™</sup>) according to the manufacturer's instructions. Ground tissue (ca. 100 mg) was resuspended in 500  $\mu\text{l}$  Trizol at room temperature for 10 min where after 100  $\mu\text{l}$  chloroform was added. After incubation at room temperature for 5 min, samples were centrifuged at 12 000  $g$  for 15 min at  $4^{\circ}\text{C}$ . The aqueous phase was recovered and the RNA precipitated with 250  $\mu\text{l}$  isopropanol. After incubation for 10 min at room temperature, tubes were centrifuged at 12 000  $g$  for 10 min at  $4^{\circ}\text{C}$  and the RNA pellet washed once with 75% (v/v) ethanol. The dried RNA was finally dissolved in 200  $\mu\text{l}$  DMPC treated water.

The RNA was treated with 5 U DNaseI (Fermentas) in 10 mM Tris-hydroxymethyl aminomethane (Tris-HCl) (pH 7.5), 2.5 mM  $\text{MgCl}_2$  and 0.1 mM  $\text{CaCl}_2$ . Samples were incubated at  $37^{\circ}\text{C}$  for 30 min where after 5 mM ethylenediaminetetraacetic acid (EDTA) was added and the samples incubated at  $65^{\circ}\text{C}$  for 10 min. After a further chloroform extraction, RNA was precipitated as previously described. The concentration of the extracted RNA was determined spectrophotometrically (Sambrook and Russell, 2000) by reading the absorbance values of diluted samples at 260 and 280 nm.

To determine the quality of the RNA, 500 ng was separated on a 1.0% (w/v) agarose gel prepared in 0.5x TAE [20 mM Tris-HCl pH 8.0, 0.28% (v/v) acetic acid, 0.5 mM EDTA] containing 0.05 g.ml<sup>-1</sup> ethidium bromide (EtBr) (Sambrook and Russell, 2000). Each 500 ng sample was diluted in DEPC water and 3' RNA loading buffer [0.25% (w/v) bromophenol blue, 0.375 M Ficoll] was added. The samples were separated for 45 min at 10 V.cm<sup>-1</sup> using 0.5x TAE as running buffer and the results photographed using the Bio-Rad gel documentation system.

#### 3.2.4.2 Reverse transcription polymerase chain reaction analysis

To evaluate the expression of selected genes following SS treatment of wheat, reverse transcription polymerase chain reaction (RT-PCR) was first used. Table 3.1 indicates a list of all genes that were tested during this study, as well as the gene specific primers used. The fragments in question were amplified using the G-Storm PCR machine (Vacutech). The RobusT II RT-PCR Kit (Finnzymes) was used for amplification according to the manufacturer's instructions. Each reaction contained 10 ng total RNA, 1x optimised reaction buffer, 10 pmol of each primer (Table 3.1), 1.5 mM MgCl<sub>2</sub>, 0.2 mM deoxynucleotide triphosphates (dNTPs), 5 U Moloney Murine Leukaemia virus RT (M-MuLVRT) and 1 U DNA polymerase (DyNAzyme).

The amplification regime was as follows: an initial reverse transcription (RT) step of 48°C for 30 min, a denaturation step of 94°C for 2 min, followed by 30 cycles of 30 sec at 94°C, 30 sec at the specific annealing temperature and 1 min at 72°C. A final elongation step at 72°C for 5 min was included. The amplified fragments were separated on a 1% (w/v) agarose gel (3.4.2.1). For semi-quantitative RT-PCR analysis, PCR cycles were interrupted at certain intervals according to the intensity of the different control bands.

#### 3.2.4.3 Northern blot analysis

Total RNA (15 g) isolated from harvested wheat tissue, was freeze-dried. The RNA was dissolved in 18 l RNA buffer [0.5 M 3-(N-morpholino)-propanesulfonic acid (MOPS), 50% (v/v) formamide, 6.5% (v/v) formaldehyde, 50 g. ml EtBr] and denatured at 65°C for 15

Table 3.1 A list of the genes and their primer sequences that were used during this study. F = forward; R =

<b>Gene</b>	<b>Name of the primers</b>	<b>Forward primer (5'&gt;3')</b>	<b>Reverse primer (5'&gt;3')</b>	<b>Tm</b>	<b>Amplified fragment length</b>
<i>18S rRNA</i>	B249 (f) B250 (r)	TCAAGAACGAAAGTTGGGGG	CTCGTTGAATACATCAGTGTAGCG	61°C	524 bp
<i>Actin</i>	B251 (f) B252 (r)	AACAGAGAGAAGATGACCCAA	CGCACTTCATGATGGAGTTGT	55°C	514 bp
<i>Glyceraldehyde-3-phosphate dehydrogenase</i>	B359 (f) B360 (r)	CAACGCTAGCTGCACCACTAACT	GACTCCTCCTTGATAGCAGCCTT	60°C	354 bp
<i>Ptr ToxA Binding Protein1</i>	B174 (f) B175 (r)	CCGAGGTACTGGACAAGCTTT	CCGGGCAGGTACTACACTT	60°C	355 bp
<i>Rubisco large subunit</i>	B231 (f) B232 (r)	GAAACTAAAGCAGGTGTTGG	AGGTGCATTTCCCAAGGA	60°C	584 bp
<i>Rubisco small subunit</i>	B229 (f) B230 (r)	TACTGGACAATGTGGAAGCTG	GCAATGAAGCTGACGCACT	60°C	155 bp
<i>Chitinase</i>	B363 (f) B364 (r)	AGAGATAAGCAAGGCCACGTC	GGTTGCTCACCAGGTCCTTC	59°C	116 bp
Oligo dT	B21	TTTTTTTTTTTTTTTTVN		37°C	
<i>Phosphoglycerate kinase</i>	B172 (f) B173 (r)	GCCGAGGTACATGAAAGGAAT	GGGCAGGTGGCTGATGTTAT	56°C	179 bp
<i>Chloroplast Inner envelope</i>	B365 (f) B366 (r)	GTCAGCAGCAACCTCATCAA	CGGAGATGCATCAGACTTCA	60°C	205 bp
<i>Chloroplast Photosystem I</i>	B367 (f) B368 (r)	ATCATCATGGTGACGACGAC	GCCGATGATCTGGTCTAGGA	60°C	194 bp

min. Two  $\mu$ l RNA loading buffer [50% (v/v) glycerol, 1 mM EDTA pH 8.0, 0.25% (w/v) bromophenol blue] was added and samples separated on a 1% (w/v) denaturing agarose gel containing 0.41 M formaldehyde for 60 min at 10 V.cm<sup>-1</sup> (Sambrook and Russell, 2000). A 10x MOPS (sodium acetate, 0.5 M EDTA, NaOH, MOPS pH8) running buffer was used. A GeneScreen Plus nylon membrane from Perkin Elmer™ was used for RNA transfer. The membrane was hydrated in water for 5 min and then in transfer buffer (3 M NaCl, 8 mM NaOH). The RNA was transferred to the membrane for 4 h by capillary action using 3 M NaCl and 8 mM NaOH as transfer buffer. After transfer, the membrane was neutralised in 100 ml neutralisation buffer (0.2 M Na<sub>2</sub>HPO<sub>4</sub>, 0.2 M NaH<sub>2</sub>PO<sub>4</sub>) and the RNA was fixed by baking the membrane at 80°C for 15 min.

The membranes were re-hydrated for 5 min in 1x SSC buffer (15 mM Na-citrate, 150 mM NaCl pH 7.0), stained in methylene blue solution (0.3 M Na-acetate, 0.02% (w/v) methylene blue) for 3 min and then destained in DEPC water for 15 min to confirm the quality of the RNA. The membranes were pre-hybridised for 2 h in 50% (v/v) formamide, 6x SSC (0.9 M NaCl, 0.09 M Na-citrate pH 7.0), 5x Denhardt's [0.1% (w/v) Ficoll 400, 0.1% (w/v) polyvinylpyrrolidone (PVP), 0.1% (w/v) bovine serum albumin (BSA)], 0.5% (w/v) SDS, 100  $\mu$ g.ml<sup>-1</sup> Salmon sperm DNA and 1  $\mu$ g.ml<sup>-1</sup> Poli-A at 42°C.

A cDNA fragment encoding the *Ptr ToxA BPI* gene was isolated from SS treated wheat using suppression subtractive hybridisation (SSH) and cloned into pGEM-T easy (Van der Merwe, 2008). This DNA probe was PCR amplified from 10 ng recombinant plasmid DNA in the presence of 10 pmol of each primer (Table 3.1) and a 1x concentration of KAPA Ready mix (KAPA Biosystems). The amplification regime included an initial denaturation step at 94°C for 30 sec, followed by 30 cycles of 94°C for 30 sec, 30 sec at the specific temperature and 72°C for 30 sec. The PCR cycle was concluded with an elongation step for 5 min at 72°C. The amplified fragment was separated on a 1% (w/v) agarose gel (3.2.4.1). After size confirmation, the DNA was purified using the FavorPrep™ PCR/Gel Purification Kit from Favorgen Biotech Corporation. The quality and quantity of the purified fragment was again determined by means of agarose gel electrophoresis.

In order to amplify the *RbcL* fragment, RT-PCR was used (3.2.4.2). The amplification regime was as mentioned above and the results were analysed by means of gel



electrophoresis (3.2.4.1). The amplified product was purified by using the FavorPrep™ PCR/Gel Purification Kit (Favorgen Biotech Corporation) where after the purified fragment was again separated on a 1% (w/v) agarose gel to determine the quality. The purified fragment was then used for hybridisation.

A High Prime DNA Labelling Kit from Roche Applied Sciences was used to label the probe radio-active according to the manufacturer's protocol using 48'Ci [ $\alpha$ -<sup>32</sup>P]-deoxycytidine triphosphate (dCTP) (Separation Scientific). Before adding the probe to the pre-heated hybridisation buffer, it was denatured at 94°C for 10 min. Hybridisation was done overnight at 42°C.

The next morning the membrane was washed twice for 5 min in wash buffer 1 [0.1% (w/v) SDS, 2x SSC (30 mM Na-citrate, 300 mM NaCl pH 7.0)] at room temperature and twice in wash buffer 2 [0.1% (w/v) SDS, 0.2x SSC (3 mM Na-citrate and 30 mM NaCl pH 7.0)] for 15 min at 68°C. The membranes were exposed to a Phosphor-imaging screen and the image developed using the Bio-Rad Personal Molecular Imager.

#### 3.2.4.4 Quantitative analysis

To further quantify the expression of photosynthetic as well as defence genes after SS treatment, quantitative PCR (qPCR) and reverse transcription quantitative PCR (RT-qPCR) analysis was performed using the CFX96 from Bio-Rad. The fluorescence threshold needed for the calculations was set automatically by the CFX96's analytical software.

##### 3.2.4.4.1 Quantitative PCR

Initially the expression of *Ptr ToxA BPI* was tested using qPCR. cDNA was synthesised using the Superscript™ III Reverse Transcriptase kit from Invitrogen. To reverse transcribe RNA to cDNA, 50' M oligo (dT)<sub>20</sub> primer was used for each reaction together with 1' g total RNA and 0.2 mM dNTP mix. The mixture was adjusted to a final volume of 13' l which was then incubated at 65°C for 5 min. The product was snap-cooled on ice and centrifuged rapidly to collect all condensate. To this was added the first-strand buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl<sub>2</sub>), 0.1 M 1.4-dithiothreitol (DTT) and 200 U SuperScript III

reverse transcriptase (200 U.l<sup>-1</sup>). Reactions were incubated at 42°C for one hour and then stopped by incubation at 70°C for 15 min. The cDNA was directly used for qPCR analysis.

To optimise the primers used during this study, a gradient and standard curve qPCR reaction was done for each primer set. The iQ<sup>TM</sup> SYBR<sup>®</sup> Green Supermix from Bio-Rad was used.

Each reaction had a total volume of 10<sup>1</sup> and contained 10 ng cDNA, 10 pmol of the gene specific primers and a 1x concentration of iQ<sup>TM</sup> SYBR<sup>®</sup> Green Supermix (Bio-Rad). The amplification regime was as follows: an initial denaturing step at 95°C for 5 min followed by 40 cycles of 95°C for 10 sec and 50-60°C for 60 sec. Finally, a melting curve increasing 0.5°C from 65°C to 95°C every 5 sec was added to ensure that no primer dimers were formed.

#### 3.2.4.4.2 Reverse transcription quantitative PCR

For RT-qPCR analysis, the iScript<sup>TM</sup> One-Step RT-PCR kit with SYBR<sup>®</sup> Green (Bio-Rad) was used. Firstly the optimisation of primers was done by running a gradient RT-qPCR reaction as well as a standard curve for each primer set. The gradient reaction contained 10 ng total RNA, 1x SYBR Green RT-PCR reaction mixture, 10 pmol of the specified primer pair and 0.2<sup>1</sup> iScript reverse transcriptase. The gradient RT-qPCR reaction included an initial reverse transcription step at 50°C for 10 min, a 95°C denaturing step for 5 min and 40 cycles of 95°C for 10 sec and 50-65°C for 30 sec. Lastly a melting curve was added incrementing 0.5°C from 65-95°C every 5 sec. The gradient results were confirmed by running the quantitative PCR product on a 1% (w/v) agarose gel (3.4.2.1). The specific temperature, at which no primer dimers but quantitative amplification occurred, was chosen as the optimal temperature for that specific primer pair.

After primer optimisation was completed, the expression of the experimental genes was tested. All reactions were done in triplicate. A 10 fold dilution series was used for the standard curve of all genes ranging from 100 ng total RNA to 0.0001 ng. All the other reactions contained 10 ng total RNA. Each RT-qPCR reaction consisted of 1x SYBR Green RT-PCR reaction mix (Bio-Rad), 10 pmol of each primer pair and 0.2<sup>1</sup> iScript reverse transcriptase. The amplification regime was as follows: an initial 50°C for 10 min RT step, a

denaturing step at 95°C for 5 min, 40 cycles of 95°C for 10 sec and 30 sec at the specific annealing temperature and lastly a melting curve from 65°C increasing every 5 sec by 0.5°C to 95°C. The amplified fragments were separated on a 1% (w/v) agarose gel (3.4.2.1).

The efficiency of each reaction was evaluated by using the following equation:

Reaction efficiency (E):  $E = 10^{(-1/\text{slope})}$  (Rasmussen, 2001).

The relative quantification for each target gene was expressed in comparison with the *GAPDH* reference gene according to the  $\Delta\Delta\text{CT}$  equation:

Ratio =  $(E_{\text{target}})^{\Delta\text{CP}_{\text{target}} [\text{control} - \text{experimental}]} / (E_{\text{reference}})^{\Delta\text{CP}_{\text{reference}} [\text{control} - \text{experimental}]}$  (Pfaffl, 2001).

### 3.2.5 DNA sequencing

The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used as the reference gene for all real-time reactions due to the fact that it is expressed constitutively throughout the wheat life cycle (Larsen, 2003). *GAPDH* primers specific for *Triticum aestivum* were designed using Primer 3 (<http://frodo.wi.mit.edu/primer3/>). However, RT-PCR amplification of the expressed gene yielded a larger DNA fragment than expected. The fragment was sequenced to confirm its identity.

The DNA fragment was amplified using 100 ng cDNA, 10 pmol primers and a 1x concentration of KAPA Ready mix (KAPA Biosystems). The amplification regime consisted of an initial 94°C step for 30 sec, followed by 30 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec and lastly a single 72°C step for 5 min. Half of the PCR product was separated on a 1% (w/v) agarose gel and visualised using the Bio-Rad gel documentation system (3.4.2.1). Twenty ng of the PCR product was sequenced using the ABI PRISM<sup>®</sup> BigDye<sup>®</sup> Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The reaction also contained 1 µl Sequencing Ready Reaction mix, 3.2 pmol of the forward or reverse primer and a 1x sequencing buffer (1x final concentration). The amplification regime consisted of a

96°C for 1 min denaturing step and 25 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min.

The sequenced products were purified by adding 5'1 of 125 mM EDTA and 60'1 100% ethanol. The solution was mixed and incubated at room temperature for 15 min. Samples were centrifuged at 12 000 g for 15 min at 4°C, the supernatant removed and the pellet washed with 60'1 70% (v/v) ethanol. The tubes were centrifuged for 5 min at 12 000 g at 4°C and the pellet air-dried in the dark. The nucleotide composition of each sample was analysed by using the Applied Biosystems 3130xl Genetic Analyser.

# **Chapter 4**

## **Results**

During a previous M.Sc study completed by Christiaan van der Merwe (2008) the influence of a novel plant activator called SS on gene expression in the Thatcher and Thatcher + *Lr34* wheat cultivars, was tested. This was done using SSH. The majority of differentially expressed genes identified during the study coded for photosynthesis related and chloroplast associated proteins, while a number of unknown genes were also isolated. One novel gene that was identified coded for the Ptr ToxA binding protein 1, a protein that could be involved in plant defence and photosynthesis.

#### **4.1 Effect of SS treatment on photosynthesis in wheat**

Chlorophyll *a* fluorescence was used to evaluate the influence of SS on photosynthesis. Following a 5 min dark adaptation, fluorescence in leaves was raised to its maximum value ( $F_m$ ) from the ground state ( $F_0$ ) when a saturating light pulse was applied. The primary electron acceptor for photosystem II (PSII), QA, is reduced and the maximum quantum efficiency of photosystem II is determined. Under normal conditions, the value given by the equation  $F_v/F_m = (F_m - F_0)/F_m$  for a healthy plant, should be close to 0.8. Photo-inhibition that occurs when plants are exposed to different stresses will cause a value of less than 0.8.

Following constant illumination, the lag phase that occurs prior to carbon fixation causes an increase in photosynthetic yield when a certain proportion of QA is reduced. When a steady state value,  $F_t$  is reached, fluorescence is subsequently quenched. Application of a second saturation flash containing actinic light, will give  $F_m'$  which ought to be lower than  $F_m$ . The photochemical part of quenching is represented by the difference between  $F_m'$  and  $F_t$ . Quenching caused by heat dissipation is represented by the difference between  $F_m$  and  $F_m'$ . This data was used for the calculation of the different fluorescence parameters.

The  $F_v/F_m$  values represent the maximum quantum efficiency of PSII and are an indicator of the plant's photosynthetic potential. The quantum yield of PSII ( $\hat{O}PSII$ ) measures the absorption of light through chlorophyll that is associated with PSII and which is used in photochemistry. NPQ measures the energy that is lost in the form of heat in the light harvesting antenna of PSII. The photochemical quenching (qP) is an indication of the open reaction centres when plants are exposed to light.

#### **4.1.1 Influence of SS treatment on photosynthesis in non-stressed wheat**

Chlorophyll *a* fluorescence was used to determine the effect of SS on normal healthy wheat. From the  $F_v/F_m$  values, it was apparent that the maximum quantum efficiency of PSII in SS treated plants gradually increased from 24 to 96 hpt, while that of the water treatment remained constant (Fig. 4.1a). Similarly, the NPQ levels for the SS treatment decreased by nearly half with a smaller decrease for the water treatment (Fig. 4.1c). In contrast, the  $\hat{O}PSII$  (Fig. 4.1b) and qP (Fig. 4.1d) levels were nearly identical for both the SS and water treatments, indicating a small positive effect of SS treatment on the photosynthetic ability of wheat.

#### **4.1.2 Influence of SS treatment on photosynthesis in water stressed wheat**

Since SS treatment of non-stressed wheat did not have a dramatic effect on photosynthetic capacity (Fig. 4.1), it was decided to investigate whether stressed plants would react stronger to the SS treatment. During this experiment, chlorophyll *a* fluorescence was used to examine the effect of SS treatment on wheat exposed to water stress. Plants were grown under normal glass house conditions and watered daily for five days. Thereafter they were watered only every third day.

The first measured parameter was  $F_v/F_m$  (Fig. 4.2a). Whereas the water treated plants had a normal basal value before treatment, the SS plants showed a much lower value, indicating a possible stress condition. This value improved dramatically 24 hpt to nearly the same as that of the water treatment. In both cases, the plant's photosynthetic potential rose until 96 hpt. When exposed to water stress, the  $F_v/F_m$  values decreased dramatically reaching the lowest level at 216 hpt. There was no clear difference between the responses of the SS and the water treated plants.

Secondly, the  $\hat{O}PSII$  values were calculated (Fig. 4.2b). Both the SS and water treatments had roughly the same pattern. A sharp initial increase was found reaching the highest values at 72 hpt where after a steady decline was evident for both the SS and water treated plants. The only real difference between the two treatments was at 240 hpt where the  $\hat{O}PSII$  value of

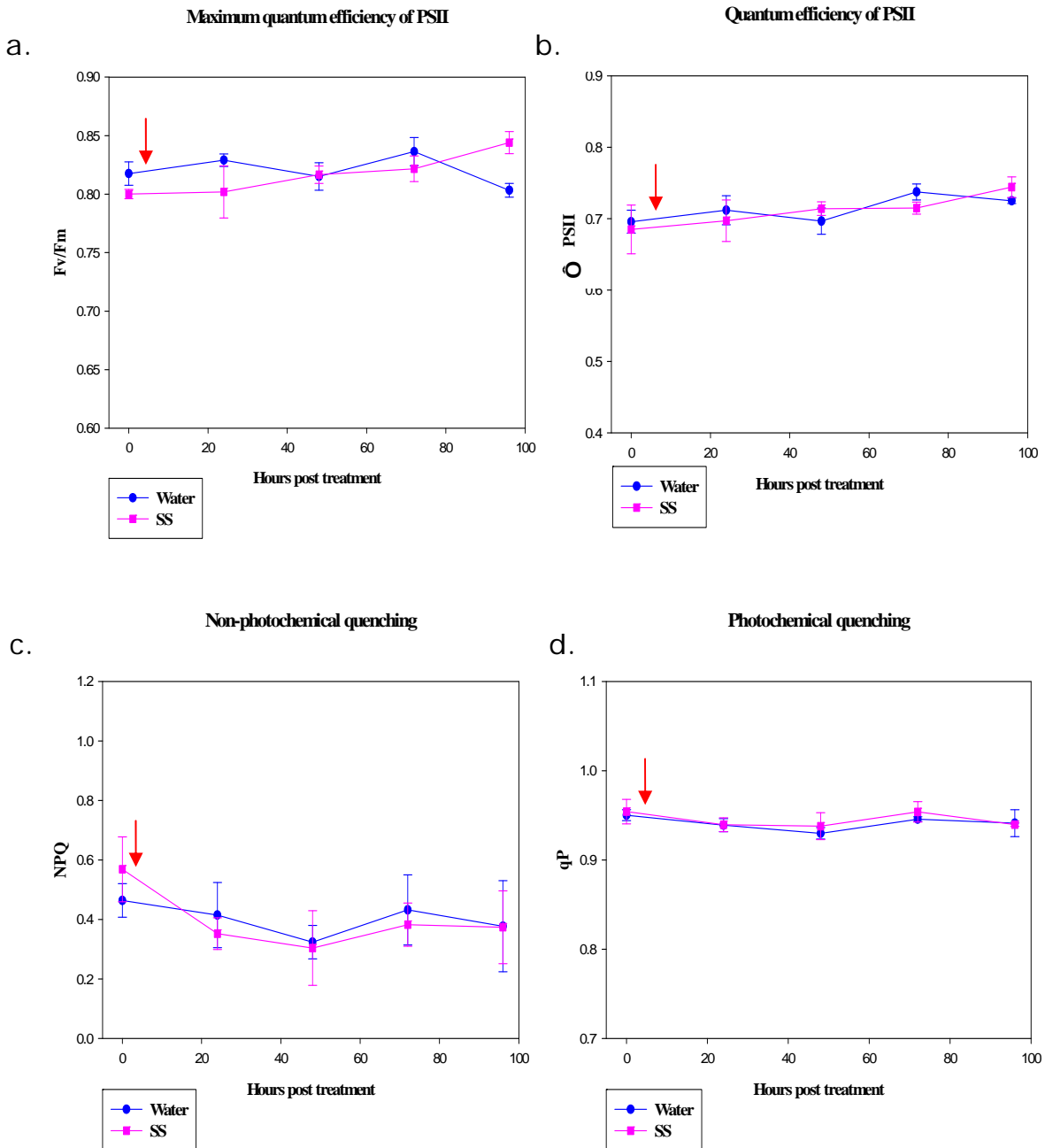


Figure 4.1 Analysis of photosynthetic capacity of healthy wheat after treatment with SS. In (a), the maximum quantum efficiency of PSII is illustrated, in (b) the quantum efficiency of PSII, in (c) the non-photochemical quenching and in (d) the photochemical quenching. Red arrows indicate when SS and water treatments were given.



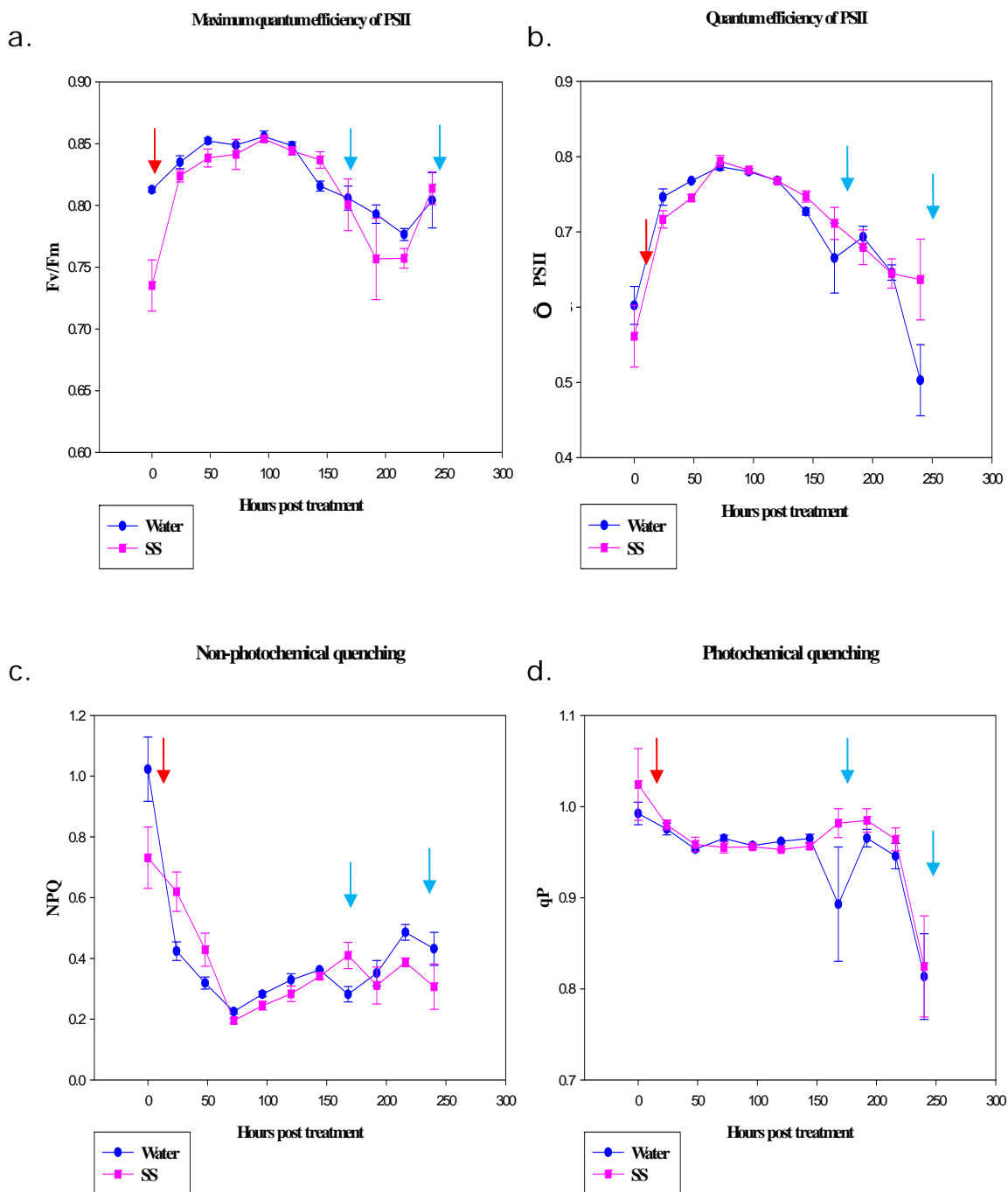


Figure 4.2 Analysis of photosynthetic capacity of water stressed plants after treatment with SS. In (a)  $F_v/F_m$  is shown, in (b) the quantum efficiency of PSII, in (c) the non-photochemical quenching and (d) photochemical quenching. Red arrows indicate the application of SS and light blue arrows indicate the times when plants were watered after the onset of water stress conditions at 96 hpt.

the SS treated plants was higher than that of the water treated plants. What was also evident was that the decline in  $\hat{O}PSII$  levels in the SS treated plants was less dramatic than that of the water treatment, levelling off at 216 hpt.

NPQ was the third parameter that was analysed (Fig. 4.2c). This graph indicated that after SS and water treatment at 0 hpt, there was a sharp decrease in heat dissipation until 72 hpt. Thereafter, the NPQ values increased until the end. The biggest decrease was found in the water treated plants.

The last parameter was qP (Fig. 4.2d). Both the SS and water treatments indicated similar levels of open reaction centres over the whole time trial with a minor difference at 168 hpt. The final stages showed a sharp decrease in qP values when plants were experiencing the water stress.

In summary, it was clear that both the SS and water treated plants were under stress at the start of the experiment as indicated by all four parameters at 0 hpt (Fig. 4.2). These values returned to more or less normal after the treatments.

#### **4.1.3 Influence of SS treatment on wheat placed under heat stress**

Heat dissipation is an important aspect of photosynthesis and exposure to heat stress affects plants in different ways. The effect of heat stress on photosynthetic potential of wheat, after the application of SS, is however unknown. During this experiment, plants were treated with SS and water and then exposed to a rise in temperature. Control plants were treated with SS and water respectively and kept at 23°C, while the heat stressed plants were treated with both SS and water and then kept at 37°C.

The  $F_v/F_m$  values indicated a clear difference in the photosynthetic capacity of control plants and those exposed to heat stress (Fig. 4.3a). Similar to Fig. 4.1a, there was little difference between the maximum quantum efficiency of SS and water treated plants incubated at 23°C. However, as soon as heat stress was applied there was a decrease in the  $F_v/F_m$  values for both SS and water treated plants. After an initial decrease at 24 hpt, the SS treated plants at 37°C showed a stable and higher level of photosynthetic potential than the control plants until 96

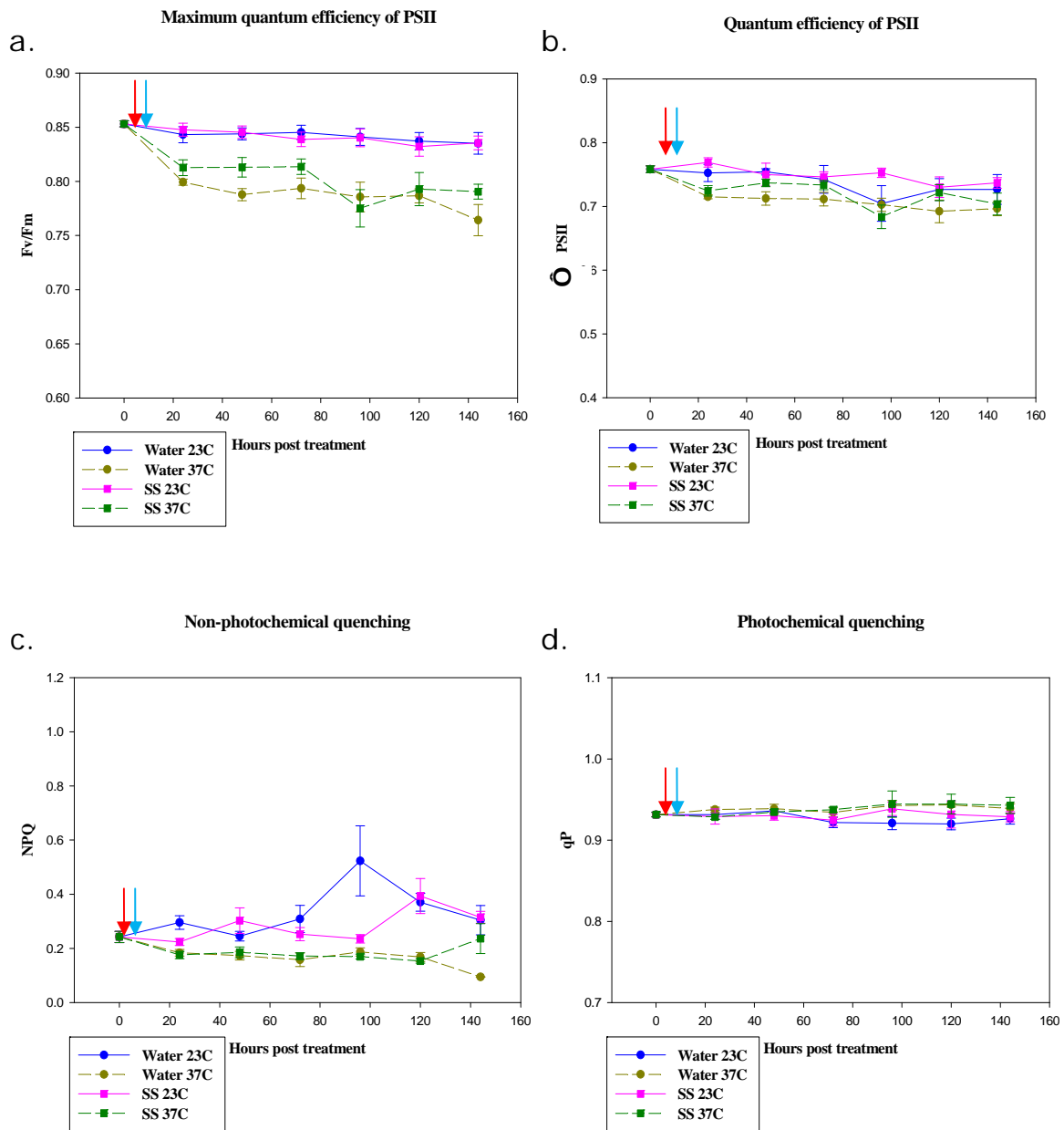


Figure 4.3 Photosynthetic capacity of wheat treated with SS and then exposed to heat stress. The maximum quantum efficiency of PSII and quantum efficiency of PSII after treatment and heat stress is indicated in (a) and (b) respectively while (c) and (d) illustrate the NPQ and qP levels. The red arrows indicate the application of SS while the blue arrows indicate the point at which the temperature was raised to 37°C.

hpt. Thereafter, a gradual decreasing trend in  $F_v/F_m$  levels for both treatments was observed with the SS treated wheat being slightly better off at 140 hpt.

A very similar result was obtained with the  $\hat{O}PSII$  calculations (Fig. 4.3b) with the plants grown at 37°C having lower PSII efficiencies than those kept at 23°C. Considering the loss of heat as indicated by NPQ levels, there was again a difference between the plants grown at 23°C and 37°C respectively (Fig. 4.3c). At 23°C, a gradual increase in NPQ levels was observed for the duration of the study in both sets of plants with little difference between them. The only difference between the SS and water treated plants occurred at 96 hpt where water treated wheat showed a transient increase in heat dissipation. Interestingly, plants grown at 37°C indicated overall lower NPQ levels compared to the control treatments at 23°C with little difference between the SS and water treatments.

Photochemical quenching for all treatments remained on the same level (Fig. 4.3d). It appears that the number of open PSII reaction centres was not affected by the heat stress.

#### **4.1.4 Photosynthetic capacity of heat stressed plants after treatment with SS**

The aim of this experiment was to determine whether plants under heat stress benefited from the application of SS. The photosynthetic ability of plants grown at 23°C and 37°C and then treated with SS and water was determined. Readings were taken at 0 and 48 h after the heat stress commenced where after SS and water treatments were given.

The  $F_v/F_m$  ratio was first calculated (Fig. 4.4a). The SS and water treatments of the control plants did not indicate any significant difference in photosynthetic capacity. Similar to the previous experiment, exposure of the plants to the elevated temperature led to an immediate decrease in maximum quantum efficiency of PSII, which did not improve, even after SS treatment. A slight recovery was seen from 72 hpt onwards, however very little difference was found between the two treatments.

The second parameter that was measured was the quantum efficiency of PSII (Fig. 4.4b). As with the  $F_v/F_m$  values there was no clear difference between the SS and water treatments of plants grown at 23°C. Both graphs indicated a decrease in  $\hat{O}PSII$  72 hpt leading to the lowest

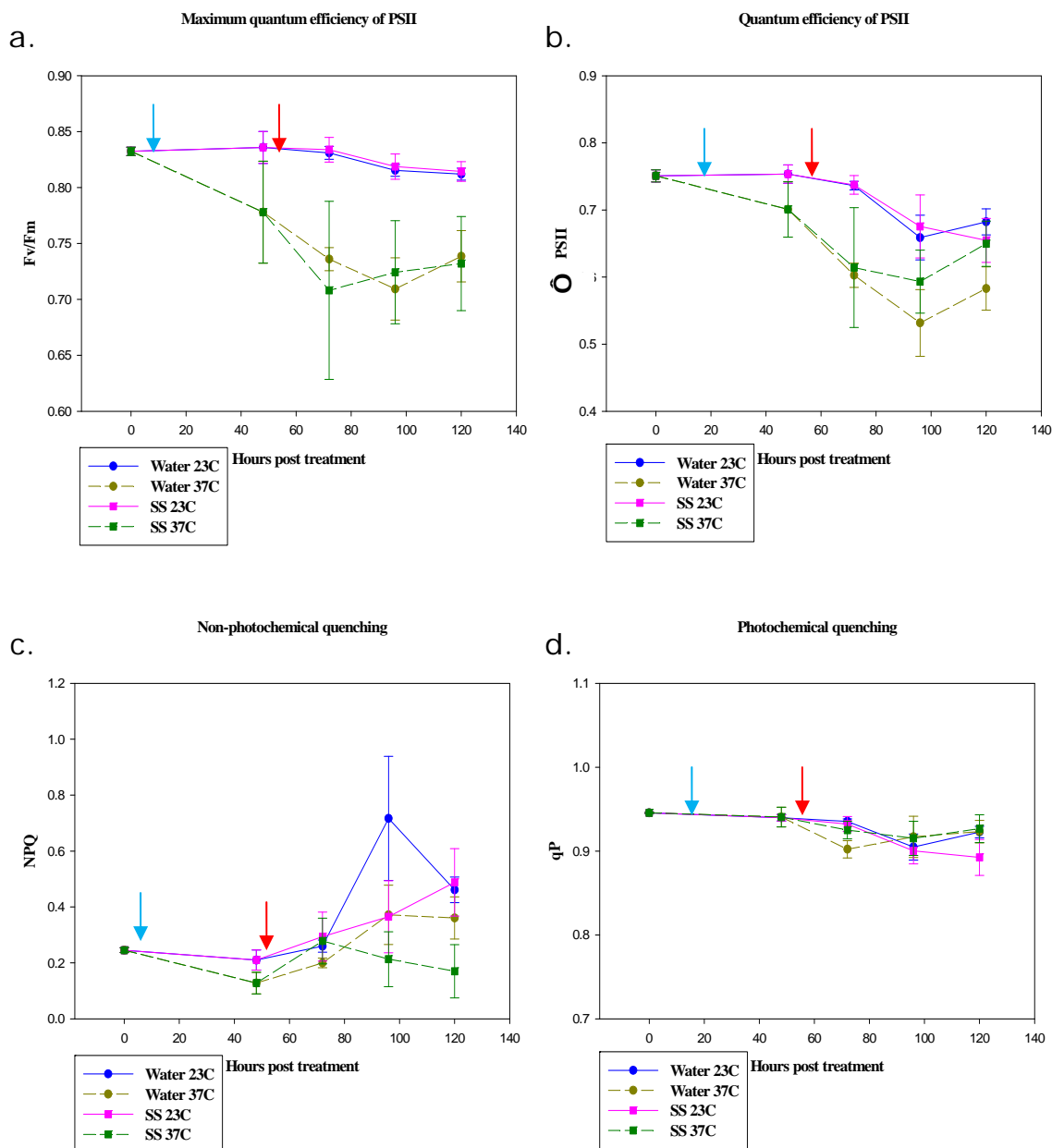


Figure 4.4 Photosynthetic capacity of wheat after exposure to heat stress followed by SS treatment. In (a) the values of  $F_v/F_m$  is shown while (b) indicates the levels of  $\hat{\Phi}_{PSII}$ . The level of NPQ and qP is shown in (c) and (d) respectively. The red arrows indicate treatment with SS and the blue arrows indicate when the temperature was increased to 37°C.

levels at 120 hpt. When analysing the experimental treatments at 37°C, both treatments indicated similar patterns as were seen in the  $F_v/F_m$  graph with no significant differences between them. The SS treated plants were however better off during the latter stages of the study improving to the levels of the plants grown at 23°C. The NPQ levels were analysed and showed that plants grown at 23°C showed an immediate increase in heat loss following the water and SS treatments at 48 hpt (Fig. 4.4c). Similar to the previous experiment, plants grown at the elevated temperature showed lower NPQ levels compared to those grown at the control temperature. The SS treated plants benefitted the most from the treatment with the lowest NPQ levels at 96 and 120 hpt.

Finally the number of open reaction centres for both treatments was almost identical with no significant differences between the SS and water treatments (Fig. 4.4d).

## **4.2 Gene expression in wheat after treatment with SS**

In order to complement the photosynthesis results, expression patterns of a number of photosynthesis related and chloroplast associated genes were determined. The first group included genes encoding the Rubisco large subunit (*RbcL*), Rubisco small subunit (*RbcS*) and Phosphoglycerate kinase (*PGK*) enzymes. The second group coded for a chloroplast inner envelope protein (*CIEP*), a putative chloroplast photosystem I (*PSI*) subunit and the thylakoid-membrane associated Ptr ToxA binding protein 1 (*Ptr ToxA BPI*). Finally, the ability of SS to activate the plant defence response was also tested by determining the expression of two *PR* genes.

### **4.2.1 Quality and quantity of RNA**

Total RNA was extracted as described in section 3.2.1. In total, 500 ng RNA from all extracts was separated on a 1% (w/v) agarose gel to determine the quantity and quality. An example of total RNA extracted from light grown wheat leaves is given in Fig. 4.5. The 28S and 18S rRNA fragments indicated in each figure were all intact which indicated that they were of good quality and that no RNA breakdown occurred. This implicated that the mRNA represented by the light smear in each sample was also intact. Furthermore, since the

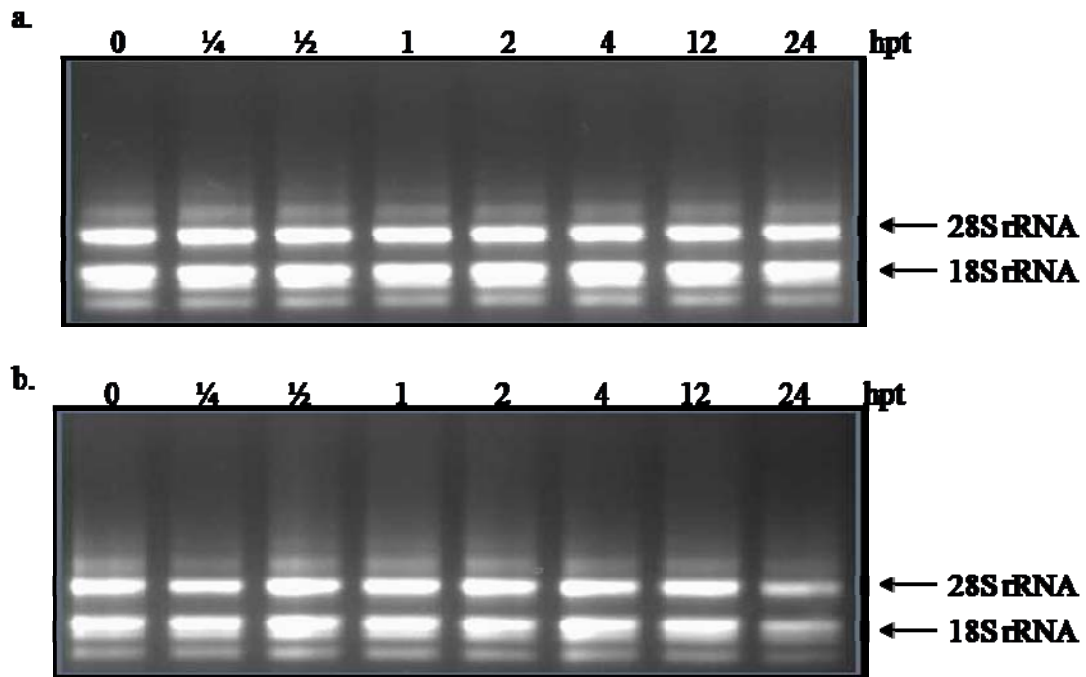


Figure 4.5 Total RNA extracted from light grown wheat plants. Total RNA extracted from plants treated with SS is indicated in (a) and from plants treated with water in (b). Time intervals are indicated.

intensities of the different samples were similar, it was concluded that the concentrations of the samples were correct. Samples that were not of good quality were re-extracted. Total RNA extracted from dark grown wheat is indicated in Fig. 4.6. It is immediately clear to see that the quality of this RNA is much lower than that of plants grown under normal glass house conditions, even though the rRNA bands were intact. Dark germinated plants moved to the light did however seem to yield better quality total RNA than those kept in complete darkness. These results are in accordance with Manning *et al.* (2007) who also noted the poorer quality of RNA extracted from dark germinated and grown wheat.

#### **4.2.2 Expression analysis of photosynthesis related genes after SS treatment**

Three different techniques were evaluated in order to confirm gene expression following SS treatment.

##### **4.2.2.1 RT-PCR analysis**

RT-PCR analysis was firstly done to determine whether SS activates the expression of three photosynthesis related genes. The *GAPDH* gene was each time used as the internal control due to the fact that this gene is expressed constitutively throughout the plant growth cycle (Larsen, 2003).

RT-PCR analysis of *RbcL* and *RbcS* did not indicate any clear induction of gene expression following SS treatment (Fig 4.7). The expression appeared to be constitutive over the four tested time intervals. The expression of *PGK* on the other hand appeared to be slightly repressed during both the SS and water treatments (Fig. 4.7c).

Plants germinated and grown in the dark (section 3.2.3.2) were used to determine the expression of *RbcL* (Fig. 4.8a) and *RbcS* (Fig. 4.8b) after SS treatment. *RbcL* gene expression in the SS treated plants decreased from 2 hpt reaching a minimum at 4 hpt with a subsequent increase at 24 hpt. The water treated samples showed a similar trend with no expression at 4 hpt. Interestingly however, the level of *RbcL* expression in the SS treated plants at 2 and 4 hpt were higher than that of the water treated plants. This confirmed that SS



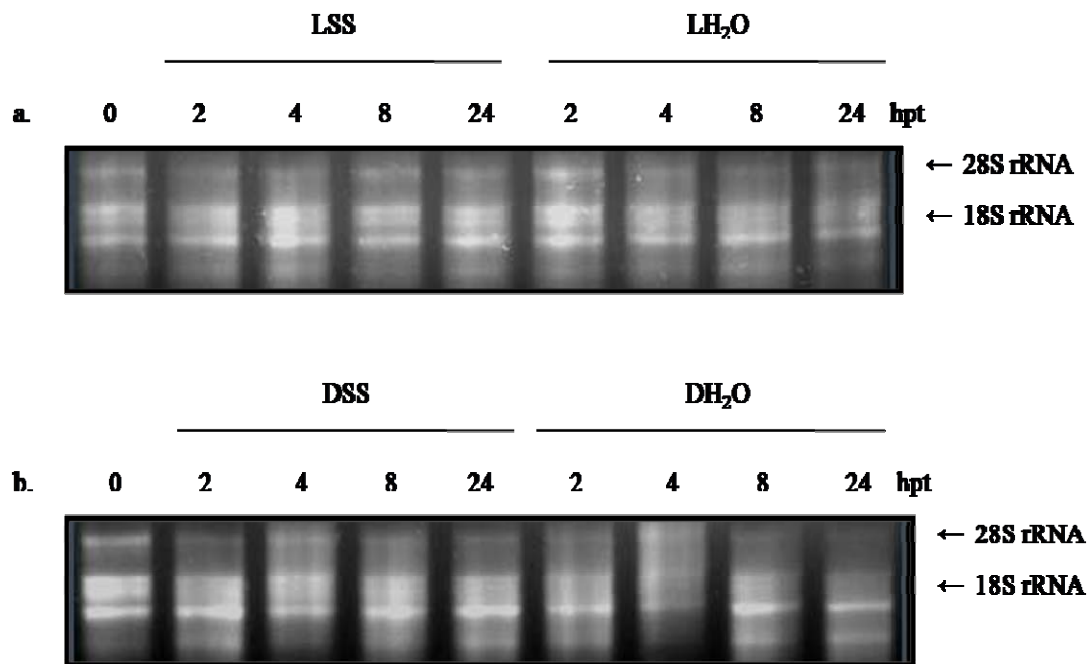


Figure 4.6 Total RNA extracted from wheat plants germinated and grown in the dark. RNA extracted from plants germinated in the dark and then moved to the light for 24 h is indicated in (a) while RNA extracted from plants germinated and grown in the dark is indicated in (b). Time intervals are as indicated. LSS: plants germinated in the dark, moved to the light and treated with SS; LH<sub>2</sub>O: plants germinated in the dark, moved to the light and treated with water; DSS: plants germinated and grown in the dark and treated with SS; DH<sub>2</sub>O: plants germinated and grown in the dark and treated with H<sub>2</sub>O.

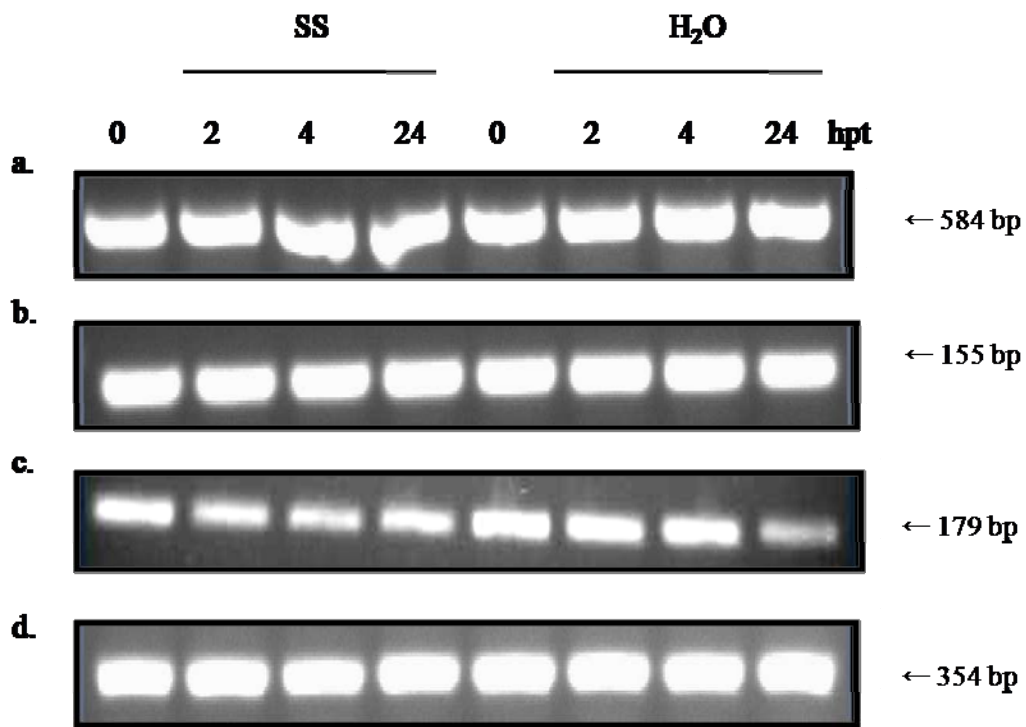


Figure 4.7 RT-PCR analysis of photosynthesis related genes after SS treatment. In (a) the expression of *RbcL* is indicated, in (b) *RbcS*, in (c) *PGK* and in (d) *GAPDH*. The time intervals and sizes of the amplified fragments are as indicated.

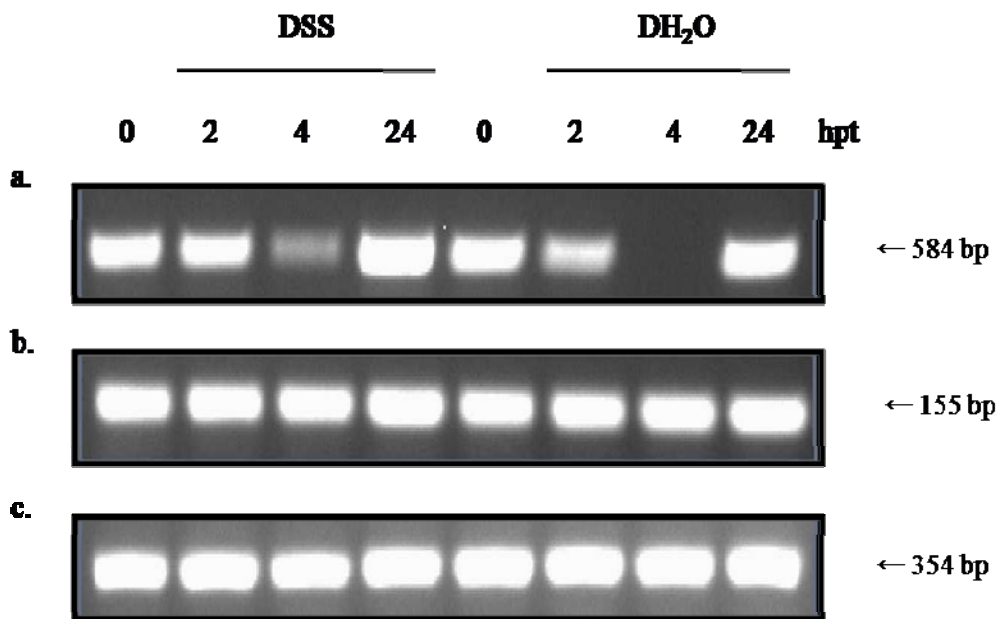


Figure 4.8 RT-PCR analysis of photosynthetic genes of wheat germinated and grown in the dark after SS treatment. The expression of *RbcL* and *RbcS* is indicated in (a) and (b) respectively while (c) illustrates *GAPDH* expression. Time intervals and fragment sizes are as indicated. DSS: plants germinated and grown in the dark and treated with SS; DH<sub>2</sub>O: plants germinated and grown in the dark and treated with H<sub>2</sub>O.

treatment did increase expression of the *RbcL* gene. In contrast, the *RbcS* expression levels for both the SS and water treatments remained unchanged. The expression of *RbcS* was therefore not influenced by both dark and SS treatments.

#### 4.2.2.2 Northern blot analysis

Since RT-PCR results cannot be quantified, the expression of the *RbcL* gene was also determined using a Northern blot. Hybridisation of total RNA from SS and water treated wheat seedlings with the *RbcL* probe resulted in a single hybridising fragment (Fig. 4.9a). The expression for the water treatment showed constitutive gene expression with a decrease from 4 hpt onward which corresponded to a decrease in light intensity due to evening approaching. The SS treated plants however showed a primary induction of expression at 0.5 hpt with a more intense secondary induction at 2 hpt. In addition, the expression of the gene during the latter stages was higher than that of the water treatment, again indicating that SS treatment induced *RbcL* gene expression.

The northern blot analysis was however stopped due to problems that were experienced when the membranes were stripped before re-probing. The stripping solution could not remove all the radio-active *RbcL* probe from the membranes, making them unsuitable for further use. It was thus decided to use RT-qPCR to quantify the expression of the three related genes.

#### 4.2.2.3 RT-qPCR analysis

All RT-qPCR analyses was done using the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines as described in Bustin *et al.* (2009). According to these guidelines the reliability of real-time results are targeted to promote the consistency between different laboratories and to increase the veracity of scientific literature.

A gradient RT-qPCR reaction was done for each primer pair to ensure that the amplification was specific with no primer dimers. The specificity of the primers was confirmed by separating the amplified fragments on an agarose gel (Fig. 4.10). The optimum temperature of *RbcL* and *RbcS* were chosen to be at 61°C, *PGK* at 56°C and that of *GAPDH* at 60°C.

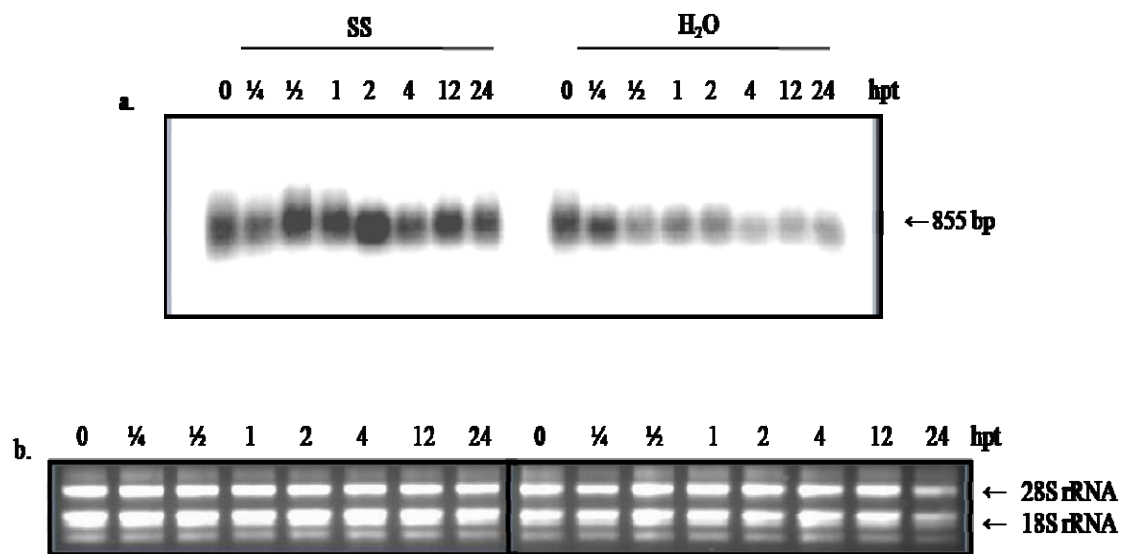


Figure 4.9 Northern blot analysis of *RbcL* expression after SS treatment. In (a) the expression of *RbcL* is shown after hybridisation and in (b) total RNA separated on the agarose gel is shown. Time intervals and molecular sizes are indicated.

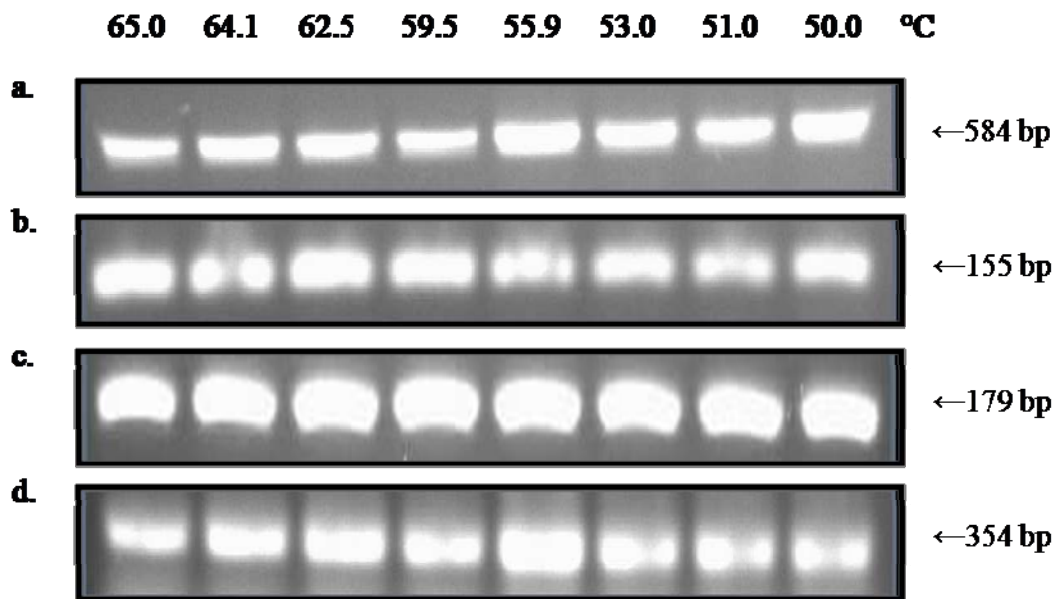


Figure 4.10 Gradient RT-qPCR analysis of photosynthesis related genes. In (a) amplification of *RbcL* is given, in (b) *RbcS*, in (c) *PGK* and in (d) the control gene, *GAPDH*. Fragment sizes and temperature intervals are as indicated.

As described in section 3.2.4.4.2, all RT-qPCR reactions were done in triplicate. Each experiment included a standard curve consisting of a 10x fold dilution series of total RNA starting at 100 ng total RNA. Also included was a reaction that contained no template RNA (NTC). After RT-qPCR, all samples were separated on a 1% (w/v) agarose gel to confirm the formation of a single amplified fragment (Fig. 4.11). The serial dilution of *RbcL* (Fig. 4.11b) showed amplification up to 10 pg total RNA while *RbcS* (Fig. 4.11d) showed amplification throughout the whole dilution series. *PGK* (Fig. 4.11f) showed amplification up to a minimum of 100 pg total RNA and the control gene throughout the whole dilution series (Fig. 4.11h). The NTC for *RbcS* (Fig. 4.11d) and *GAPDH* (Fig. 4.11h) did however show amplification which could indicate a possible contamination. To ensure that RNA was not contaminated, a PCR reaction was done (data not shown) which did not indicate any contaminating agents.

To establish the efficiency of the RT-qPCR reactions, the standard curves for all four genes were plotted (Fig. 4.12). The amplification efficiencies should be between 90 and 110%, while  $R^2$ -values should be close to 1. An efficiency value within these parameters indicates a good amplification with no primer dimer formation. The standard curve analysis of *RbcL* (Fig. 4.12a) indicated an efficiency of only 65.1% but a  $R^2$ -value of 0.997. All unknown samples, save one, fell between the third and fourth dilution factor. This point was excluded from analysis as all reactions were done in triplicate. The efficiency of *RbcS* amplification was 93.8% with the  $R^2$ -value being 0.999 (Fig. 4.12b). All unknown samples fell between the fourth and fifth dilution factor. The reaction efficiency of *PGK* (Fig. 4.12c) was 123.6% with a  $R^2$ -value of 0.979. All unknown samples were present on the standard curve and grouped together very closely. Standard curve analysis of *GAPDH* (Fig. 4.12d) indicated an efficiency of 97.2% and a  $R^2$ -value of 0.999. All unknown samples were present between dilution factor two and three except for one.

In Fig. 4.13 the melting curves for all four tested genes are indicated. As is evident, the melting curves of all four genes indicated no primer dimer or secondary product formation, except for *RbcL* (Fig. 4.13a). This secondary product represents one of the water treated samples at 24 hpt which also did not group together with the other unknown samples on the standard curve (Fig. 4.12a). The melting temperature for the remaining fragments was 80°C.

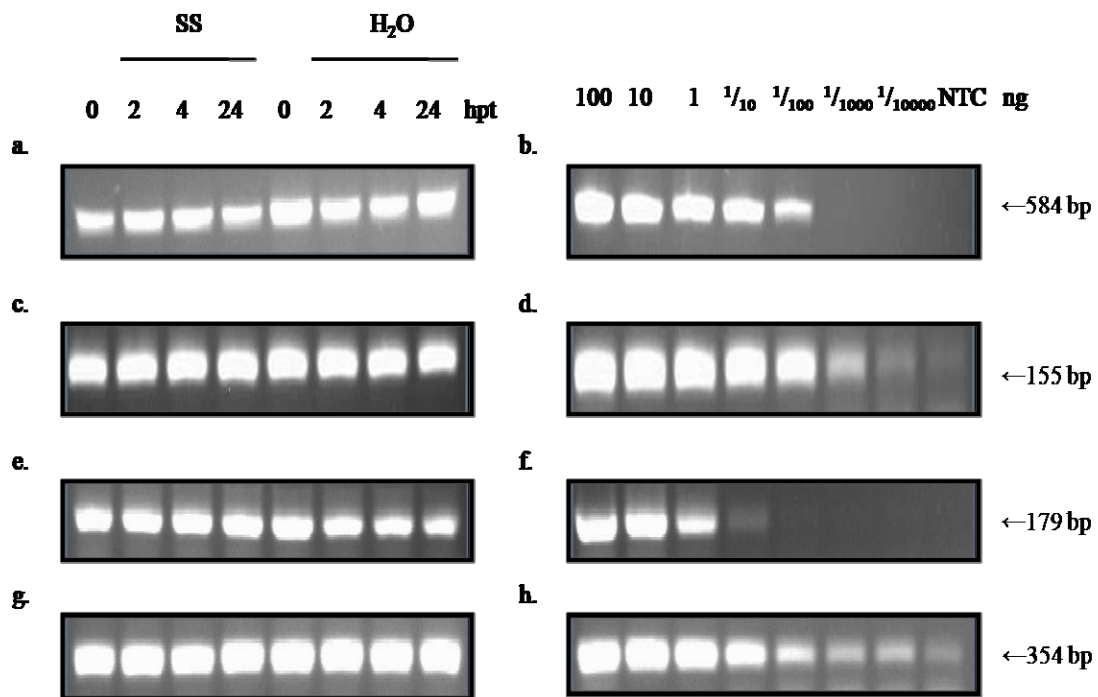
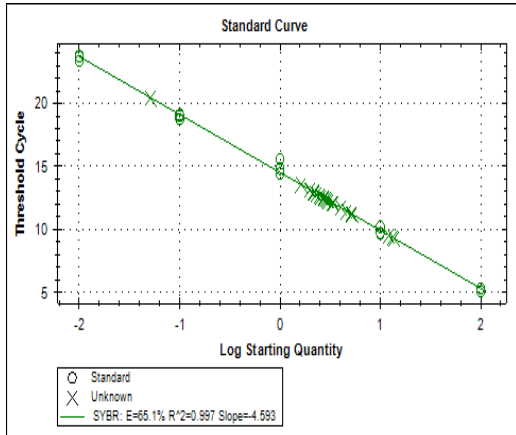


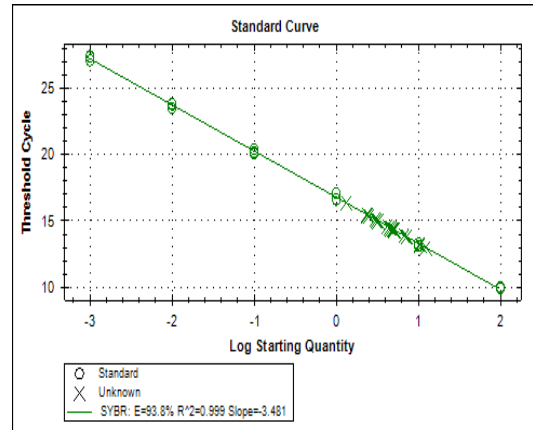
Figure 4.11 Agarose gel electrophoresis of the RT-qPCR products of three different photosynthesis related and one control gene. The four amplified gene fragments of (a) *RbcL*, (c) *RbcS*, (e) *PGK* and (g) *GAPDH* are indicated on the left and the corresponding standard curves (b, d, f and h) on the right. Fragment sizes, time intervals and template amounts are given.



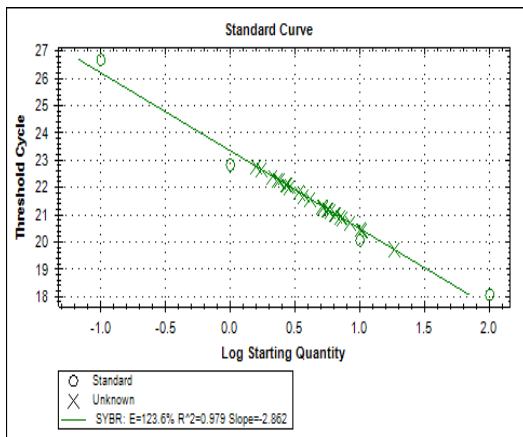
a.



b.



c.



d.

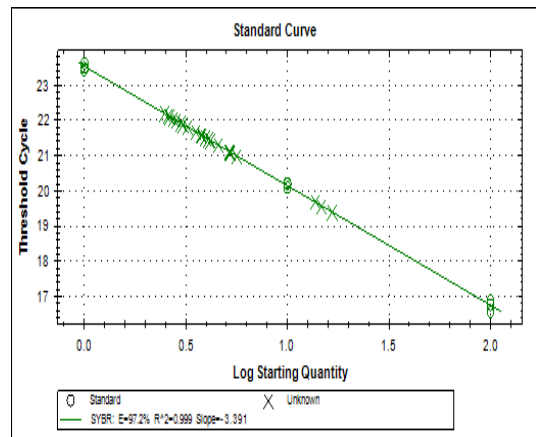
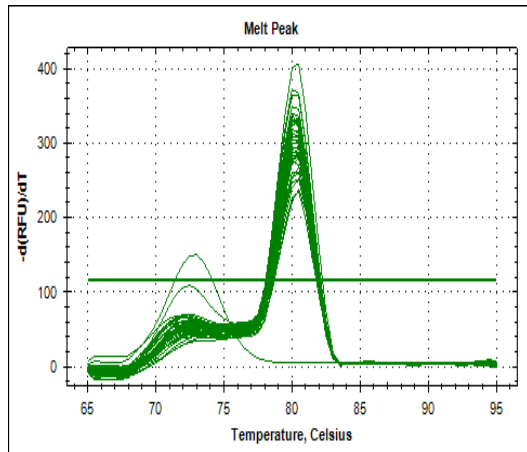
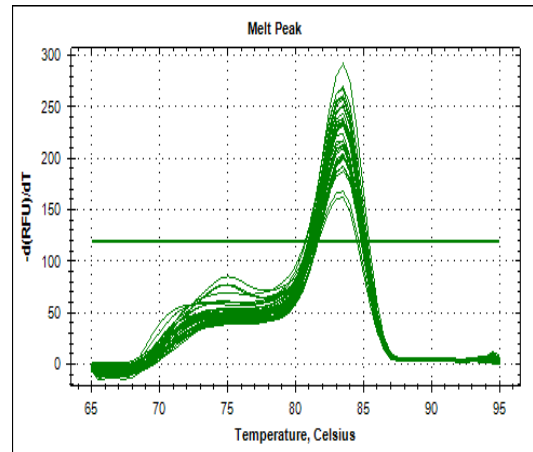


Figure 4.12 Standard curve analysis of threshold cycle vs. the log of the Cq value for three photosynthesis related and one control gene. In (a) the standard curve of *RbcL* amplification is indicated, in (b) *RbcS*, in (c) *PGK* and in (d) *GAPDH*.

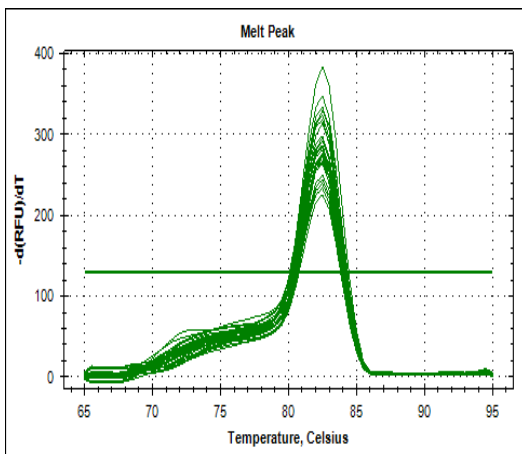
a.



b.



c.



d.

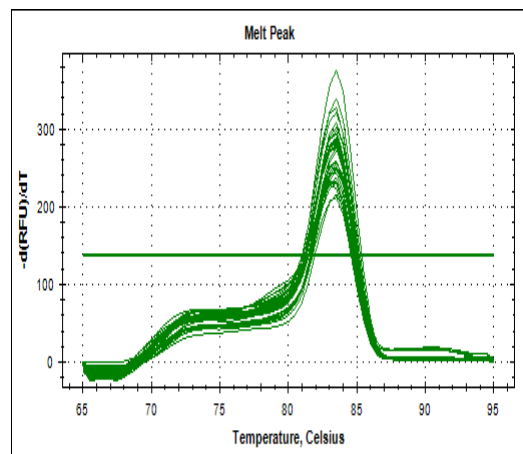


Figure 4.13 Melting curve analysis of three photosynthesis related and one control gene. In (a) *RbcL*, in (b) *RbcS*, in (c) *PGK* and in (d) *GAPDH* melt curves are shown.

The melting curves for *RbcS*, *PGK* and *GAPDH* indicated melting temperatures of 83.5°C (Fig. 4.13b), 82.5°C (Fig. 4.13c) and 83.5°C respectively (Fig. 4.13d).

The expression levels of the photosynthesis related genes were finally determined using the  $\Delta$ Cq method described by Pfaffl (2001). The Cq values of the different genes in the SS treated plant samples were firstly expressed relative to Cq values of the water treatments. These values were then expressed relative to that of the *GAPDH* reference gene. Any induced expression levels were then expressed relative to 0 hpt.

*RbcL* expression indicated an initial decrease up to 4 hpt with a final 1.5 fold increase in expression at 24 hpt (Fig. 4.14a). *RbcS* on the other hand showed a statistically insignificant initial increase in expression (Fig. 4.14b) followed by decreased expression at 24 hpt. Finally, *PGK* expression (Fig. 4.14c) indicated a statistically significant 1.75 fold increase in expression at 24 hpt. It was interesting to note that the expression profiles of *RbcL* and *PGK* were similar over the duration of the study, but differed from that of *RbcS*.

### **4.2.3 Expression analysis of chloroplast associated genes after SS treatment**

#### 4.2.3.1 Influence of SS on the expression of *Ptr ToxA BPI*, a chloroplast associated gene

##### 4.2.3.1.1 RT-PCR analysis

The expression of *Ptr ToxA BPI* in wheat treated with SS was tested using the *18S RNA* gene as control (Fig. 4.15). The expression of the *18S rRNA* gene in wheat treated with SS (Fig 4.15b) and water (Fig 4.15d) was constitutive. No visible induced expression of *Ptr ToxA BPI* was found (Fig. 4.15a and c). However, it was clear that the concentration of all the RNA samples was not accurately determined with the 1 hpt sample containing much less RNA.

After the RNA concentrations of all the samples were recalculated, the expression of both genes were tested again. This time the expression of the *18S rRNA* gene was constitutive (Fig. 4.15f) for the duration of the study, while *Ptr ToxA BPI* showed a strong induction at 1 hpt (Fig. 4.15e), indicating the activation of this gene's expression by SS.

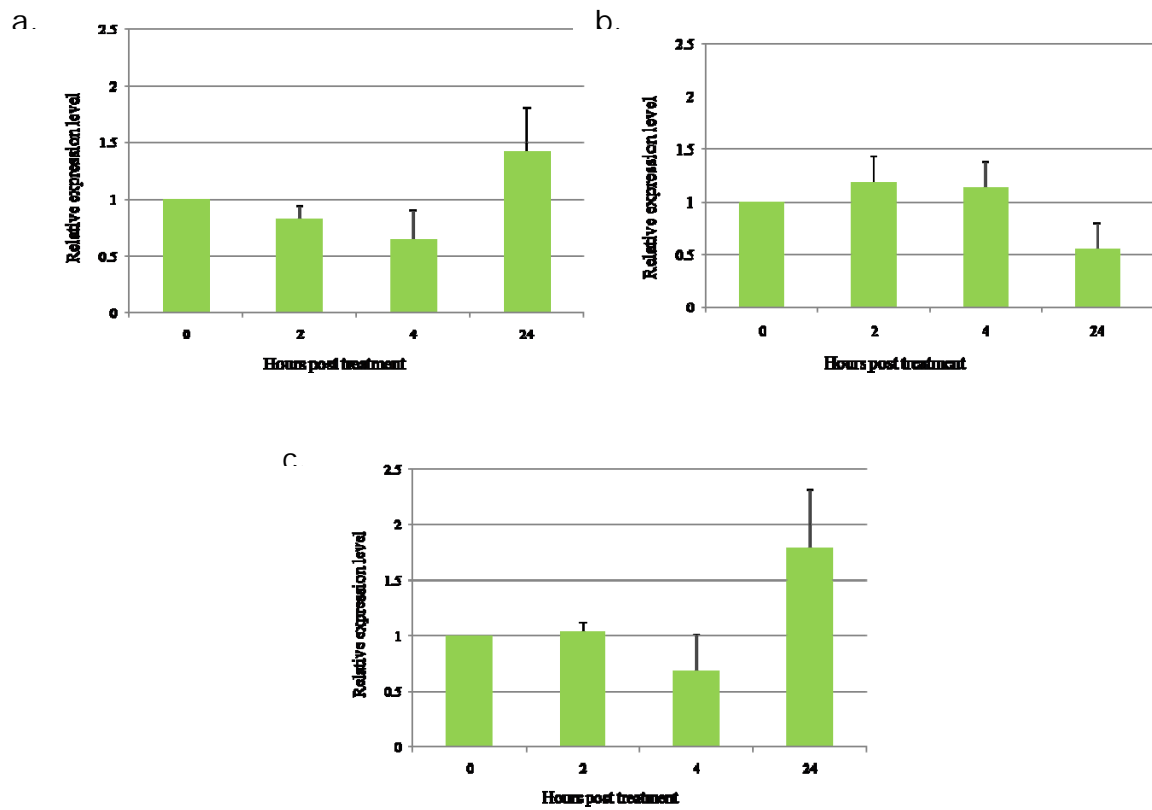


Figure 4.14 Real-time expression analyses of three different photosynthesis related genes following SS treatment. The expression of *RbcL* (a), *RbcS* (b) and *PGK* (c) are as indicated. The x-axis represents the four different time intervals that were used while the y-axis represents the induced expression levels of the three genes relative to that of *GAPDH*.

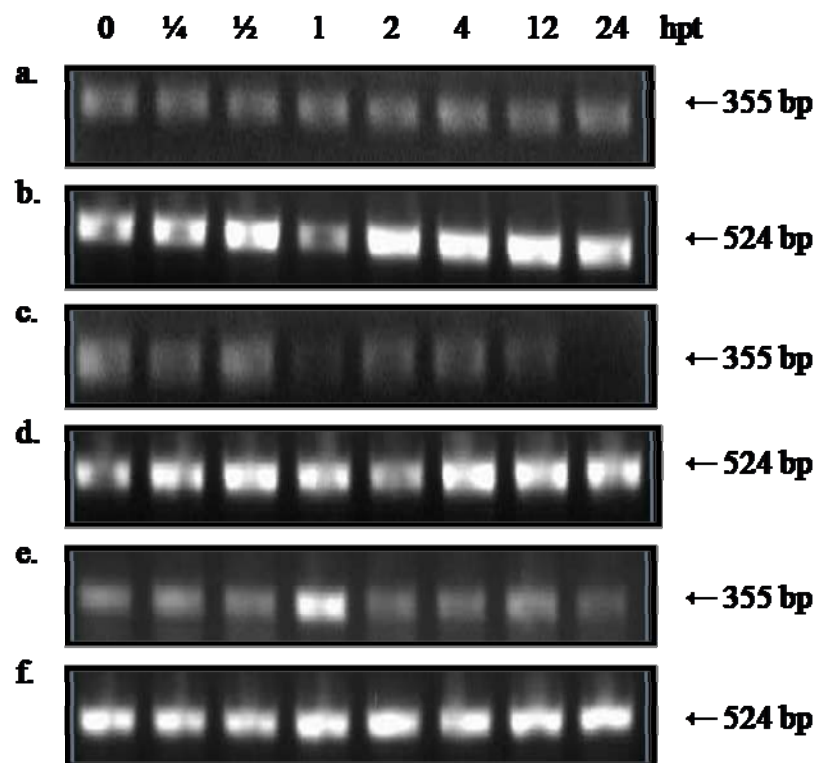


Figure 4.15 Expression of the *Ptr ToxA BPI* and *18S rRNA* genes after SS and water treatment. Plants treated with SS is shown in (a), (b), (e) and (f), while those treated with water is shown in (c) and (d). The amplification of the *Ptr ToxA BPI* (a + c) and *18S rRNA* (b + d) genes in wheat treated with SS and water respectively, is shown. The expression of the *Ptr ToxA BPI* and *18S rRNA* genes after the recalculation of RNA concentration is finally given in (e) and (f) respectively. Fragment sizes and time intervals are as indicated.

#### 4.2.3.1.2 Semi-quantitative RT-PCR

It is widely known that RT-PCR analysis is not quantitative due to the fact that the end-product of a RT-PCR reaction is normally a representation of the stationary phase. This entails that one or more of the PCR reaction components may become depleted during the logarithmic phase of amplification resulting in non-linear amplification during the latter cycles. The result is thus not a quantitative representation of the expression of the gene or fragment in question. In an attempt to overcome these restrictions, semi-quantitative RT-PCR analyses were done.

The first step was to determine at which cycle the exponential amplification of the *Ptr ToxA BPI* and *18S rRNA* genes occurred. The PCR reactions were interrupted at 15, 18, 21, 24, 27 and 30 cycles. After separation on an agarose gel, it was determined that exponential amplification still occurred at 27 cycles for *Ptr ToxA BPI* and at 24 cycles for *18S rRNA* (results not shown). While there were clear differences in the semi-quantitative amplification of the *18S rRNA* gene, it was still possible to see an induced expression pattern for the *Ptr ToxA BPI* gene at ½ hpt after SS application (Fig. 4.16).

When wheat plants were grown under normal light conditions and then transferred to the dark, a difference in the expression of *Ptr ToxA BPI* was seen (Fig. 4.17). Semi-quantitative RT-PCR again indicated that the *18S rRNA* gene was expressed constitutively in both light and dark incubated plants (Fig. 4.17b and d).

Light incubated wheat showed a constant expression of the *Ptr ToxA BPI* gene but once SS was applied, a clear induction of expression was evident at 8 hpt (Fig. 4.17a). A small increase in expression was also visible in the light grown water treated plants. Plants transferred to the dark showed no induced expression, but after the application of SS an increase in *Ptr ToxA BPI* expression from 4 until 24 hpt was again evident (Fig. 4.17c). A small increase in expression was also seen in the water treated plants.

A similar induction of *Ptr ToxA BPI* expression was found in wheat plants germinated in the dark and then transferred to the light (Fig. 4.18). The expression of the *18S rRNA* gene was mostly constitutive with the exceptions being 24 hpt of light grown wheat treated with water

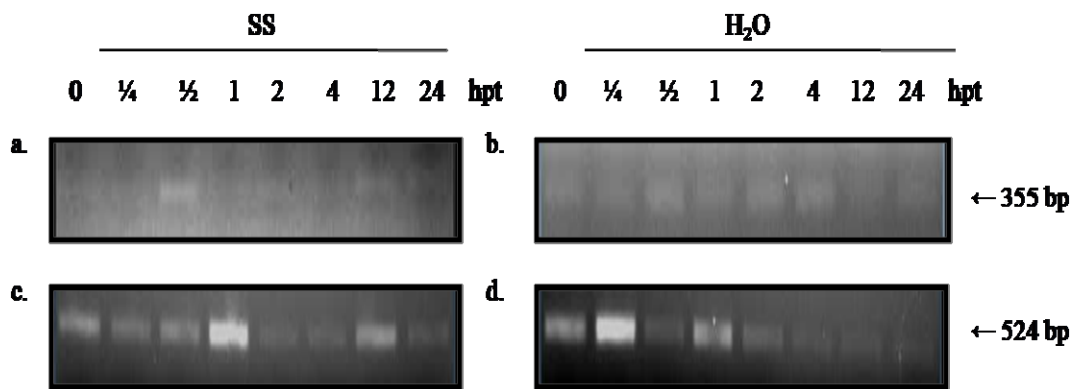


Figure 4.16 Semi-quantitative RT-PCR analyses of the *Ptr ToxA BPI* and *18S rRNA* genes in SS and water treated wheat. In (a) and (b) *Ptr ToxA BPI* amplification is indicated after SS and water treatments respectively, while in (c) and (d) that of *18S rRNA* is shown. Time intervals and size markers are as indicated.

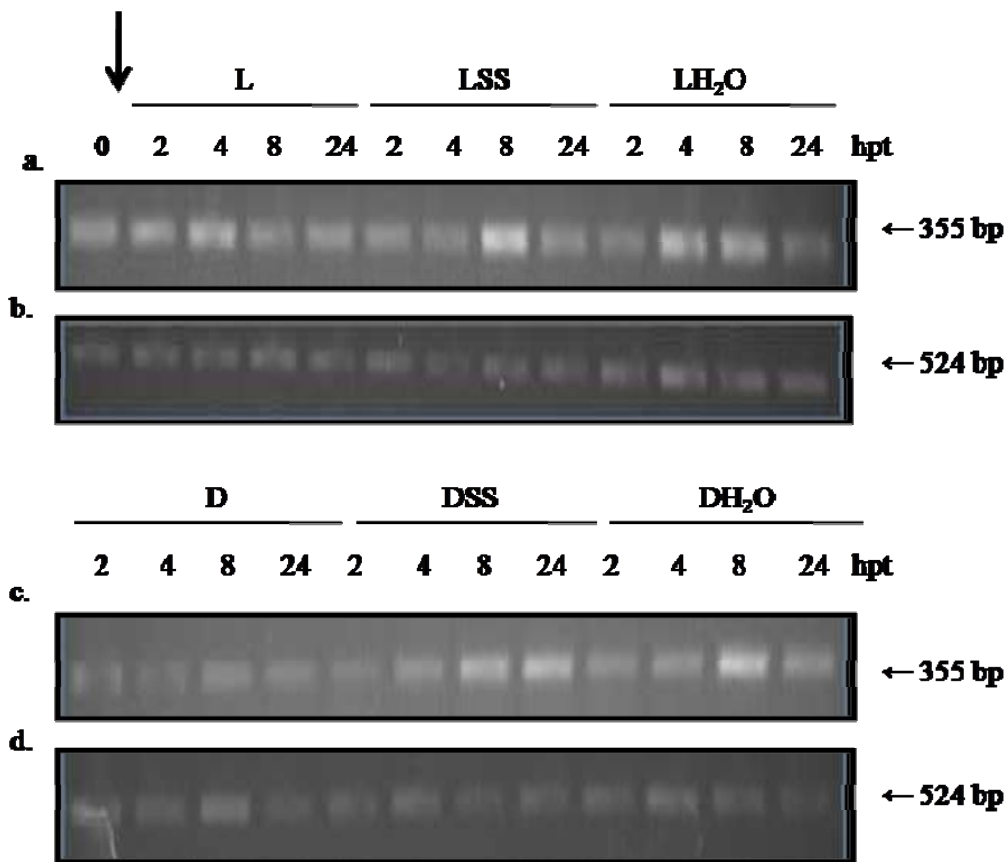


Figure 4.17 Influence of SS treatment on *Ptr ToxA BPI* gene expression in light grown wheat transferred to the dark. In (a) and (c) the expression of *Ptr ToxA BPI* in light and dark grown is indicated while in (b) and (d) the expression of the *18S rRNA* gene is indicated. All expression levels were compared to the 0 hpt levels indicated in (a) and (b). Fragment sizes and time intervals are as indicated. L: plants grown in the light; LSS: plants grown in the light and treated with SS; LH<sub>2</sub>O: plants grown in the light and treated with H<sub>2</sub>O; D: plants grown in the light and moved to the dark for 24 hours; DSS: plants grown in the light, moved to the dark for 24 hours and treated with SS; DH<sub>2</sub>O: plants grown in the light, moved to the dark for 24 hours and treated with water. Arrow indicates when SS and water was applied.



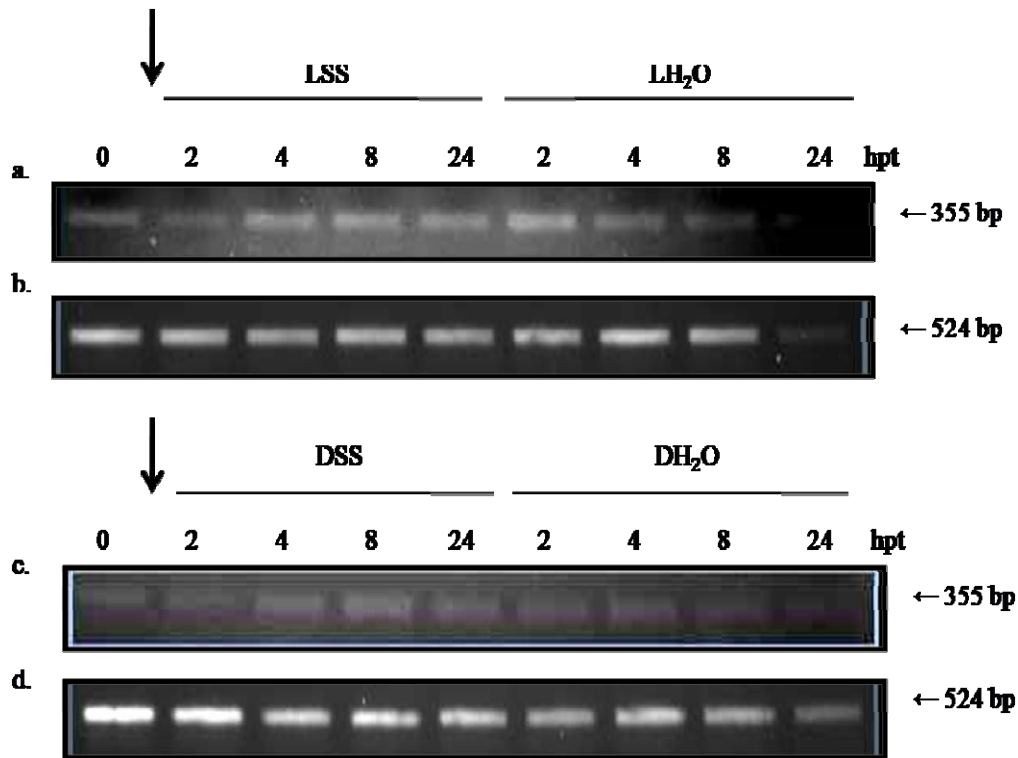


Figure 4.18 Semi-quantitative RT-PCR analysis of *Ptr ToxA BPI* and *18S rRNA* gene expression in wheat germinated in the dark. The expression of *Ptr ToxA BPI* is shown in (a) and (c) and that of the *18S* control gene in (b) and (d) respectively. Fragment sizes and time intervals are as indicated. LSS: plants germinated in the dark, moved to the light and treated with SS; LH<sub>2</sub>O: plants germinated in the dark, moved to the light and treated with water; DSS: plants germinated and grown in the dark and treated with SS; DH<sub>2</sub>O: plants germinated and grown in the dark and treated with H<sub>2</sub>O. Arrows indicate when SS and water treatment was applied.

and the first two time intervals of the dark grown plants treated with SS. Plants transferred from the dark to the light and then treated with SS showed increased gene expression of *Ptr ToxA BPI* from 4 hpt until 24 hpt (Fig. 4.18a). Plants left in the dark and then treated with SS did show induced expression of *Ptr ToxA BPI* from 4 hpt until 24 hpt. This induction will be even higher if the *18S rRNA* levels in the DSS plants at 0 hpt and 2 hpt is taken into consideration. These results indicated that the expression of *Ptr ToxA BPI* is light dependent, but can also be induced by SS, even in the absence of light.

#### 4.2.3.1.3 Northern blot analysis

The induced expression of *Ptr ToxA BPI* by SS application was further confirmed using a Northern blot (Fig. 4.19). When total RNA was first hybridised with the *Ptr ToxA BPI* probe, no hybridisation was evident (results not shown). This indicated that the expression of *Ptr ToxA BPI* occurs at low levels within wheat. The Northern blot was then repeated using mRNA purified by means of oligo dT cellulose. It was assumed that by using a known and constant amount of total RNA, the purified mRNA would also be present in equal quantities.

When purified mRNA was used for the Northern blot, a single hybridising fragment was evident in both the SS and water treated samples (Fig. 4.19). The 1 hpt mRNA sample of SS treated wheat was lost and was thus excluded from hybridisation analysis. As indicated, SS treatment led to the induction of *Ptr ToxA BPI* expression at  $\frac{1}{4}$  hpt. The expression level then gradually decreased. In the water treated plants the expression of this gene was more or less constitutive.

#### 4.2.3.1.4 RT-qPCR

At first a gradient RT-qPCR reaction was done to ensure that no primer dimer formation occurred and that the gene of interest was amplified at the correct annealing temperature. Gel electrophoresis showed amplification of the fragment at the different temperature intervals (Fig. 4.20). Amplification of *Ptr ToxA BPI* was done at 60°C.

Reactions were again done in triplicate and a standard curve as well as a NTC was included in each reaction. The standard curve was done using a fourfold dilution series starting at 100

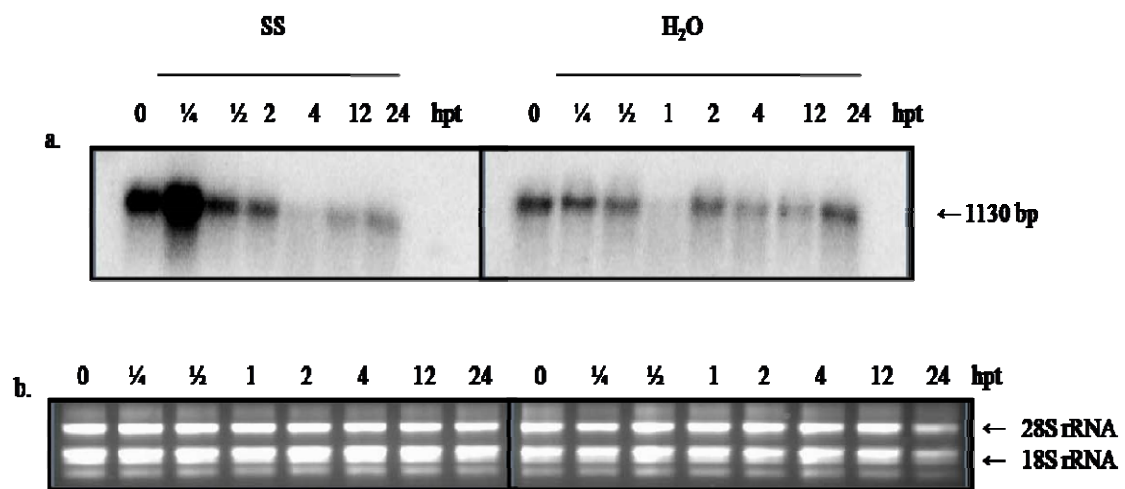


Figure 4.19 Northern blot analysis of *Ptr ToxA BPI* expression after SS and water treatment. In (a) *Ptr ToxA BPI* hybridisation is shown and in (b) the total RNA used for the Northern blot. Time intervals as well as molecular fragment sizes are as indicated.

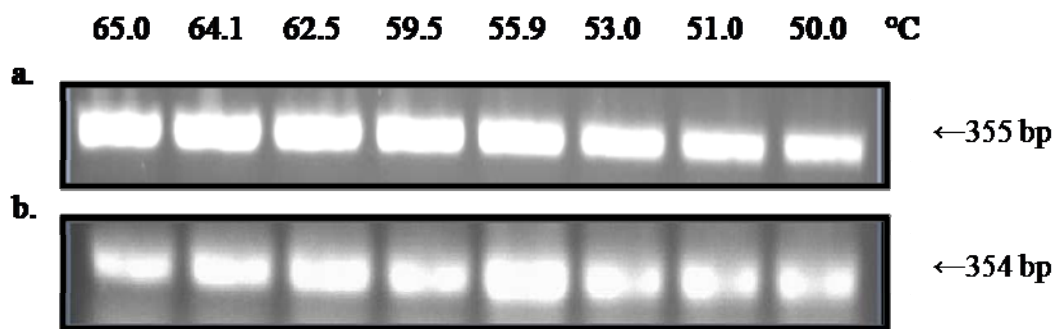


Figure 4.20 Gradient RT-qPCR optimisation of *Ptr ToxA BPI*. In (a) the gradient amplification of *Ptr ToxA BPI* is shown and in (b) that of *GAPDH*. The different temperature intervals as well as the fragment sizes are shown.

ng total RNA. Initial analysis was done using a tenfold dilution series, but due to the fact that *Ptr ToxA BPI* is expressed at very low levels, the four fold dilution series gave better results. The standard curve for *Ptr ToxA BPI* indicated amplification up to 0.4 ng total RNA (Fig. 4.21b). In Fig. 4.21 (a) and (c) the amplification of *Ptr ToxA BPI* and GAPDH, respectively, is more or less constitutive.

To ensure good reaction efficiency the standard curve was plotted (Fig. 4.22a). All unknown samples were present between dilution factor two and four except for one. The reaction efficiency was given as 135.6% and a R<sup>2</sup>-value of 1 was found. The melting curve (Fig. 4.22b) was also analysed and a single peak was seen at 81.5°C for all unknown samples. Thus no primer dimers or secondary products were found.

Gene expression analysis of *Ptr ToxA BPI* indicated a 5.25 fold increase two hours after SS treatment (Fig. 4.23). Even though the standard deviation for this sample was high, the induction was significant. There after the expression of this gene decreased dramatically. This result confirmed the previous induced expression results of *Ptr ToxA BPI* that was found using RT-PCR, semi-quantitative RT-PCR and the Northern blot.

#### 4.2.3.2 Expression analysis of two other chloroplast associated genes

From the initial SSH study completed by Van der Merwe (2008), two other chloroplast associated genes were identified as expressed sequence tags (ESTs). BLAST analysis indicated that the first clone showed homology with a chloroplast inner envelope protein (*CIEP*) encoding gene, while the second shared homology with a putative chloroplast *PSI* subunit encoding gene. The expression of these genes was analysed, since together with *Ptr ToxA BPI*, the encoded proteins are implicated to play a role in chloroplast stability.

##### 4.2.3.2.1 RT-PCR analysis

The expression levels of the two genes differed dramatically with the *CIEP* gene being expressed at high levels and the *PSI* subunit gene at low levels that are comparable to that of *Ptr ToxA BPI* (Fig. 4.24). While *CIEP* expression appeared to be unaffected by SS treatment (Fig. 4.24a), the expression of the *PSI* subunit gene did show differences in

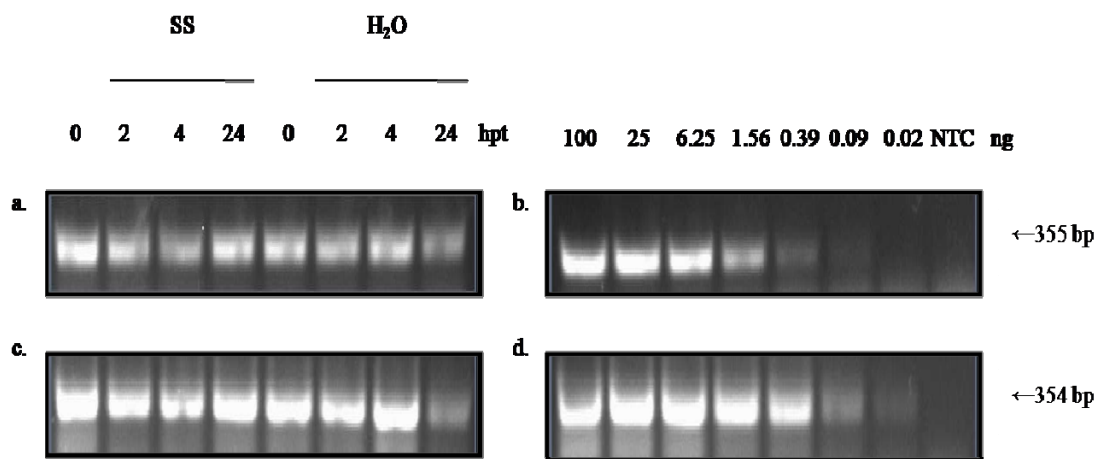


Figure 4.21 Agarose gel electrophoresis of the RT-qPCR products of the *Ptr ToxA BPI* and *GAPDH* genes. The amplified fragment (a) and standard curve amplification (b) for *Ptr ToxA BPI* is as shown, while that for *GAPDH* is shown in (c) and (d) respectively. Time intervals, amount of template and fragment sizes are as indicated.

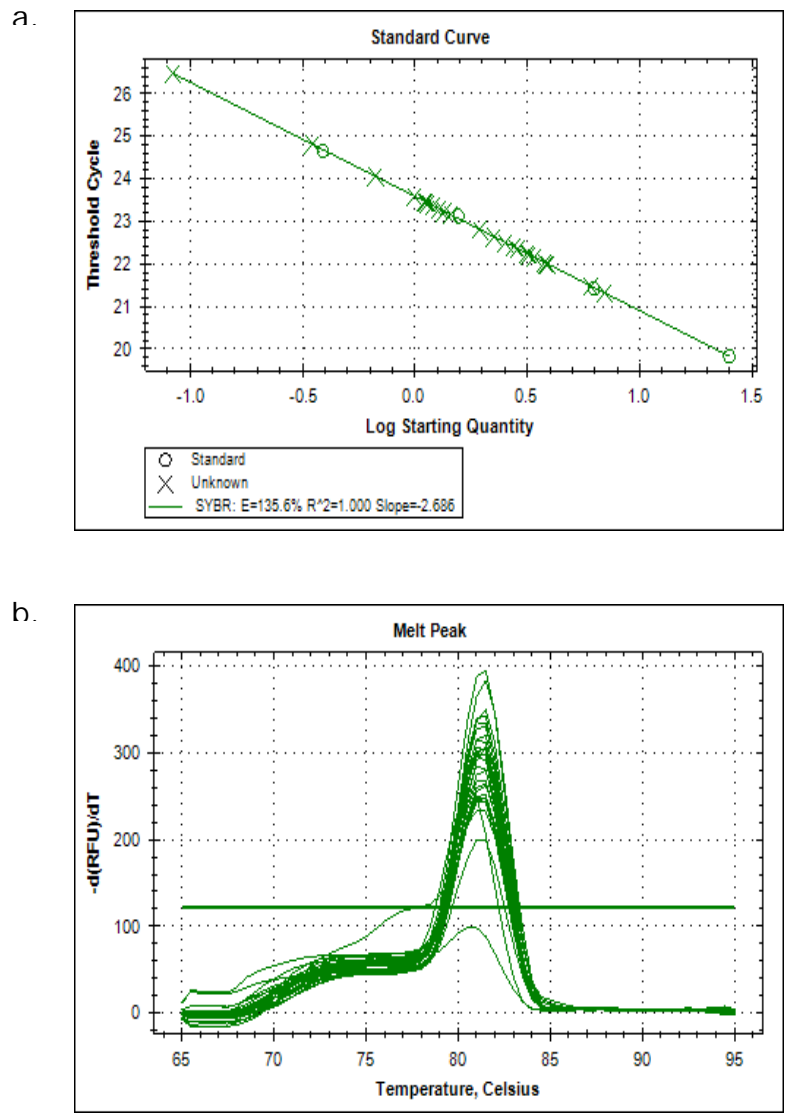


Figure 4.22 Melting curve analysis of *Ptr ToxA BPI*. In (a) the standard curve and in (b) the melt curve of *Ptr ToxA BPI* is shown.

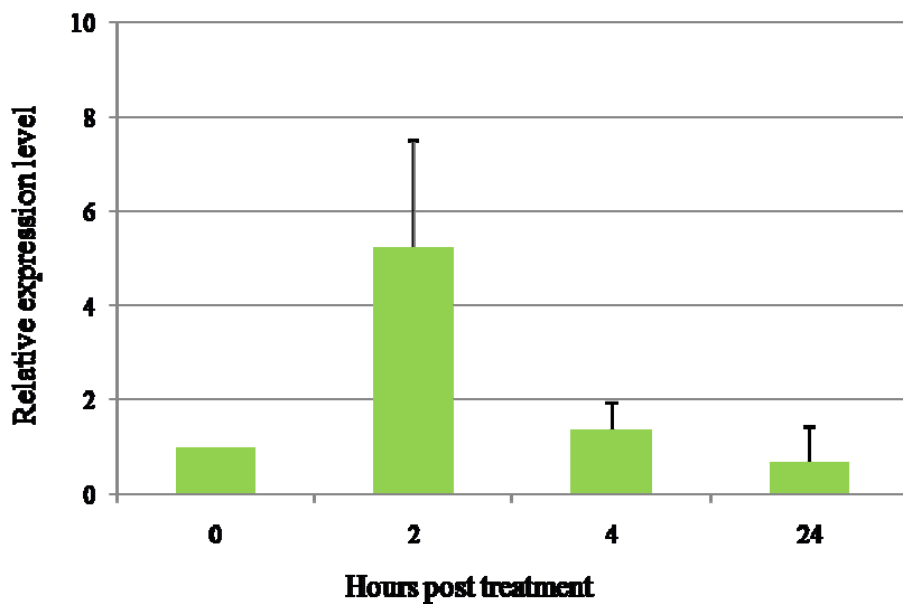


Figure 4.23 Real-time expression analysis of *Ptr ToxA BPI* in wheat after SS treatment. The x-axis represents the four different time intervals that were used while the y-axis represents the induced expression level of *Ptr ToxA BPI* relative to that of *GAPDH*.



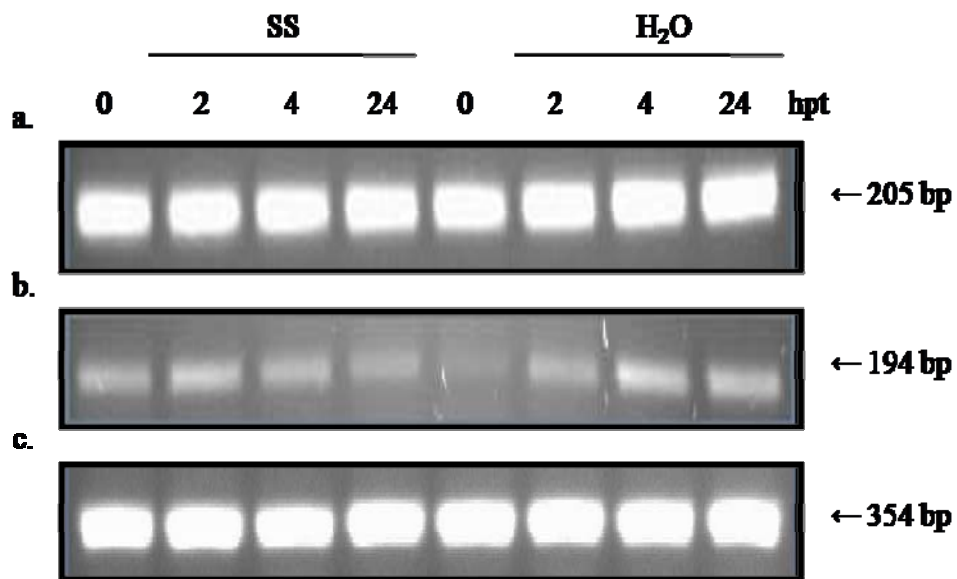


Figure 4.24 RT-PCR expression analyses of two putative chloroplast associated genes following SS treatment. In (a) the expression of *CIEP* is shown, in (b) the expression of *PSI* and in (c) the expression of *GAPDH*. Time intervals and fragment sizes are as indicated.

expression (Fig. 4.24b). Similar changes were however also visible in the water treated wheat.

#### 4.2.3.2.2 RT-qPCR

The expression of the *CIEP* and *PSI* subunit genes was finally quantified using RT-qPCR. For the standard curves, 10x serial dilutions starting at 100 ng total RNA were used. The *GAPDH* gene was used as a reference gene. Gel electrophoresis indicated that the expression of *CIEP* was very strong (Fig. 4.25a) as was seen with normal RT-PCR analysis (Fig. 4.24a). Amplification of *CIEP* was found in all dilutions of the standard curve, except in the 1 pg dilution where no product was found, most probably due to a pipeting error. This point was excluded from analysis. The NTC was however contaminated since it produced an amplified fragment. The *PSI* subunit gene was also expressed at high levels (Fig. 4.25c). When analysing the *PSI* subunit standard curve (Fig. 4.25d) an amplified fragment was seen using as little as 1 ng total RNA.

For *CIEP* all the unknown samples fell between dilution factors three and five on the standard curve (Fig. 4.26a). The efficiency of this reaction was 78.1% with an  $R^2$ -value of 0.995. The efficiency of the *PSI* subunit gene (Fig. 4.26b) expression was 81.4% while the obtained  $R^2$ -value was 0.945. The melt curve peaks of the two genes differed considerably. The melting temperature for *CIEP* was 79.5°C (Fig. 4.27a) while that of the *PSI* subunit gene was 90.5°C (Fig. 4.27b).

Even though *CIEP* expression almost doubled 2 h after SS treatment, a very large standard deviation meant that the induced expression was not significant (Fig 4.28a). Thereafter the expression remained largely unaffected. The expression profile of the *PSI* subunit gene (Fig. 4.28b) was the same as that of the *RbcL* and *PGK* genes (Fig. 4.14), with the exception that the *PSI* subunit gene showed no induced expression above the 0 hpt for the duration of the study.

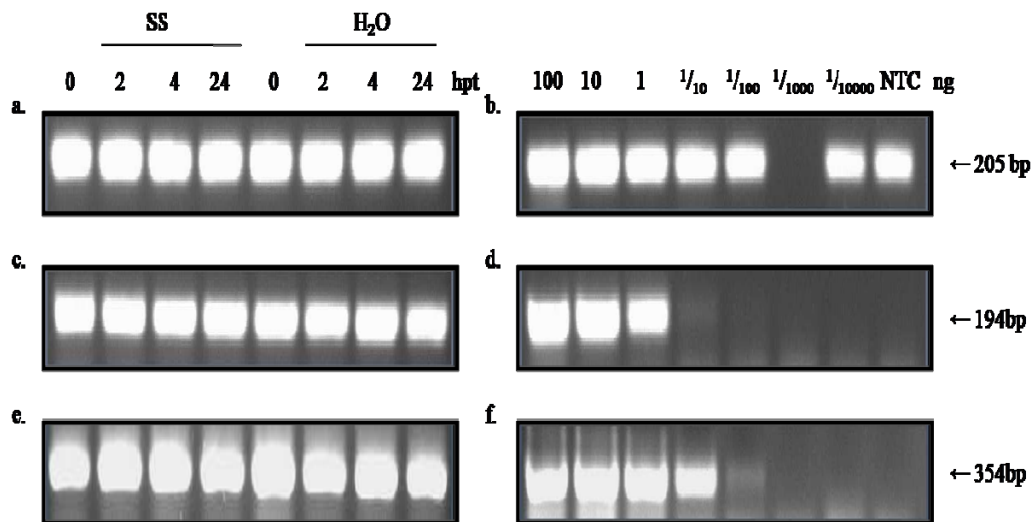


Figure 4.25 Agarose gel electrophoresis of the RT-qPCR products of two chloroplast associated and the *GAPDH* control gene. In (a), (c) and (e) the amplification products of the *CIEP*, *PSI* subunit and *GAPDH* genes are shown while the standard curve amplifications of the three respective genes are indicated in (b), (d) and (f) respectively. Time intervals, amounts of total RNA as well as molecular sizes are given.

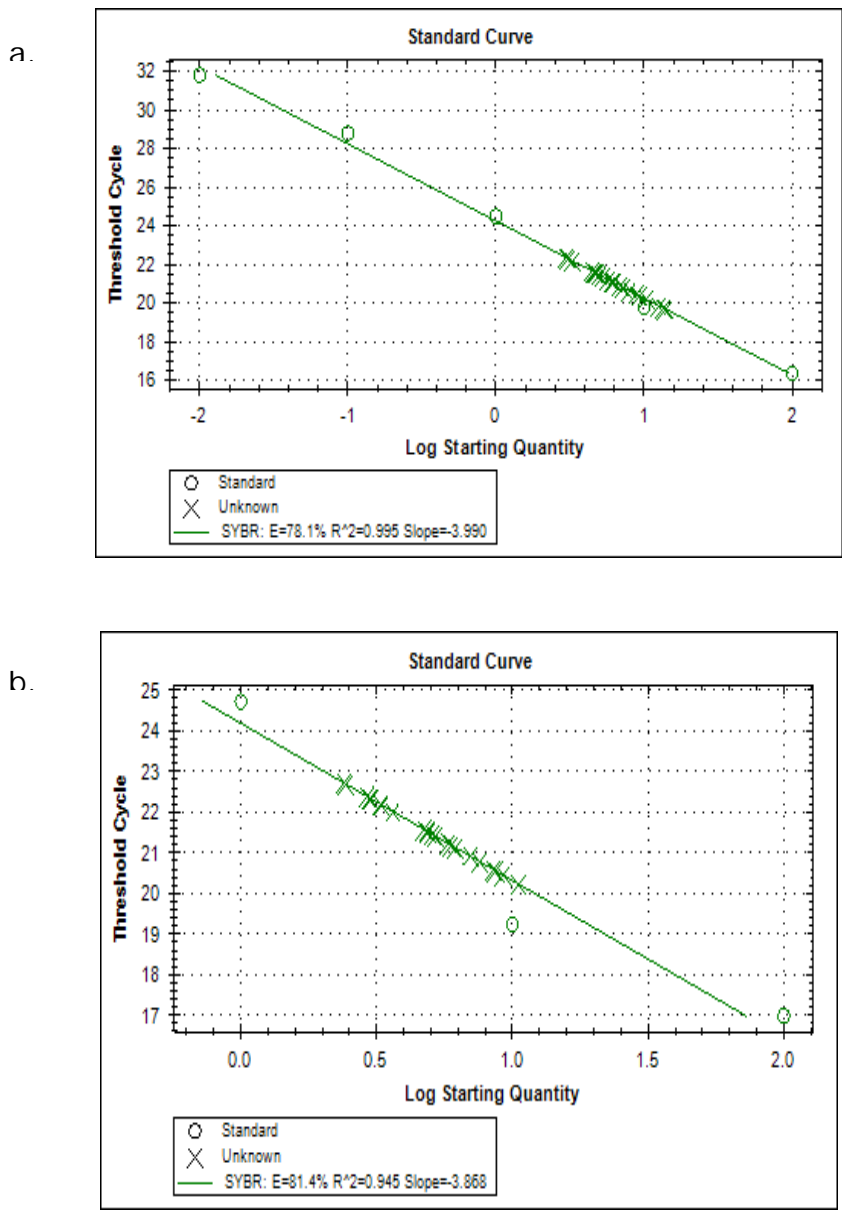


Figure 4.26 Standard curve analysis of two chloroplast associated genes. In (a) the standard curve for *CIEP* is shown while (b) illustrates the standard curve of the *PSI* subunit gene.

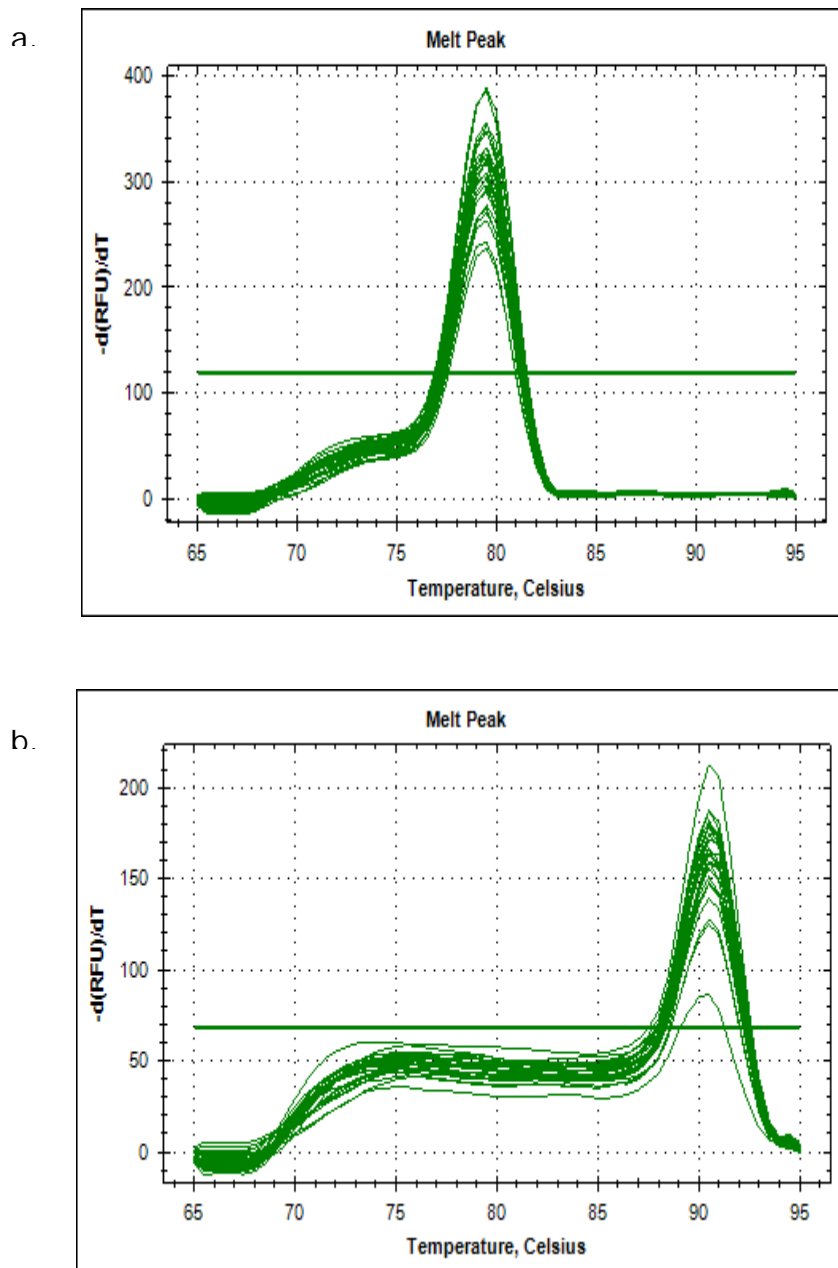
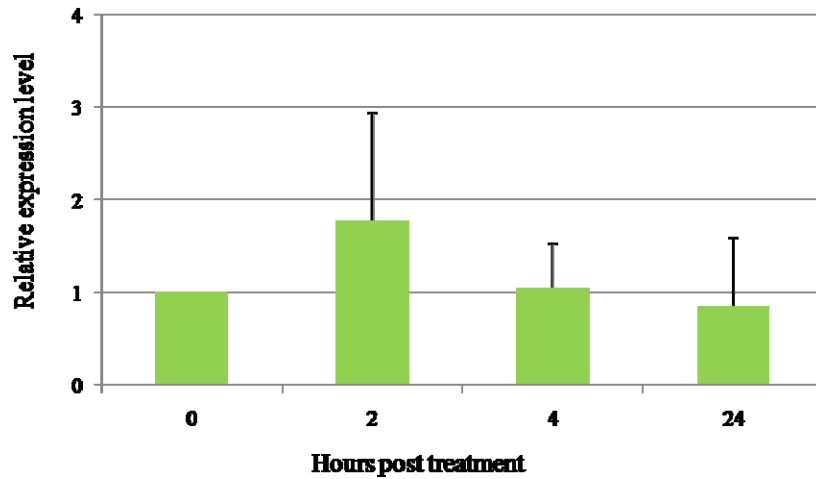


Figure 4.27 Melt curve analysis for two novel chloroplast genes. The melt curve for *CIEP* is shown in (a) and that for *PSI* subunit in (b).

a.



b.

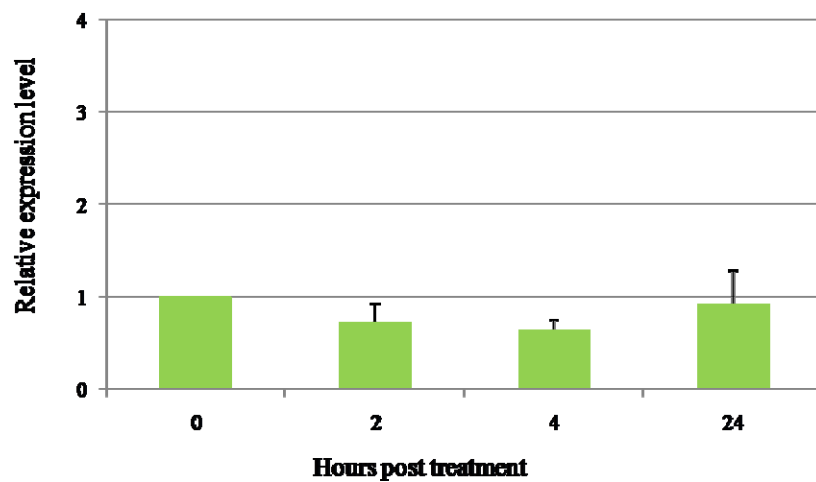


Figure 4.28 Influence of SS on the expression levels of two novel chloroplast genes. In (a) the expression level of the *CIEP* gene is indicated, while the *PSI* subunit gene level is indicated in (b). The x-axis represents the four different time intervals that were used while the y-axis represents the induced expression levels of the two genes relative to that of *GAPDH*.

#### 4.2.4 Expression analysis of defence related genes

Finally, the effect of SS application on the expression of two wheat *PR* genes, namely *PR2* and *PR3*, was tested.

##### 4.2.4.1 RT-PCR

Using RT-PCR analysis, a small increase in *PR2* expression was found in the SS treated plants at ½ and 1 hpt while the expression in the water treated plants was constitutive (Fig. 4.29a and b). A similar increase in *PR3* expression was evident at 1 and 2 hpt (Fig. 4.29c) with constitutive expression in the water treated plants (Fig. 4.29d). The amplification profiles of these two genes were however poor with multiple bands being amplified.

##### 4.2.4.2 RT-qPCR

For the real-time RT-PCR analysis, it was decided to analyse the expression of only the *PR3* gene. New primers for the real-time amplification of *PR3* were designed. The optimal annealing temperature was found to be 59°C (results not shown) and was thus used for the RT-qPCR. A tenfold serial dilution starting at 100 ng total RNA was done. Gel electrophoresis of the *PR3* amplified fragments indicated a single fragment in all the samples (Fig. 4.30a). The standard curve indicated successful amplification of the fragment up to the 10 pg total RNA dilution (Fig. 4.30b). Standard curve analysis indicated that all unknown samples fell between dilution factor two and three (Fig. 4.31a). The reaction efficiency was 101.4% and the R<sup>2</sup>-value 0.994. Both of these values were between the given parameters. Melt curve analysis indicated a single peak at 83°C with no primer dimers being formed (Fig. 4.31b). When the expression level of *PR3* was calculated, a near doubling in *PR3* gene expression was found at 2 and 4 hpt (Fig. 4.32). This expression finally decreased to about half that of the 0 hpt value.

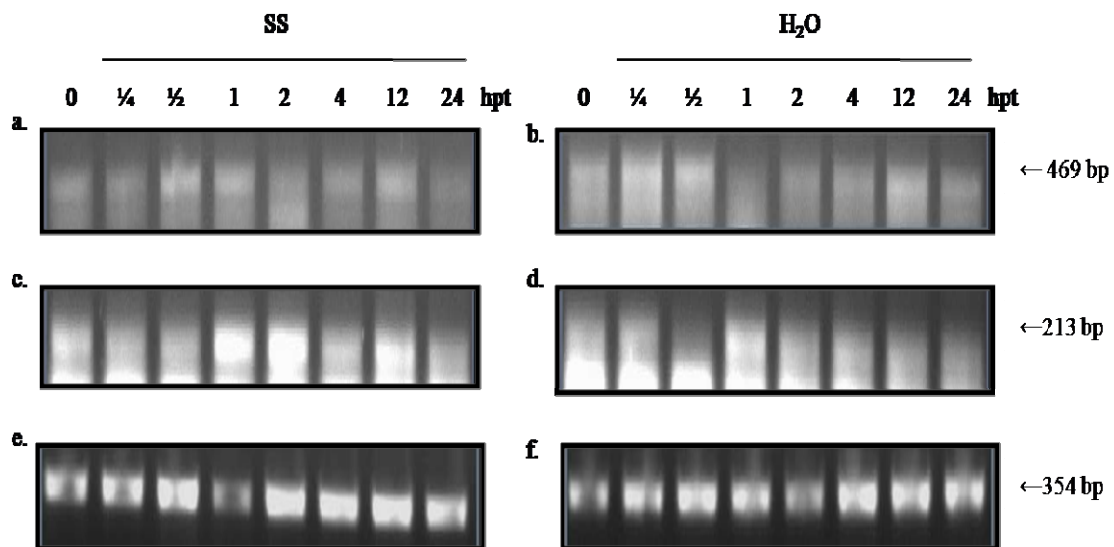


Figure 4.29 RT-PCR analysis of two *PR* genes in wheat treated with SS and water. In (a, b) and (c, d) the expression of *PR2* and *PR3* is illustrated respectively, while *GAPDH* is shown in (e) and (f). Time intervals and molecular sizes are as indicated.



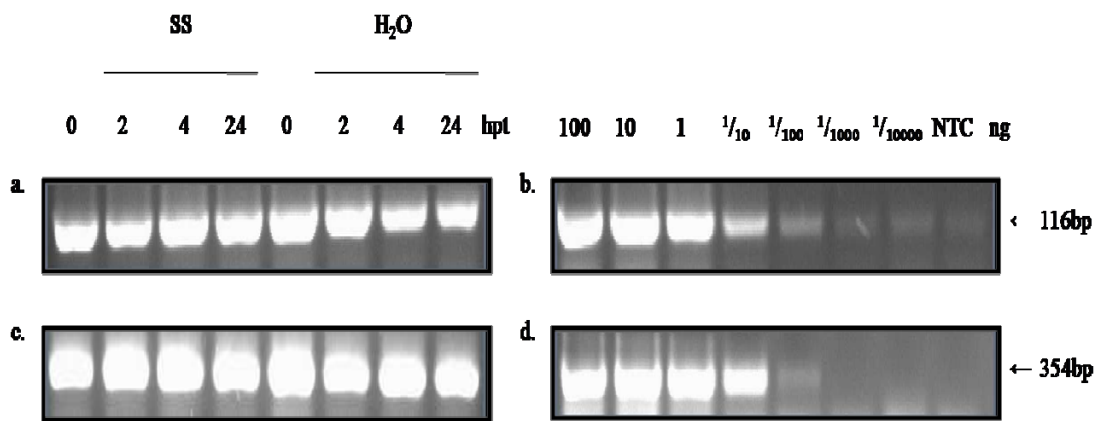


Figure 4.30 Agarose gel electrophoresis of the *PR3* amplified product after RT-qPCR amplification. The expression of *PR3* and *GAPDH* is given in (a) and (c) respectively. The dilution series for both these genes are indicated in (b) and (d). Time intervals and molecular fragment sizes are as shown.

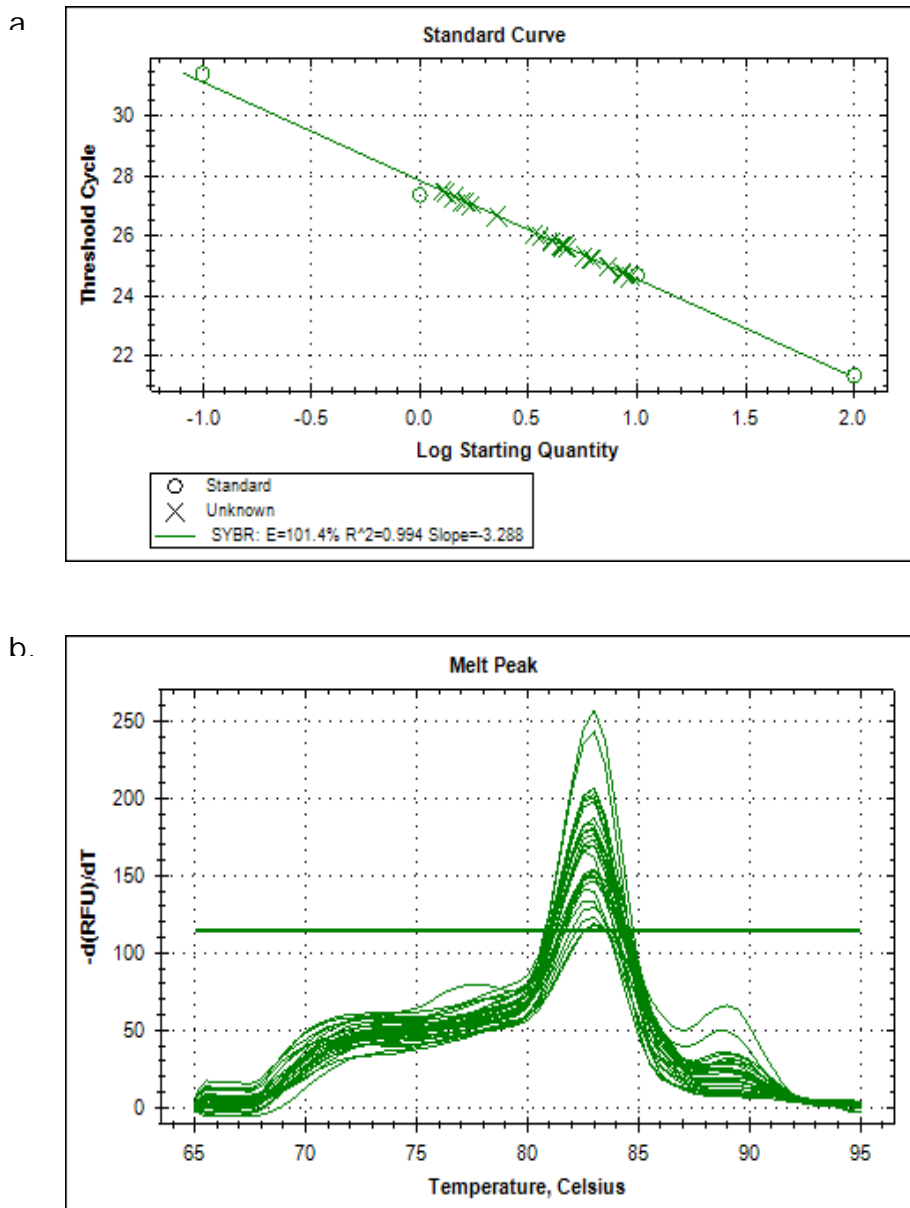


Figure 4.31 Standard and melting curve analyses of *PR3* amplification. The standard curve for *PR3* is shown in (a) and the melt curve in (b).

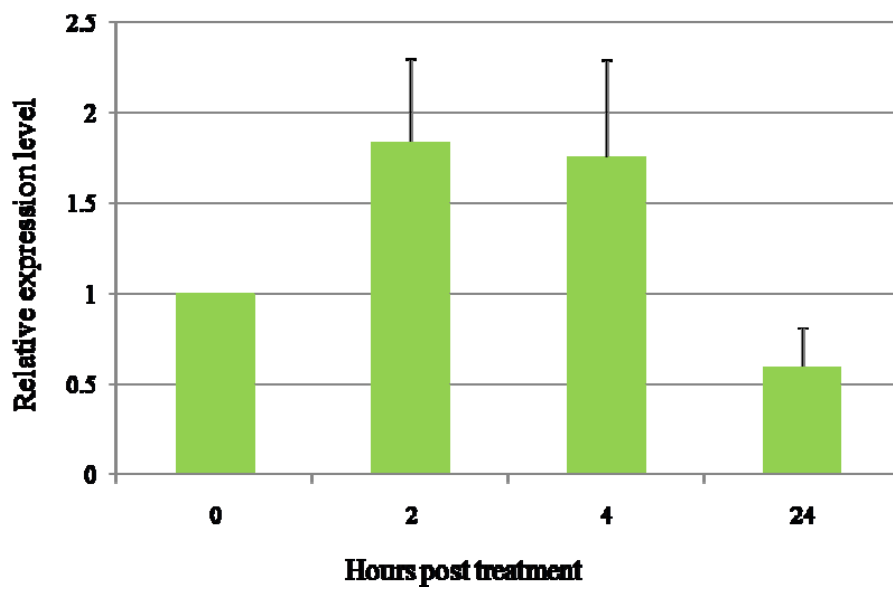


Figure 4.32 Influence of SS on the expression level of the *PR3* gene. The x-axis represents the four different time intervals that were used while the y-axis represents the induced expression levels of *PR3* relative to that of *GAPDH*.

# **Chapter 5**

## **Discussion**

The constant increase in the world's human population demands an increase in crop production in order to feed them. In 2008, the total world population reached 6.7 billion and is still increasing (Fujiwara and Matoh, 2009). Taken together, the increase in population and decrease in available farmland are of cardinal value. To increase yield and maintain the resources necessary to provide food worldwide, the nutritional value of plants is of importance (Fujiwara and Matoh, 2009).

The use of fertilisers to aid increased crop production has risen dramatically ([http://www.icrof.org/pdf/Halberg\\_food\\_security.pdf](http://www.icrof.org/pdf/Halberg_food_security.pdf)) and is continuing to do so (Beman *et al.*, 2005). However, the use of fertilisers may be more harmful than expected, affecting not only the direct environment but also the surrounding area (Yosef and Deyrup, 1998). This has urged farmers and scientists to search for new products that will effectively and safely improve the yield of crops.

The future production of crops will also be influenced by climate changes (Lobell and Field, 2007). In most cases, human activities are responsible for these climate changes (Sun *et al.*, 2009). With an increase in temperature over the next few years, an increase in variability in rainfall will no doubt also be seen. This will especially have a negative effect on Africa with the eastern parts where most crop production occurs, becoming drier.

To decrease the use of fertilisers, farmers are turning to more natural approaches for improving crops. One such approach is the use of plant activators. Plant activators can be divided into natural or synthetic activators and have been shown to have a positive effect on crop production and ultimately yield (Bishnoi and Payyavula, 2004). Examples of natural plant activators include harpin and ComCat<sup>®</sup>. Harpin is a heat-stable glycine-rich type III secreted protein from bacteria (Perino *et al.*, 1999) that as a plant activator is known to induce the hypersensitive defence response in plants (Krause and Durner, 2004).

ComCat<sup>®</sup> is a plant activator that is able to improve growth, quality and yield of crops after treatment (Meaza *et al.*, 2007). The active ingredients in ComCat<sup>®</sup> are brassinosteroids, a plant steroid hormone. Brassinosteroids were shown to promote growth and thermotolerance, while the exogenous application of brassinosteroids increased both the yield and fat content of groundnut with about 50% (Vardhini and Rao, 1998). The application of 24-

epibrassinolide (EBR), a brassinosteroid, to heat stressed tomatoes alleviated photosynthetic inhibition and reduced the total amount of H<sub>2</sub>O<sub>2</sub> formed during the stress (Ogweno *et al.*, 2008). During that study it was found that the F<sub>v</sub>/F<sub>m</sub> values of control plants declined after four days of heat stress, while the plants treated with EBR showed a constant F<sub>v</sub>/F<sub>m</sub> level. The NPQ value of EBR treated plants was also unaffected by heat exposure. This indicated that brassinosteroids have a positive effect on the photosynthetic machinery and thus suggests that ComCat<sup>®</sup>, as a plant activator, aids plants by protecting the plant during stress conditions.

The synthetic compound BTH is a plant activator that was released in 1996 and trade under names like BION<sup>®</sup>, Actigard<sup>®</sup> and BOOST<sup>®</sup>. In a study done by Lang *et al.* (2007), they indicated that the application of BTH decreased the severity of *Xanthomonas* leaf blight infection in onion by 50%. However, weekly applications of BTH could not be linked to increased bulb size or yield improvement. In a separate study done by Gent and Schwartz (2005) ten weekly applications of BTH to uninfected onions actually decreased the bulb yield by up to 27%.

A novel plant activator whose positive effects have not yet been properly described, is SS. SS is a *L. albus* seed suspension that was prepared to determine whether it has a stimulatory effect on seed germination and seedling growth of field crops (Van der Watt, 2005). Foliar application induced the yield of three different crops significantly. Beetroot yield increased with 9.3 ton/ha while lettuce and carrots increased by 20.0 and 24.3 ton/ha respectively. SS also increased wheat yield by 2.6 ton/ha. The active ingredient in SS is a triglyceride called glycerol trilinoleate. The current study thus focused on the response of wheat as an important crop upon treatment with SS, since it was found that SS improves growth and yield (Van der Watt, 2005).

The first aspect that was studied was the influence of SS on the photosynthetic ability of both normal growing plants, as well as stressed plants. Photosynthesis is perhaps the single most important aspect of plant growth and survival. Photosynthesis occurs in the chloroplasts and uses energy from the sun to generate chemical energy in the form of carbohydrates (Slater *et al.*, 2003). This serves as the energy resource the plant needs to maintain other vital

processes and pathways. A by-product of photosynthesis besides sugars, is the production of oxygen.

In order to test the effect of SS on photosynthesis, chlorophyll *a* fluorescence was used (Maxwell and Johnson, 2000). The advantage of this technique is that when measuring photosynthetic yield, information is also obtained regarding the photochemistry and heat dissipation within the leaves (Maxwell and Johnson, 2000). Chlorophyll *a* fluorescence measurement is further a non-invasive method of analysing the effect of certain biotic stresses on photosynthesis. In this study the plants were treated with SS while a water treatment was used as control.

Under normal glass house conditions it was clear that photosynthetic potential as indicated by the  $F_v/F_m$  values of SS treated plants increased over the duration of the study while the water control stayed more or less constant (Fig. 4.1a). In addition, when analysing the NPQ curve, it appeared that SS treatment restricted heat loss due to photo-inhibition by nearly a third (Fig. 4.1c). However, with no stress applied to the plants, no real differences were found for both the actual efficiency of PSII (Fig. 4.1b) and the number of open reaction centres (Fig. 4.1d) for both treatments. It was previously shown by Beckett *et al.* (2000) that, after treatment with ABA, which is also a plant activator, the value of  $F_v/F_m$  increased in moss (*Atrichum undulatum*). They indicated that after desiccation the  $F_v/F_m$  values for ABA treated plants decreased, but as soon as rehydration occurred, NPQ and  $\hat{O}PSII$  increased much faster after ABA treatment as opposed to untreated plants. They concluded by stating that ABA treatment positively changes the characteristics of PSII in *A. undulatum* plants by dissipating excess energy.

Based on the previous findings, it was decided to expose wheat to different stress factors to determine the effect of SS during these different stress responses. In higher plants the rate of photosynthesis is regulated by ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) (Chaitanya *et al.*, 2002). Rubisco catalyses the assimilation of CO<sub>2</sub> by means of ribulose-1,5-bisphosphate (Bloom, 2009). Even though Rubisco is present in high quantities in plants, its activity can be inhibited (Sun *et al.*, 2009). This was shown in a study done by Hudson *et al.* (1992) where they illustrated that after cloning *RbcS* from tobacco (*Nicotiana tabacum*) and fusing it with a cauliflower mosaic virus 35S promoter in the antisense orientation, the

reduced Rubisco levels limited photosynthesis of antisense plants by reducing the rate of CO<sub>2</sub> assimilation by 63%. The stomatal conductance was however not reduced, indicating that the total Rubisco activity in the leaf functions independent of stomatal conductance.

It is well known that drought reduces carbon assimilation while water stress reduces Rubisco activity (Flexas *et al.*, 2006). Water stress thus limits the diffusion of CO<sub>2</sub> from the air into carboxylation sites which ultimately decreases photosynthesis (Lawlor and Cornic, 2002). When stress in the form of drought was applied to wheat seedlings, no significant differences were observed in the number of open reaction centres between the SS and control treatments (Fig. 4.2d). These levels did however decrease after the onset of drought conditions which clearly also affected  $\hat{O}PSII$  (Fig. 4.2b). It was however evident that SS treatment led to an improved quantum efficiency compared to the water treatment during the latter stages of the study (Fig. 4.2b), even though the number of open reaction centres decreased dramatically. This was accompanied with lower NPQ levels as compared to the water treatment (Fig. 4.2c).

It was also evident that before the two treatments were applied, both the experimental and control plants were under some form of unintended stress as indicated by both the  $\hat{O}PSII$  (Fig. 4.2b) and the NPQ levels (Fig. 4.2c). Both sets of plants showed improved  $\hat{O}PSII$ , as well as reduced heat loss shortly after treatment with no real differences between the two treatments. SS treatment did however have a positive effect on the photosynthetic potential ( $F_v/F_m$ ) of wheat (Fig. 4.2a), similar to that found in Fig. 4.1a. Overall the SS treated plants were able to respond slightly better to the water stress in comparison to the control treatment.

The decrease in the quantum efficiency of PSII (Fig. 4.2b) indicated that the water stress caused photo-inhibitory damage to the plant. Similar results were obtained in a study done by Pastenes *et al.* (2005). They illustrated that para-heliotropism plays a role in photo-inhibition and that the capacity of  $F_v/F_m$  to recover post water-stress, is not higher than well watered plants. They also found that in bean leaves where para-heliotropism was restrained, a significant reduction in both qP and  $F_v/F_m$  was found. Their data indicated that the restriction of para-heliotropism affects CO<sub>2</sub> assimilation in well watered plants.



Field trials indicated that the application of SS to crops increased both the growth and yield (Van der Watt, 2005). The effect of this activator on plants placed under heat stress is however unknown and it was subsequently investigated (Fig. 4.3).

While the  $F_v/F_m$  levels of the plants grown at 23°C were almost identical for both treatments and remained unchanged for the duration of the study, the heat stressed plants showed an immediate decrease in both the SS and water treatments (Fig. 4.3a). The photosynthetic potential of the SS treated wheat was however significantly higher than that of the water treated plants for the duration of the study, except at 108 hpt. A similar profile was found for the actual photosynthetic rate (Fig. 4.3b). These results were supported by a previous study that indicated that the foliar application of SA increased both the  $F_v/F_m$  values and the actual photosynthetic rate as given by  $\hat{O}PSII$  after 36 h of heat stress and recovery (Shi *et al.*, 2006). The researchers observed that foliar application of SA was successful in removing  $H_2O_2$  and reducing heat stress.

Law and Crafts-Brandner (1999) indicated more or less similar results. They indicated that by increasing the leaf temperature of cotton (*Gossypium hirsutum*) and wheat (*Triticum aestivum*) plants, a decrease in the light saturated  $CO_2$  exchange rate (CER) was seen. A decline in the latter correlated with a decrease in Rubisco activity. Even though the correlation of Rubisco activity was stronger in relation to CER, decreased  $F_v/F_m$  values also signified a decrease in Rubisco activity.

Interesting to note was that the NPQ levels, an indication of heat dissipation, decreased immediately after the onset of the heat stress with no difference between the SS and water treated plants (Fig. 4.3c). This was exactly the opposite than what was expected since the plants were placed under stress. The number of open PSII reaction centres was unaffected by the heat stress treatments (Fig. 4.3d). Even though plants were stressed, the heat-induced effect on photosynthesis can be reversible. Oak trees exposed to high temperatures indicated a decrease in the net photosynthetic  $CO_2$  assimilation rate ( $P_n$ ) of almost 90%. The inhibition of  $P_n$  was fully reversible and the levels of Rubisco returned to normal once the temperature was normalised (Haldimann and Feller, 2004).

In the previous experiment, SS was applied before the plants were exposed to heat stress. It was decided to test whether the time of application of SS to stressed plants, is important. Wheat plants were first exposed to the heat stress, and only then were they treated with SS (Fig. 4.4). The obtained results were similar to the previous experiment (Fig. 4.3). Heat stressed plants indicated a rapid decrease in photosynthetic potential (Fig. 4.4a), the actual photosynthetic rate (Fig. 4.4b) and heat dissipation (Fig. 4.4c). When comparing the  $\hat{O}PSII$  (Fig. 4.4b) and NPQ graphs (Fig. 4.4c), the last two time intervals are of importance. In the  $\hat{O}PSII$  graph, SS treated wheat in comparison with the water treated plants showed improved photosynthetic activity starting at 96 hpt which was accompanied by decreased levels of heat dissipation. This was a clear indication that photosynthesis in the SS treated plant was working more efficiently than the water treated plants. No real differences between the two treatments were seen for  $F_v/F_m$  (Fig. 4.4a) or the number of open reaction centres (Fig. 4.4d).

In conclusion, SS application benefits the photosynthetic potential and actual photosynthetic ability of wheat. When no stress is applied, the benefits are not as evident as when the plants are placed under stress. However, during drought and heat stress, the actual photosynthetic ability of wheat improved after treatment with SS. This was also accompanied by a reduction in dissipated heat. In contrast, SS treatment had no effect on the number of open PSII reaction centres.

A critical factor influencing improved photosynthesis and ultimately yield is the fixation of  $CO_2$  via the Calvin cycle. There are many factors that can impair  $CO_2$  fixation with elevated temperatures and water stress being two of them (Van Rensen *et al.*, 1999). Another inhibiting factor is the accumulation of carbohydrates in  $Mg^{2+}$ -deficient leaves (Cakmak and Kirkby, 2008). A decrease in stomatal conductance of  $Mg^{2+}$ -deficient leaves is associated with a decline in photosynthetic capacity (Laing *et al.*, 2000). The latter then leads to a decline in enzyme activity involved in  $CO_2$  fixation. The acclimation of plants to different stress conditions therefore relies on stomatal conductance (Mateo *et al.*, 2004). The stomatal closure decreases the  $CO_2/O_2$  ratio within the plants which increases photorespiration and  $H_2O_2$  production (Wingler *et al.*, 2000).

In order to better characterise the effect of SS on plants, molecular analysis of gene expression was done. The expression of three different sets of genes was analysed. The first

group included three photosynthesis related genes, the second group three chloroplast associated genes while the final group included two defence related genes. These genes were all represented in the SSH generated cDNA library prepared from wheat treated with SS (Van der Merwe, 2008). It is important to note that during the course of the study, several different SS treatments were done since large amounts of total RNA were needed. Even though the SS treatments as well as the time intervals when tissue was harvested remained the same throughout, variations in the time intervals when the genes showed induced expression were evident.

Various techniques were used to determine the expression of the different genes. Included were RT-PCR, semi-quantitative RT-PCR, Northern blots and RT-qPCR. RT-PCR is however not a quantitative technique, while the latter three are. RT-qPCR offers numerous advantages over conventional RT-PCR and Northern blots (Bustin *et al.*, 2005). The first is that RT-qPCR measures the kinetics of the reaction after each cycle of amplification, while conventional RT-PCR only detects the end-point of the reaction. Secondly RT-qPCR is able to measure the quantitative relationship between the initial template sample and the amount of product after any given cycle, while conventional RT-PCR can not. Thirdly, real-time reactions measure the quantitation cycle where fluorescence intensity is above that of the background. RT-qPCR is also much safer than Northern blot analysis as the latter often uses radio-active components and the sensitivity is much lower in comparison with RT-qPCR. Thus in order to quantify expression, RT-qPCR analysis was ultimately used because of all these advantages. Using RT-qPCR, clinical diagnosis of non-A-C acute hepatitis patients indicated that 30.5% of tested individuals were positive for this disease, while only 20% tested positive with conventional RT-PCR analysis (Zhao *et al.*, 2007), indicating the sensitivity of this technique.

All RT-qPCR analysis was done according to the MIQE guidelines (Bustin *et al.*, 2009). MIQE is the minimum information that is necessary for the evaluation of qPCR results. These guidelines include a checklist that researchers need to include in publications in order to promote the repeatability of reactions between different laboratories and to ensure the veracity of scientific literature (Bustin *et al.*, 2009). The current study complies with all these guidelines.

The three photosynthesis related genes that were analysed were *RbcL*, *RbcS* and *PGK*. Rubisco, which constitutes 50% of all soluble leaf protein and 25% of leaf nitrogen, catalyses the assimilation of CO<sub>2</sub> through the carboxylation of rubulose-1,5-biphosphate (RuBP) during the Calvin cycle (Reynolds *et al.*, 2009). Rubisco consist of two different subunits with eight small and eight large subunits [(L<sub>2</sub>)<sub>4</sub>(S<sub>4</sub>)<sub>2</sub>] forming this hexa-decamer (Tabita, 2007). On a transcriptional level, the expression of *RbcL* is regulated by the availability of the Rubisco small subunit protein in the chloroplast (Suzuki *et al.*, 2009). In *RbcS* antisense tobacco, the mRNA levels of *RbcS* and *RbcL* simultaneously decreased (Suzuki *et al.*, 2009). The factors that regulate synchronised gene expression between the *RbcL* and *RbcS* is however unknown. The expression of both the large and small subunit genes of Rubisco was initially analysed. RT-PCR analysis did not indicate any differential gene expression for both *RbcL* and *RbcS* after SS treatment (Fig. 4.7). This could have been due to the fact that these genes are normally expressed at high levels (Oey *et al.*, 2008) and RT-PCR, due to its limitations, is unable to detect any subtle changes. Northern blot analysis of *RbcL* on the other hand indicated induced expression at ½ hpt with a second stronger induction at 2 hpt after SS treatment (Fig. 4.9a). RT-qPCR analysis supported this finding by indicating a near 1.5 fold increase in expression (Fig. 4.14). The expression of *RbcS* was unaffected by SS treatment (Fig. 4.7, 4.8 and 4.14).

The expression of *RbcL* and *RbcS* in dark germinated plants was also analysed (Fig. 4.8). The expression of *RbcS* seems to be unaffected by the dark and SS treatment, but *RbcL* not. The latter indicated a constitutive expression up to 2 h post SS treatment. There after, at 4 hpt, a decrease in expression was seen in both SS and water treated plants. It was however clear that the expression of *RbcL* in the SS treated plants was still higher than that in the water treated plants, indicating the stimulatory effect of SS treatment on *RbcL* expression.

The third photosynthesis associated gene that was analysed was *PGK*. The enzyme uses ATP during the first reduction step of the Calvin cycle to form 1,3-bisphosphoglycerate (Parker *et al.*, 2006). Even though not clear using RT-PCR (Fig. 4.7), RT-qPCR results of *PGK* showed a near doubling of gene expression 24 h after SS treatment (Fig. 4.14c). A recent study found that *PGK* expression increased after salt stress in rice (*Oryza sativa*) (Parker *et al.*, 2006). They also noticed a rise in the expression of the *ATP synthase* gene and postulated that this might be associated with increased photosynthetic rates as well as *PGK* expression.

Thus, even though the induction levels of the three tested genes were not as high as anticipated, the induced expression of *RbcL* and *PGK* indicated that SS could increase carbon assimilation during the dark phase of photosynthesis by increasing protein levels through induced gene expression.

Since photosynthesis depends on fully functional chloroplasts, the influence of SS on the expression of three genes whose encoded proteins are chloroplast associated was tested. It is known that pathogen infection regularly targets the photosynthetic machinery in the chloroplasts causing changes in chloroplast protein complexes and photosynthetic enzymes involved in the disease response (Balachandran *et al.*, 1997). One pathogen that is known to do so is *P. syringae* (Jones *et al.*, 2006). HopI1, a *P. syringae* specific effector targets the chloroplasts (Jalenska *et al.*, 2007) where it inhibits the chloroplast mediated defence response while remodelling the thylakoid. The latter suppresses the production and transport of SA in the chloroplasts which may ultimately lead to the colonisation and growth of the pathogen within the host.

Abiotic stresses also influences chloroplast stability and thus also yield. Climate changes, drought, light and nutrition are amongst the most important (Pinheiro *et al.*, 2004; Wagner *et al.*, 2008). When a winter wheat cultivar was exposed to three different temperatures, the severity of chloroplast damage differed (Xu *et al.*, 2005). Exposure to 38°C caused chloroplasts and thylakoids to become swollen. At 42°C, chloroplasts disappeared completely and thylakoids were in serious disorganisation. However, plants that were acclimatised at 30°C indicated less damage to the chloroplast machinery. The latter was slightly swollen at 38°C and slightly ruptured at 42°C (Xu *et al.*, 2005). Another study indicated that chloroplast and thylakoid membrane damage is closely correlated to heat stress (Ristic *et al.*, 2007). They found that with increased thylakoid damage, more chlorophyll was lost.

One putative key protein that could influence thylakoid membrane stability, is Ptr ToxA BP, a chloroplast located protein that has not yet been thoroughly investigated (Manning *et al.*, 2007). Ptr ToxA BP is a homolog of the *Arabidopsis* Thf1 protein (Wang *et al.*, 2004) which is important in the formation of thylakoids suggesting that Ptr ToxA BP might play a similar role (Manning *et al.*, 2007). In *Arabidopsis*, the presence of Thf1 is found in both the

chloroplast envelope (Peltier *et al.*, 2004) and stroma (Peltier *et al.*, 2006) while in wheat Ptr ToxA BP was also found in these sites (Manning *et al.*, 2007). Ptr ToxA BP and ToxA, a proteinaceous toxin from *P. tritici-repentis*, interact (Manning *et al.*, 2007; 2008) when ToxA is internalised into the mesophyll cells of susceptible wheat (Kwon *et al.*, 1996). Once inside the host cell, it is translocated to the chloroplasts (Manning *et al.*, 2007) where transcriptional as well as translational changes occur which lead to cell death. The interaction between *P. tritici-repentis* and *Arabidopsis* is comparable to the interaction between *Stagonospora nodorum* and wheat where both deliver cell death via host selective toxins (HSTs) (Liu *et al.*, 2009).

Following SS treatment, RT-PCR showed that the expression of *Ptr ToxA BP1* increased at 1 hpt (Fig. 4.15e). This induced expression was confirmed using semi-quantitative RT-PCR (Fig. 4.16) and Northern blot analysis using purified mRNA (Fig. 4.19). Finally, RT-qPCR confirmed a fivefold induction of this gene 2 h after SS treatment (Fig. 4.23). This induced expression is also light independent, since the expression was induced by SS treatment, even in the dark (Fig. 4.17 and 4.18).

Two other chloroplast associated genes that were analysed were *CIEP* and the *PSI* subunit gene. Chloroplasts have three membrane structures, namely the outer envelope membrane, the inner envelope membrane and the thylakoid membrane (Teng *et al.*, 2006). Protein insertion into the outer envelope membrane has been described in a range of studies (Tu *et al.*, 2004; Hofmann and Theg, 2005) but those inserted in the inner envelope is poorly described. In a study done by Chiu and Li (2008) it was indicated that two chloroplast inner envelope proteins, Tic40 and Tic110, are imported from the cytosol to the chloroplast stroma to the inner envelope membrane via multiple pathways. One such pathway includes a one megadalton protein complex that creates a protein channel that aids in protein translocation into the inner envelope of both *Arabidopsis* and *Pisum sativum* (Kikuchi *et al.*, 2009). These two inner envelope proteins possibly aid in the translocation of proteins into the chloroplast and the cleavage of peptide sequences from their precursor proteins (Dávila-Aponte *et al.*, 2003; Park *et al.*, 2007). A similar function for *CIEP* could be envisaged.

While the expression of the *CIEP* gene showed an almost doubling in gene expression, no induced gene expression was evident for the *PSI* subunit gene (Fig. 4.28). A large standard

deviation however meant that the induced *CIEP* expression was not significant. Even though SS can therefore be linked to the induced expression of the *Ptr ToxA BP1* gene and thus also to thylakoid membrane stability within the chloroplast, it could not be linked to the expression of the other two membrane associated genes.

Finally, the ability of SS treatment to activate a defence response in wheat was tested by determining the gene expression profiles of two *PR* genes. Proteins encoded by *PR* genes have antimicrobial activities which make these genes important in the development of an HR and SAR (Shah *et al.*, 1999). These genes are expressed at high levels once it has been stimulated by either a pathogen or a plant activator (Uknes *et al.*, 1992). Ward *et al.* (1991) observed that SA, which is important in the establishment of a defence response, induced the expression of *PR2*, *PR3* and *PR4* 24 hours after treatment. In a study done by Whan *et al.* (2009), it was shown that when cotton seeds were soaked in BION<sup>®</sup>, germinated and then infected with *Fusarium oxysporum* f.sp. *vasinfectum* (Fov), the level of three *PR* genes were induced. The roots and shoots showed the greatest increase in chitinase (*PR2*),  $\alpha$ -1, 3-glucanase (*PR3*) and peroxidase (*PR9*) activity.

The last group of genes that were thus analysed in this study included *PR2* and *PR3*. While RT-PCR analysis of *PR2* expression did not indicate any induction, *PR3* expression showed an induction at 1 and 2 hpt (Fig. 4.29). Based on this, new primers were designed for the *PR3* gene which was used for RT-qPCR analysis. As with most of the other analysed genes, *PR3* gene expression was induced 2 h after treatment with SS (Fig. 4.32). This indicated that SS application has the ability to induce a defence response within wheat.

In conclusion, where previous work showed that the application of SS to field crops led to increased yield, this study implicated both photosynthesis and chloroplast stability as key factors influencing the increased yield following SS treatment. Furthermore, improved photosynthesis in combination with induced *PR* gene expression indicates the value of SS as a general plant activator. SS application thus benefits the plant on both a photosynthetic and molecular level, making it a very valuable commodity.

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# Summary

The use of plant activators offers an exciting new approach for the enhancement of growth and yield in crops and ornamental plants. During a previous study, the effect of a *Lupinus albus* L. seed suspension (SS), a novel plant activator, was tested on different crops. Field trials indicated that SS application could be linked to improved growth and yield of agricultural as well as horticultural crops. The aim of this study was thus to confirm the results of Van der Watt (2005) by studying the effects of SS on photosynthesis and gene expression in wheat.

When applied to Thatcher + *Lr34* wheat, chlorophyll *a* fluorescence measurements indicated that unstressed plants were not dramatically affected by SS application. However, when placed under water and heat stress, SS treatment improved the actual photosynthetic ability of the plants by reducing photo-inhibition as measured by heat loss as opposed to the control treatment.

On molecular level, three different techniques were used to confirm the expression of selected genes after SS treatment, with RT-qPCR analysis ultimately being the most effective. RT-qPCR confirmed the induced expression by SS of two photosynthesis related genes (*RbcL* and *PGK*), one chloroplast associated gene (*Ptr ToxA BPI*) and one defence gene (*PR3*). The expression of these genes indicated that SS is an important activator and that its main target site in wheat is related to the photosynthetic system. In this system SS improves membrane stability but also aid in the delivery of electrons across the chloroplast membrane. SS is able to induce a PR defence response that is able to protect field crops against pathogenic infection.

This study thus confirmed that SS does indeed function as an effective plant activator that when applied, would benefit the plant on various different levels.

**Keywords:** Gene expression, *Lupinus albus* L. seed suspension, photosynthesis, *Ptr ToxA BPI*, RT-qPCR.

# **Opsomming**

Die gebruik van plant aktiveerders bied 'n opwindende nuwe benadering tot die bevordering van groei en opbrengs in gewasse en sierplante. Tydens 'n vorige studie is die invloed van *Lupinus albus* L. seed suspension (SS), 'n potensiële plant aktiveerder, op verskillende gewasse getoets. Veldproewe het aangedui dat SS toediening aan verbeterde groei en opbrengs van beide landbou en tuinbougewasse gekoppel kan word. Die doel van hierdie studie was dus om die resultate van Van der Watt (2005) te bevestig deur die invloed van SS toediening op fotosintese en geenuitdrukking in koring te bestudeer.

Met die toediening van SS aan Thatcher + *Lr34* koring, het chlorofil *a* fluoresensiemetings aangedui dat plante wat nie onder spanning verkeer nie, nie dramaties daardeur geaffekteer word nie. Wanneer die plante egter onder water en hitte-spanning geplaas is, het die behandeling met SS die fotosintetiese vermoë van plante verbeter deur foto-inhibisie, soos gemeet deur hitteverlies, te verminder.

Op molekulêre vlak is drie verskillende tegnieke getoets om die uitdrukking van geselekteerde gene na SS behandeling te bepaal. Op die ou einde was die RT-qPCR tegniek die mees effektiefste. Met hierdie tegniek is die geïnduseerde uitdrukking van twee fotosinteties-verwante gene (*RbcL* en *PGK*), een chloroplas-verwante gene (*Ptr ToxA BPI*) en een verdedigingsgeen (*PR3*) na SS behandeling, bevestig. Die uitdrukking van hierdie gene het aangetoon dat SS 'n belangrike aktiveerder is en dat dit veral die fotosintetiese-sisteem in koring teiken. In hierdie sisteem bevorder SS membraanstabieliteit asook die vervoer van elektrone oor die chloroplasmembraan. SS is ook instaat om 'n patogeenverwante verdedigingsrespons te aktiveer wat gewasse teen patogeen-infeksie kan beskerm.

Hierdie studie bewys dus dat SS as 'n effektiewe plant aktiveerder funksioneer wat na toediening, plante op verskillende vlakke bevoordeel.

**Sleutelwoorde:** Fotosintese, geenuitdrukking, *Lupinus albus* L. seed suspension, *Ptr ToxA BPI*, RT-qPCR.

# **Appendices**

# **Appendix 1**



## Internet references

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## **Appendix 2**

Well	Fluor	Content	Target	Sample	Quantitation Cycle (Cq)	Cq Mean	Cq Std. Dev	Starting Quantity (SQ)	Log Starting Quantity	SQ Mean	SQ Std. Dev
A01	SYBR	Std-01	GAPDH		16.15	16.12	0.127	100.00000	2.000	100.00000	0.00000
A02	SYBR	Std-01	GAPDH		15.99	16.12	0.127	100.00000	2.000	100.00000	0.00000
A03	SYBR	Std-01	GAPDH		16.24	16.12	0.127	100.00000	2.000	100.00000	0.00000
A04	SYBR	Unkn-01	GAPDH	Water 0h	21.07	21.23	0.146	3.55623	0.551	3.21499	0.31789
A05	SYBR	Unkn-01	GAPDH	Water 0h	21.25	21.23	0.146	3.16150	0.500	3.21499	0.31789
A06	SYBR	Unkn-01	GAPDH	Water 0h	21.36	21.23	0.146	2.92723	0.466	3.21499	0.31789
A07	SYBR	Std-08	RbcL		6.18	6.33	0.144	100.00000	2.000	100.00000	0.00000
A08	SYBR	Std-08	RbcL		6.35	6.33	0.144	100.00000	2.000	100.00000	0.00000
A09	SYBR	Std-08	RbcL		6.47	6.33	0.144	100.00000	2.000	100.00000	0.00000
A10	SYBR	Unkn-09	RbcL	Water 0h	13.18	13.27	0.091	3.14033	0.497	2.99333	0.14011
A11	SYBR	Unkn-09	RbcL	Water 0h	13.28	13.27	0.091	2.97835	0.474	2.99333	0.14011
A12	SYBR	Unkn-09	RbcL	Water 0h	13.36	13.27	0.091	2.86132	0.457	2.99333	0.14011
B01	SYBR	Std-02	GAPDH		19.58	19.52	0.083	10.00000	1.000	10.00000	0.00000
B02	SYBR	Std-02	GAPDH		19.43	19.52	0.083	10.00000	1.000	10.00000	0.00000
B03	SYBR	Std-02	GAPDH		19.56	19.52	0.083	10.00000	1.000	10.00000	0.00000
B04	SYBR	Unkn-02	GAPDH	Water 2h	20.81	20.65	0.179	4.24181	0.628	4.75620	0.57962
B05	SYBR	Unkn-02	GAPDH	Water 2h	20.68	20.65	0.179	4.64256	0.667	4.75620	0.57962
B06	SYBR	Unkn-02	GAPDH	Water 2h	20.46	20.65	0.179	5.38422	0.731	4.75620	0.57962
B07	SYBR	Std-09	RbcL		10.67	10.84	0.323	10.00000	1.000	10.00000	0.00000
B08	SYBR	Std-09	RbcL		11.21	10.84	0.323	10.00000	1.000	10.00000	0.00000
B09	SYBR	Std-09	RbcL		10.63	10.84	0.323	10.00000	1.000	10.00000	0.00000

B10	SYBR	Unkn-10	RbcL	Water 2h	12.83	12.80	0.167	3.74407	0.573	3.81262	0.32827
B11	SYBR	Unkn-10	RbcL	Water 2h	12.62	12.80	0.167	4.16976	0.620	3.81262	0.32827
B12	SYBR	Unkn-10	RbcL	Water 2h	12.95	12.80	0.167	3.52404	0.547	3.81262	0.32827
C01	SYBR	Std-03	GAPDH		22.85	22.96	0.112	1.00000	0.000	1.00000	0.00000
C02	SYBR	Std-03	GAPDH		22.96	22.96	0.112	1.00000	0.000	1.00000	0.00000
C03	SYBR	Std-03	GAPDH		23.08	22.96	0.112	1.00000	0.000	1.00000	0.00000
C04	SYBR	Unkn-03	GAPDH	Water 4h	20.41	20.39	0.078	5.55344	0.745	5.64628	0.30029
C05	SYBR	Unkn-03	GAPDH	Water 4h	20.45	20.39	0.078	5.40337	0.733	5.64628	0.30029
C06	SYBR	Unkn-03	GAPDH	Water 4h	20.30	20.39	0.078	5.98202	0.777	5.64628	0.30029
C07	SYBR	Std-10	RbcL		16.41	15.76	0.598	1.00000	0.000	1.00000	0.00000
C08	SYBR	Std-10	RbcL		15.25	15.76	0.598	1.00000	0.000	1.00000	0.00000
C09	SYBR	Std-10	RbcL		15.61	15.76	0.598	1.00000	0.000	1.00000	0.00000
C10	SYBR	Unkn-11	RbcL	Water 4h	12.38	12.22	0.140	4.71650	0.674	5.13241	0.36035
C11	SYBR	Unkn-11	RbcL	Water 4h	12.13	12.22	0.140	5.35098	0.728	5.13241	0.36035
C12	SYBR	Unkn-11	RbcL	Water 4h	12.14	12.22	0.140	5.32976	0.727	5.13241	0.36035
D04	SYBR	Unkn-04	GAPDH	Water 24h	21.27	21.42	0.125	3.10566	0.492	2.82504	0.24307
D05	SYBR	Unkn-04	GAPDH	Water 24h	21.49	21.42	0.125	2.67948	0.428	2.82504	0.24307
D06	SYBR	Unkn-04	GAPDH	Water 24h	21.49	21.42	0.125	2.69000	0.430	2.82504	0.24307
D07	SYBR	Std-11	RbcL		19.55	19.74	0.174	0.10000	-1.000	0.10000	0.00000
D08	SYBR	Std-11	RbcL		19.89	19.74	0.174	0.10000	-1.000	0.10000	0.00000
D09	SYBR	Std-11	RbcL		19.77	19.74	0.174	0.10000	-1.000	0.10000	0.00000
D10	SYBR	Unkn-	RbcL	Water	13.68	13.86	0.170	2.43178	0.386	2.22338	0.19478

		12		24h							
D11	SYBR	Unkn-12	RbcL	Water 24h	13.88	13.86	0.170	2.19241	0.341	2.22338	0.19478
D12	SYBR	Unkn-12	RbcL	Water 24h	14.02	13.86	0.170	2.04594	0.311	2.22338	0.19478
E04	SYBR	Unkn-05	GAPDH	SS 0h	19.02	18.99	0.130	14.12870	1.150	14.44517	1.27727
E05	SYBR	Unkn-05	GAPDH	SS 0h	19.11	18.99	0.130	13.35588	1.126	14.44517	1.27727
E06	SYBR	Unkn-05	GAPDH	SS 0h	18.85	18.99	0.130	15.85092	1.200	14.44517	1.27727
E07	SYBR	Std-12	RbcL		24.19	24.43	0.204	0.01000	-2.000	0.01000	0.00000
E08	SYBR	Std-12	RbcL		24.55	24.43	0.204	0.01000	-2.000	0.01000	0.00000
E09	SYBR	Std-12	RbcL		24.54	24.43	0.204	0.01000	-2.000	0.01000	0.00000
E10	SYBR	Unkn-13	RbcL	SS 0h	10.45	10.34	0.113	12.63399	1.102	13.38423	0.77400
E11	SYBR	Unkn-13	RbcL	SS 0h	10.35	10.34	0.113	13.33873	1.125	13.38423	0.77400
E12	SYBR	Unkn-13	RbcL	SS 0h	10.23	10.34	0.113	14.17999	1.152	13.38423	0.77400
F04	SYBR	Unkn-06	GAPDH	SS 2h	20.97	20.87	0.137	3.80731	0.581	4.09781	0.38728
F05	SYBR	Unkn-06	GAPDH	SS 2h	20.92	20.87	0.137	3.94861	0.596	4.09781	0.38728
F06	SYBR	Unkn-06	GAPDH	SS 2h	20.71	20.87	0.137	4.53750	0.657	4.09781	0.38728
F10	SYBR	Unkn-14	RbcL	SS 2h	13.28	13.48	0.273	2.98573	0.475	2.71366	0.36333
F11	SYBR	Unkn-14	RbcL	SS 2h	13.36	13.48	0.273	2.85419	0.455	2.71366	0.36333
F12	SYBR	Unkn-14	RbcL	SS 2h	13.79	13.48	0.273	2.30106	0.362	2.71366	0.36333
G04	SYBR	Unkn-07	GAPDH	SS 4h	21.01	21.05	0.085	3.69752	0.568	3.59966	0.20298
G05	SYBR	Unkn-	GAPDH	SS 4h	21.00	21.05	0.085	3.73517	0.572	3.59966	0.20298

		07									
G06	SYBR	Unkn-07	GAPDH	SS 4h	21.15	21.05	0.085	3.36629	0.527	3.59966	0.20298
G10	SYBR	Unkn-15	RbcL	SS 4h	14.33	13.98	0.341	1.74349	0.241	2.10464	0.36225
G11	SYBR	Unkn-15	RbcL	SS 4h	13.96	13.98	0.341	2.10246	0.323	2.10464	0.36225
G12	SYBR	Unkn-15	RbcL	SS 4h	13.65	13.98	0.341	2.46798	0.392	2.10464	0.36225
H04	SYBR	Unkn-08	GAPDH	SS 24h	21.34	21.41	0.134	2.97196	0.473	2.84192	0.24866
H05	SYBR	Unkn-08	GAPDH	SS 24h	21.32	21.41	0.134	2.99860	0.477	2.84192	0.24866
H06	SYBR	Unkn-08	GAPDH	SS 24h	21.56	21.41	0.134	2.55520	0.407	2.84192	0.24866
H10	SYBR	Unkn-16	RbcL	SS 24h	13.20	16.12	4.817	3.09769	0.491	1.94035	1.65815
H11	SYBR	Unkn-16	RbcL	SS 24h	13.49	16.12	4.817	2.68263	0.429	1.94035	1.65815
H12	SYBR	Unkn-16	RbcL	SS 24h	21.68	16.12	4.817	0.04073	-1.390	1.94035	1.65815

Well	Fluor	Content	Target	Sample	Quanitation Cycle (Cq)	Cq Mean	Cq Std. Dev	Starting Quantity (SQ)	Log SQ	SQ Mean	SQ Std. Dev
A01	SYBR	Std-01	GAPDH		16.34	16.40	0.166	100.00000	2.000	100.00000	0.00000
A02	SYBR	Std-01	GAPDH		16.28	16.40	0.166	100.00000	2.000	100.00000	0.00000
A03	SYBR	Std-01	GAPDH		16.59	16.40	0.166	100.00000	2.000	100.00000	0.00000
A04	SYBR	Unkn-01	GAPDH	Water 0h	21.09	21.13	0.155	3.54369	0.549	3.45512	0.36901
A05	SYBR	Unkn-01	GAPDH	Water 0h	21.00	21.13	0.155	3.77178	0.577	3.45512	0.36901
A06	SYBR	Unkn-01	GAPDH	Water 0h	21.30	21.13	0.155	3.04989	0.484	3.45512	0.36901
A07	SYBR	Std-08	Rbc S		10.26	10.29	0.052	100.00000	2.000	100.00000	0.00000
A08	SYBR	Std-08	Rbc S		10.26	10.29	0.052	100.00000	2.000	100.00000	0.00000
A09	SYBR	Std-08	Rbc S		10.35	10.29	0.052	100.00000	2.000	100.00000	0.00000
A10	SYBR	Unkn-09	Rbc S	Water 0h	15.02	14.88	0.144	4.02109	0.604	4.40749	0.42159
A11	SYBR	Unkn-09	Rbc S	Water 0h	14.73	14.88	0.144	4.85713	0.686	4.40749	0.42159
A12	SYBR	Unkn-09	Rbc S	Water 0h	14.90	14.88	0.144	4.34425	0.638	4.40749	0.42159
B01	SYBR	Std-02	GAPDH		19.45	19.52	0.174	10.00000	1.000	10.00000	0.00000
B02	SYBR	Std-02	GAPDH		19.40	19.52	0.174	10.00000	1.000	10.00000	0.00000
B03	SYBR	Std-02	GAPDH		19.72	19.52	0.174	10.00000	1.000	10.00000	0.00000
B04	SYBR	Unkn-02	GAPDH	Water 2h	21.21	21.03	0.210	3.26509	0.514	3.71558	0.56426
B05	SYBR	Unkn-02	GAPDH	Water 2h	20.80	21.03	0.210	4.34848	0.638	3.71558	0.56426
B06	SYBR	Unkn-02	GAPDH	Water 2h	21.09	21.03	0.210	3.53315	0.548	3.71558	0.56426
B07	SYBR	Std-09	Rbc S		13.39	13.48	0.144	10.00000	1.000	10.00000	0.00000
B08	SYBR	Std-09	Rbc S		13.42	13.48	0.144	10.00000	1.000	10.00000	0.00000

B09	SYBR	Std-09	Rbc S		13.65	13.48	0.144	10.00000	1.000	10.00000	0.00000
B10	SYBR	Unkn-10	Rbc S	Water 2h	14.83	14.90	0.062	4.55642	0.659	4.35920	0.17992
B11	SYBR	Unkn-10	Rbc S	Water 2h	14.91	14.90	0.062	4.31713	0.635	4.35920	0.17992
B12	SYBR	Unkn-10	Rbc S	Water 2h	14.95	14.90	0.062	4.20404	0.624	4.35920	0.17992
C01	SYBR	Std-03	GAPDH		22.83	22.94	0.108	1.00000	0.000	1.00000	0.00000
C02	SYBR	Std-03	GAPDH		23.05	22.94	0.108	1.00000	0.000	1.00000	0.00000
C03	SYBR	Std-03	GAPDH		22.94	22.94	0.108	1.00000	0.000	1.00000	0.00000
C04	SYBR	Unkn-03	GAPDH	Water 4h	20.63	20.66	0.026	4.88718	0.689	4.81421	0.08696
C05	SYBR	Unkn-03	GAPDH	Water 4h	20.65	20.66	0.026	4.83746	0.685	4.81421	0.08696
C06	SYBR	Unkn-03	GAPDH	Water 4h	20.68	20.66	0.026	4.71799	0.674	4.81421	0.08696
C07	SYBR	Std-10	Rbc S		17.02	17.15	0.235	1.00000	0.000	1.00000	0.00000
C08	SYBR	Std-10	Rbc S		17.00	17.15	0.235	1.00000	0.000	1.00000	0.00000
C09	SYBR	Std-10	Rbc S		17.42	17.15	0.235	1.00000	0.000	1.00000	0.00000
C10	SYBR	Unkn-11	Rbc S	Water 4h	14.71	14.70	0.076	4.93887	0.694	4.95327	0.24802
C11	SYBR	Unkn-11	Rbc S	Water 4h	14.78	14.70	0.076	4.71277	0.673	4.95327	0.24802
C12	SYBR	Unkn-11	Rbc S	Water 4h	14.63	14.70	0.076	5.20818	0.717	4.95327	0.24802
D01	SYBR	Std-04	GAPDH		26.16	26.14	0.172	0.10000	-1.000	0.10000	0.00000
D02	SYBR	Std-04	GAPDH		25.96	26.14	0.172	0.10000	-1.000	0.10000	0.00000
D03	SYBR	Std-04	GAPDH		26.30	26.14	0.172	0.10000	-1.000	0.10000	0.00000
D04	SYBR	Unkn-04	GAPDH	Water 24h	21.54	21.64	0.199	2.56900	0.410	2.42120	0.32646
D05	SYBR	Unkn-04	GAPDH	Water 24h	21.50	21.64	0.199	2.64761	0.423	2.42120	0.32646
D06	SYBR	Unkn-04	GAPDH	Water 24h	21.87	21.64	0.199	2.04697	0.311	2.42120	0.32646



D07	SYBR	Std-11	Rbc S		20.49	20.56	0.213	0.10000	-1.000	0.10000	0.00000
D08	SYBR	Std-11	Rbc S		20.39	20.56	0.213	0.10000	-1.000	0.10000	0.00000
D09	SYBR	Std-11	Rbc S		20.80	20.56	0.213	0.10000	-1.000	0.10000	0.00000
D10	SYBR	Unkn-12	Rbc S	Water 24h	14.13	14.17	0.045	7.23755	0.860	7.05323	0.20772
D11	SYBR	Unkn-12	Rbc S	Water 24h	14.16	14.17	0.045	7.09399	0.851	7.05323	0.20772
D12	SYBR	Unkn-12	Rbc S	Water 24h	14.22	14.17	0.045	6.82815	0.834	7.05323	0.20772
E04	SYBR	Unkn-05	GAPDH	SS 0h	19.26	19.21	0.044	12.91021	1.111	13.32987	0.41398
E05	SYBR	Unkn-05	GAPDH	SS 0h	19.21	19.21	0.044	13.34147	1.125	13.32987	0.41398
E06	SYBR	Unkn-05	GAPDH	SS 0h	19.17	19.21	0.044	13.73793	1.138	13.32987	0.41398
E07	SYBR	Std-12	Rbc S		24.13	24.06	0.158	0.01000	-2.000	0.01000	0.00000
E08	SYBR	Std-12	Rbc S		23.88	24.06	0.158	0.01000	-2.000	0.01000	0.00000
E09	SYBR	Std-12	Rbc S		24.18	24.06	0.158	0.01000	-2.000	0.01000	0.00000
E10	SYBR	Unkn-13	Rbc S	SS 0h	13.52	13.45	0.155	10.84433	1.035	11.35375	1.18935
E11	SYBR	Unkn-13	Rbc S	SS 0h	13.56	13.45	0.155	10.50395	1.021	11.35375	1.18935
E12	SYBR	Unkn-13	Rbc S	SS 0h	13.28	13.45	0.155	12.71297	1.104	11.35375	1.18935
F04	SYBR	Unkn-06	GAPDH	SS 2h	21.05	21.11	0.060	3.64656	0.562	3.49615	0.14843
F05	SYBR	Unkn-06	GAPDH	SS 2h	21.17	21.11	0.060	3.34978	0.525	3.49615	0.14843
F06	SYBR	Unkn-06	GAPDH	SS 2h	21.11	21.11	0.060	3.49212	0.543	3.49615	0.14843
F07	SYBR	Std-13	Rbc S		27.41	27.64	0.207	0.00100	-3.000	0.00100	0.00000
F08	SYBR	Std-13	Rbc S		27.74	27.64	0.207	0.00100	-3.000	0.00100	0.00000
F09	SYBR	Std-13	Rbc S		27.79	27.64	0.207	0.00100	-3.000	0.00100	0.00000
F10	SYBR	Unkn-14	Rbc S	SS 2h	15.36	15.36	0.070	3.20052	0.505	3.19763	0.14870
F11	SYBR	Unkn-14	Rbc S	SS 2h	15.44	15.36	0.070	3.04750	0.484	3.19763	0.14870
F12	SYBR	Unkn-14	Rbc S	SS 2h	15.30	15.36	0.070	3.34486	0.524	3.19763	0.14870
G04	SYBR	Unkn-07	GAPDH	SS 4h	21.11	21.20	0.186	3.48506	0.542	3.30587	0.41832
G05	SYBR	Unkn-07	GAPDH	SS 4h	21.07	21.20	0.186	3.60473	0.557	3.30587	0.41832

G06	SYBR	Unkn-07	GAPDH	SS 4h	21.41	21.20	0.186	2.82781	0.451	3.30587	0.41832
G10	SYBR	Unkn-15	Rbc S	SS 4h	15.76	15.84	0.071	2.46578	0.392	2.33673	0.11184
G11	SYBR	Unkn-15	Rbc S	SS 4h	15.88	15.84	0.071	2.27639	0.357	2.33673	0.11184
G12	SYBR	Unkn-15	Rbc S	SS 4h	15.88	15.84	0.071	2.26802	0.356	2.33673	0.11184
H04	SYBR	Unkn-08	GAPDH	SS 24h	21.60	21.49	0.097	2.47966	0.394	2.68340	0.17991
H05	SYBR	Unkn-08	GAPDH	SS 24h	21.41	21.49	0.097	2.82040	0.450	2.68340	0.17991
H06	SYBR	Unkn-08	GAPDH	SS 24h	21.45	21.49	0.097	2.75014	0.439	2.68340	0.17991
H10	SYBR	Unkn-16	Rbc S	SS 24h	16.73	15.80	0.815	1.29244	0.111	2.61706	1.15401
H11	SYBR	Unkn-16	Rbc S	SS 24h	15.38	15.80	0.815	3.15376	0.499	2.61706	1.15401
H12	SYBR	Unkn-16	Rbc S	SS 24h	15.27	15.80	0.815	3.40498	0.532	2.61706	1.15401

Well	Fluor	Content	Target	Sample	Quantitation Cycle (Cq)	Cq Mean	Cq Std. Dev	Starting Quantity (SQ)	Log SQ	SQ Mean	SQ Std. Dev
A03	SYBR	Std-01	GAPDH		17.15	17.15	0.000	100.00000	2.000	100.00000	0.00000
A04	SYBR	Unkn-01	GAPDH	Water 0h	21.77	21.73	0.118	2.78341	0.445	2.87434	0.26241
A05	SYBR	Unkn-01	GAPDH	Water 0h	21.83	21.73	0.118	2.66949	0.426	2.87434	0.26241
A06	SYBR	Unkn-01	GAPDH	Water 0h	21.60	21.73	0.118	3.17012	0.501	2.87434	0.26241
A07	SYBR	Std-08	PGK		18.01	18.01	0.000	100.00000	2.000	100.00000	0.00000
A08	SYBR	Unkn-09	PGK	Water 0h	21.08	21.24	0.235	5.87143	0.769	5.22008	0.93211
A09	SYBR	Unkn-09	PGK	Water 0h	21.13	21.24	0.235	5.63645	0.751	5.22008	0.93211
A10	SYBR	Unkn-09	PGK	Water 0h	21.51	21.24	0.235	4.15236	0.618	5.22008	0.93211
B03	SYBR	Std-02	GAPDH		19.94	19.94	0.000	10.00000	1.000	10.00000	0.00000
B04	SYBR	Unkn-02	GAPDH	Water 2h	21.42	21.48	0.074	3.64009	0.561	3.47274	0.19427
B05	SYBR	Unkn-02	GAPDH	Water 2h	21.47	21.48	0.074	3.51842	0.546	3.47274	0.19427
B06	SYBR	Unkn-02	GAPDH	Water 2h	21.57	21.48	0.074	3.25969	0.513	3.47274	0.19427
B07	SYBR	Std-09	PGK		20.01	20.01	0.000	10.00000	1.000	10.00000	0.00000
B08	SYBR	Unkn-10	PGK	Water 2h	21.14	21.35	0.266	5.58936	0.747	4.79074	0.97244
B09	SYBR	Unkn-10	PGK	Water 2h	21.26	21.35	0.266	5.07502	0.705	4.79074	0.97244
B10	SYBR	Unkn-10	PGK	Water 2h	21.65	21.35	0.266	3.70783	0.569	4.79074	0.97244
C03	SYBR	Std-03	GAPDH		23.19	23.19	0.000	1.00000	0.000	1.00000	0.00000
C04	SYBR	Unkn-03	GAPDH	Water 4h	21.18	21.15	0.096	4.38814	0.642	4.48804	0.33334
C05	SYBR	Unkn-03	GAPDH	Water	21.04	21.15	0.096	4.85990	0.687	4.48804	0.33334

				4h							
C06	SYBR	Unkn-03	GAPDH	Water 4h	21.23	21.15	0.096	4.21608	0.625	4.48804	0.33334
C07	SYBR	Std-10	PGK		22.76	22.76	0.000	1.00000	0.000	1.00000	0.00000
C08	SYBR	Unkn-11	PGK	Water 4h	20.85	20.92	0.065	7.08664	0.850	6.68328	0.35453
C09	SYBR	Unkn-11	PGK	Water 4h	20.97	20.92	0.065	6.42109	0.808	6.68328	0.35453
C10	SYBR	Unkn-11	PGK	Water 4h	20.95	20.92	0.065	6.54209	0.816	6.68328	0.35453
D04	SYBR	Unkn-04	GAPDH	Water 24h	20.61	20.62	0.114	6.75173	0.829	6.70409	0.57463
D05	SYBR	Unkn-04	GAPDH	Water 24h	20.52	20.62	0.114	7.25341	0.861	6.70409	0.57463
D06	SYBR	Unkn-04	GAPDH	Water 24h	20.74	20.62	0.114	6.10712	0.786	6.70409	0.57463
D07	SYBR	Std-11	PGK		26.62	26.62	0.000	0.10000	-1.000	0.10000	0.00000
D08	SYBR	Unkn-12	PGK	Water 24h	20.64	20.89	0.305	8.38130	0.923	6.98222	1.61946
D09	SYBR	Unkn-12	PGK	Water 24h	20.80	20.89	0.305	7.35720	0.867	6.98222	1.61946
D10	SYBR	Unkn-12	PGK	Water 24h	21.23	20.89	0.305	5.20817	0.717	6.98222	1.61946
E04	SYBR	Unkn-05	GAPDH	SS 0h	19.77	19.90	0.136	12.81102	1.108	11.64035	1.19299
E05	SYBR	Unkn-05	GAPDH	SS 0h	20.04	19.90	0.136	10.42623	1.018	11.64035	1.19299
E06	SYBR	Unkn-05	GAPDH	SS 0h	19.89	19.90	0.136	11.68380	1.068	11.64035	1.19299
E08	SYBR	Unkn-13	PGK	SS 0h	19.66	20.15	0.424	18.42712	1.265	12.94006	4.75222
E09	SYBR	Unkn-13	PGK	SS 0h	20.39	20.15	0.424	10.24833	1.011	12.94006	4.75222
E10	SYBR	Unkn-13	PGK	SS 0h	20.40	20.15	0.424	10.14472	1.006	12.94006	4.75222
F04	SYBR	Unkn-06	GAPDH	SS 2h	21.49	21.54	0.053	3.44085	0.537	3.31742	0.13268
F05	SYBR	Unkn-06	GAPDH	SS 2h	21.60	21.54	0.053	3.17711	0.502	3.31742	0.13268
F06	SYBR	Unkn-06	GAPDH	SS 2h	21.54	21.54	0.053	3.33431	0.523	3.31742	0.13268
F08	SYBR	Unkn-14	PGK	SS 2h	21.75	22.01	0.232	3.41967	0.534	2.81627	0.54130

F09	SYBR	Unkn-14	PGK	SS 2h	22.21	22.01	0.232	2.37338	0.375	2.81627	0.54130
F10	SYBR	Unkn-14	PGK	SS 2h	22.07	22.01	0.232	2.65575	0.424	2.81627	0.54130
G04	SYBR	Unkn-07	GAPDH	SS 4h	21.69	21.62	0.061	2.96564	0.472	3.12958	0.14373
G05	SYBR	Unkn-07	GAPDH	SS 4h	21.59	21.62	0.061	3.18916	0.504	3.12958	0.14373
G06	SYBR	Unkn-07	GAPDH	SS 4h	21.58	21.62	0.061	3.23395	0.510	3.12958	0.14373
G08	SYBR	Unkn-15	PGK	SS 4h	22.59	22.55	0.187	1.74532	0.242	1.81321	0.27817
G09	SYBR	Unkn-15	PGK	SS 4h	22.35	22.55	0.187	2.11905	0.326	1.81321	0.27817
G10	SYBR	Unkn-15	PGK	SS 4h	22.71	22.55	0.187	1.57527	0.197	1.81321	0.27817
H04	SYBR	Unkn-08	GAPDH	SS 24h	22.03	21.93	0.121	2.29497	0.361	2.47912	0.23236
H05	SYBR	Unkn-08	GAPDH	SS 24h	21.97	21.93	0.121	2.40219	0.381	2.47912	0.23236
H06	SYBR	Unkn-08	GAPDH	SS 24h	21.79	21.93	0.121	2.74018	0.438	2.47912	0.23236
H08	SYBR	Unkn-16	PGK	SS 24h	21.97	22.08	0.136	2.86899	0.458	2.63975	0.28241
H09	SYBR	Unkn-16	PGK	SS 24h	22.03	22.08	0.136	2.72599	0.436	2.63975	0.28241
H10	SYBR	Unkn-16	PGK	SS 24h	22.23	22.08	0.136	2.32427	0.366	2.63975	0.28241

Well	Fluor	Content	Target	Sample	Quantitaion Cycle (Cq)	Cq Mean	Cq Std. Dev	Starting Quantity (SQ)	Log SQ	SQ Mean	SQ Std. Dev
A04	SYBR	Unkn-01	GAPDH	Water 0h	20.00	20.13	0.132	4.56275	0.659	4.19198	0.37349
A05	SYBR	Unkn-01	GAPDH	Water 0h	20.27	20.13	0.132	3.81584	0.582	4.19198	0.37349
A06	SYBR	Unkn-01	GAPDH	Water 0h	20.13	20.13	0.132	4.19734	0.623	4.19198	0.37349
A08	SYBR	Unkn-09	Ptr ToxA BP1	Water 0h	25.40	25.45	0.047	4.58860	0.662	4.36702	0.19315
A09	SYBR	Unkn-09	Ptr ToxA BP1	Water 0h	25.47	25.45	0.047	4.27819	0.631	4.36702	0.19315
A10	SYBR	Unkn-09	Ptr ToxA BP1	Water 0h	25.48	25.45	0.047	4.23427	0.627	4.36702	0.19315
B03	SYBR	Std-02	GAPDH		17.57	17.57	0.000	25.00000	1.398	25.00000	0.00000
B04	SYBR	Unkn-02	GAPDH	Water 2h	21.23	21.13	0.081	1.99651	0.300	2.12750	0.11474
B05	SYBR	Unkn-02	GAPDH	Water 2h	21.08	21.13	0.081	2.21020	0.344	2.12750	0.11474
B06	SYBR	Unkn-02	GAPDH	Water 2h	21.10	21.13	0.081	2.17580	0.338	2.12750	0.11474
B07	SYBR	Std-09	Ptr ToxA BP1		23.67	23.67	0.000	25.00000	1.398	25.00000	0.00000
B08	SYBR	Unkn-10	Ptr ToxA BP1	Water 2h	27.16	26.97	0.167	0.88307	-0.054	1.06604	0.15867
B09	SYBR	Unkn-10	Ptr ToxA BP1	Water 2h	26.86	26.97	0.167	1.16579	0.067	1.06604	0.15867
B10	SYBR	Unkn-10	Ptr ToxA BP1	Water 2h	26.88	26.97	0.167	1.14925	0.060	1.06604	0.15867
C03	SYBR	Std-03	GAPDH		19.44	19.44	0.000	6.25000	0.796	6.25000	0.00000
C04	SYBR	Unkn-03	GAPDH	Water 4h	20.69	20.62	0.106	2.87037	0.458	3.01484	0.22047
C05	SYBR	Unkn-03	GAPDH	Water 4h	20.67	20.62	0.106	2.90556	0.463	3.01484	0.22047

C06	SYBR	Unkn-03	GAPDH	Water 4h	20.50	20.62	0.106	3.26861	0.514	3.01484	0.22047
C07	SYBR	Std-10	Ptr ToxA BP1		24.82	24.82	0.000	6.25000	0.796	6.25000	0.00000
C08	SYBR	Unkn-11	Ptr ToxA BP1	Water 4h	26.47	26.78	0.277	1.67992	0.225	1.29129	0.34626
C09	SYBR	Unkn-11	Ptr ToxA BP1	Water 4h	26.85	26.78	0.277	1.17834	0.071	1.29129	0.34626
C10	SYBR	Unkn-11	Ptr ToxA BP1	Water 4h	27.01	26.78	0.277	1.01562	0.007	1.29129	0.34626
D03	SYBR	Std-04	GAPDH		21.52	21.52	0.000	1.56250	0.194	1.56250	0.00000
D04	SYBR	Unkn-04	GAPDH	Water 24h	20.21	20.26	0.083	3.96002	0.598	3.84878	0.21259
D05	SYBR	Unkn-04	GAPDH	Water 24h	20.20	20.26	0.083	3.98267	0.600	3.84878	0.21259
D06	SYBR	Unkn-04	GAPDH	Water 24h	20.35	20.26	0.083	3.60366	0.557	3.84878	0.21259
D07	SYBR	Std-11	Ptr ToxA BP1		26.80	26.80	0.000	1.56250	0.194	1.56250	0.00000
D08	SYBR	Unkn-12	Ptr ToxA BP1	Water 24h	25.69	26.10	0.379	3.48854	0.543	2.49030	0.90295
D09	SYBR	Unkn-12	Ptr ToxA BP1	Water 24h	26.16	26.10	0.379	2.25187	0.353	2.49030	0.90295
D10	SYBR	Unkn-12	Ptr ToxA BP1	Water 24h	26.44	26.10	0.379	1.73050	0.238	2.49030	0.90295
E03	SYBR	Std-05	GAPDH		23.71	23.71	0.000	0.39063	-0.408	0.39063	0.00000
E04	SYBR	Unkn-05	GAPDH	SS 0h	19.69	19.36	0.281	5.64476	0.752	7.10760	1.26806
E05	SYBR	Unkn-05	GAPDH	SS 0h	19.21	19.36	0.281	7.78371	0.891	7.10760	1.26806
E06	SYBR	Unkn-05	GAPDH	SS 0h	19.19	19.36	0.281	7.89432	0.897	7.10760	1.26806
E07	SYBR	Std-12	Ptr ToxA BP1		27.95	27.95	0.000	0.39063	-0.408	0.39063	0.00000
E08	SYBR	Unkn-13	Ptr ToxA BP1	SS 0h	25.09	25.35	0.225	6.13626	0.788	4.88945	1.07992

E09	SYBR	Unkn-13	Ptr ToxA BP1	SS 0h	25.47	25.35	0.225	4.28415	0.632	4.88945	1.07992
E10	SYBR	Unkn-13	Ptr ToxA BP1	SS 0h	25.48	25.35	0.225	4.24794	0.628	4.88945	1.07992
F04	SYBR	Unkn-06	GAPDH	SS 2h	21.23	21.30	0.180	1.98557	0.298	1.90282	0.22368
F05	SYBR	Unkn-06	GAPDH	SS 2h	21.51	21.30	0.180	1.64955	0.217	1.90282	0.22368
F06	SYBR	Unkn-06	GAPDH	SS 2h	21.17	21.30	0.180	2.07333	0.317	1.90282	0.22368
F08	SYBR	Unkn-14	Ptr ToxA BP1	SS 2h	25.71	26.17	0.671	3.42230	0.534	2.48822	1.23990
F09	SYBR	Unkn-14	Ptr ToxA BP1	SS 2h	25.87	26.17	0.671	2.96086	0.471	2.48822	1.23990
F10	SYBR	Unkn-14	Ptr ToxA BP1	SS 2h	26.94	26.17	0.671	1.08152	0.034	2.48822	1.23990
G04	SYBR	Unkn-07	GAPDH	SS 4h	19.47	19.29	0.162	6.51535	0.814	7.41986	0.78544
G05	SYBR	Unkn-07	GAPDH	SS 4h	19.18	19.29	0.162	7.92969	0.899	7.41986	0.78544
G06	SYBR	Unkn-07	GAPDH	SS 4h	19.21	19.29	0.162	7.81452	0.893	7.41986	0.78544
G08	SYBR	Unkn-15	Ptr ToxA BP1	SS 4h	25.67	25.93	0.225	3.54616	0.550	2.82884	0.62446
G09	SYBR	Unkn-15	Ptr ToxA BP1	SS 4h	26.09	25.93	0.225	2.40661	0.381	2.82884	0.62446
G10	SYBR	Unkn-15	Ptr ToxA BP1	SS 4h	26.03	25.93	0.225	2.53376	0.404	2.82884	0.62446
H04	SYBR	Unkn-08	GAPDH	SS 24h	21.33	21.64	0.389	1.85454	0.268	1.53991	0.37773
H05	SYBR	Unkn-08	GAPDH	SS 24h	21.51	21.64	0.389	1.64421	0.216	1.53991	0.37773
H06	SYBR	Unkn-08	GAPDH	SS 24h	22.08	21.64	0.389	1.12099	0.050	1.53991	0.37773
H08	SYBR	Unkn-16	Ptr ToxA BP1	SS 24h	27.36	28.44	1.392	0.73157	-0.136	0.40454	0.33542



H09	SYBR	Unkn-16	Ptr ToxA BP1	SS 24h	30.01	28.44	1.392	0.06132	-1.212	0.40454	0.33542
H10	SYBR	Unkn-16	Ptr ToxA BP1	SS 24h	27.95	28.44	1.392	0.42074	-0.376	0.40454	0.33542

Well	Fluor	Content	Target	Sample	Quantitaion Cycle (Cq)	Cq Mean	Cq Std. Dev	Starting Quantity (SQ)	Log SQ	SQ Mean	SQ Std. Dev
A03	SYBR	Std-01	GAPDH		16.97	16.97	0.000	100.00000	2.000	100.00000	0.00000
A04	SYBR	Unkn-01	GAPDH	Water 0h	21.70	21.55	0.145	3.92634	0.594	4.34081	0.39672
A05	SYBR	Unkn-01	GAPDH	Water 0h	21.41	21.55	0.145	4.71700	0.674	4.34081	0.39672
A06	SYBR	Unkn-01	GAPDH	Water 0h	21.53	21.55	0.145	4.37909	0.641	4.34081	0.39672
A07	SYBR	Std-08	Chl I.E protein		16.41	16.41	0.000	100.00000	2.000	100.00000	0.00000
A08	SYBR	Unkn-09	Chl I.E protein	Water 0h	21.16	21.28	0.229	6.18401	0.791	5.82213	0.73795
A09	SYBR	Unkn-09	Chl I.E protein	Water 0h	21.13	21.28	0.229	6.30930	0.800	5.82213	0.73795
A10	SYBR	Unkn-09	Chl I.E protein	Water 0h	21.54	21.28	0.229	4.97310	0.697	5.82213	0.73795
B03	SYBR	Std-02	GAPDH		19.50	19.50	0.000	10.00000	1.000	10.00000	0.00000
B04	SYBR	Unkn-02	GAPDH	Water 2h	22.11	21.63	0.420	3.02037	0.480	4.20034	1.02461
B05	SYBR	Unkn-02	GAPDH	Water 2h	21.41	21.63	0.420	4.71576	0.674	4.20034	1.02461
B06	SYBR	Unkn-02	GAPDH	Water 2h	21.36	21.63	0.420	4.86491	0.687	4.20034	1.02461
B07	SYBR	Std-09	Chl I.E protein		19.89	19.89	0.000	10.00000	1.000	10.00000	0.00000
B08	SYBR	Unkn-10	Chl I.E protein	Water 2h	21.29	21.33	0.086	5.75231	0.760	5.61753	0.27312
B09	SYBR	Unkn-10	Chl I.E protein	Water 2h	21.27	21.33	0.086	5.79707	0.763	5.61753	0.27312
B10	SYBR	Unkn-10	Chl I.E protein	Water 2h	21.43	21.33	0.086	5.30322	0.725	5.61753	0.27312
C03	SYBR	Std-03	GAPDH		24.22	24.22	0.000	1.00000	0.000	1.00000	0.00000
C04	SYBR	Unkn-03	GAPDH	Water 4h	21.06	21.01	0.076	5.88167	0.770	6.10973	0.29918
C05	SYBR	Unkn-03	GAPDH	Water 4h	20.92	21.01	0.076	6.44849	0.809	6.10973	0.29918

C06	SYBR	Unkn-03	GAPDH	Water 4h	21.03	21.01	0.076	5.99901	0.778	6.10973	0.29918
C07	SYBR	Std-10	Chl I.E protein		24.55	24.55	0.000	1.00000	0.000	1.00000	0.00000
C08	SYBR	Unkn-11	Chl I.E protein	Water 4h	20.72	20.87	0.132	7.96063	0.901	7.34223	0.56409
C09	SYBR	Unkn-11	Chl I.E protein	Water 4h	20.90	20.87	0.132	7.21015	0.858	7.34223	0.56409
C10	SYBR	Unkn-11	Chl I.E protein	Water 4h	20.98	20.87	0.132	6.85590	0.836	7.34223	0.56409
D04	SYBR	Unkn-04	GAPDH	Water 24h	21.95	21.98	1.453	3.35853	0.526	4.27373	3.53118
D05	SYBR	Unkn-04	GAPDH	Water 24h	23.45	21.98	1.453	1.29025	0.111	4.27373	3.53118
D06	SYBR	Unkn-04	GAPDH	Water 24h	20.55	21.98	1.453	8.17242	0.912	4.27373	3.53118
D07	SYBR	Std-11	Chl I.E protein		28.87	28.87	0.000	0.10000	-1.000	0.10000	0.00000
D08	SYBR	Unkn-12	Chl I.E protein	Water 24h	20.24	20.40	0.146	10.52590	1.022	9.61249	0.81898
D09	SYBR	Unkn-12	Chl I.E protein	Water 24h	20.44	20.40	0.146	9.36790	0.972	9.61249	0.81898
D10	SYBR	Unkn-12	Chl I.E protein	Water 24h	20.52	20.40	0.146	8.94368	0.952	9.61249	0.81898
E04	SYBR	Unkn-05	GAPDH	SS 0h	19.52	19.80	0.475	15.67189	1.195	13.54466	3.68177
E05	SYBR	Unkn-05	GAPDH	SS 0h	20.34	19.80	0.475	9.29331	0.968	13.54466	3.68177
E06	SYBR	Unkn-05	GAPDH	SS 0h	19.52	19.80	0.475	15.66877	1.195	13.54466	3.68177
E07	SYBR	Std-12	Chl I.E protein		31.89	31.89	0.000	0.01000	-2.000	0.01000	0.00000
E08	SYBR	Unkn-13	Chl I.E protein	SS 0h	19.76	19.84	0.096	13.88205	1.142	13.26851	0.72742
E09	SYBR	Unkn-13	Chl I.E protein	SS 0h	19.81	19.84	0.096	13.45854	1.129	13.26851	0.72742
E10	SYBR	Unkn-13	Chl I.E protein	SS 0h	19.95	19.84	0.096	12.46494	1.096	13.26851	0.72742
F04	SYBR	Unkn-06	GAPDH	SS 2h	23.29	22.16	0.983	1.42888	0.155	3.28543	1.60819

F05	SYBR	Unkn-06	GAPDH	SS 2h	21.60	22.16	0.983	4.17927	0.621	3.28543	1.60819
F06	SYBR	Unkn-06	GAPDH	SS 2h	21.58	22.16	0.983	4.24815	0.628	3.28543	1.60819
F08	SYBR	Unkn-14	Chl I.E protein	SS 2h	21.67	21.67	0.011	4.61369	0.664	4.62050	0.03059
F09	SYBR	Unkn-14	Chl I.E protein	SS 2h	21.68	21.67	0.011	4.59389	0.662	4.62050	0.03059
F10	SYBR	Unkn-14	Chl I.E protein	SS 2h	21.66	21.67	0.011	4.65393	0.668	4.62050	0.03059
G04	SYBR	Unkn-07	GAPDH	SS 4h	22.16	21.84	0.352	2.93581	0.468	3.64407	0.83539
G05	SYBR	Unkn-07	GAPDH	SS 4h	21.46	21.84	0.352	4.56535	0.659	3.64407	0.83539
G06	SYBR	Unkn-07	GAPDH	SS 4h	21.91	21.84	0.352	3.43106	0.535	3.64407	0.83539
G08	SYBR	Unkn-15	Chl I.E protein	SS 4h	22.23	22.34	0.097	3.34081	0.524	3.14374	0.17733
G09	SYBR	Unkn-15	Chl I.E protein	SS 4h	22.42	22.34	0.097	2.99706	0.477	3.14374	0.17733
G10	SYBR	Unkn-15	Chl I.E protein	SS 4h	22.36	22.34	0.097	3.09336	0.490	3.14374	0.17733
H04	SYBR	Unkn-08	GAPDH	SS 24h	22.02	21.87	0.143	3.20493	0.506	3.53576	0.31947
H05	SYBR	Unkn-08	GAPDH	SS 24h	21.85	21.87	0.143	3.55984	0.551	3.53576	0.31947
H06	SYBR	Unkn-08	GAPDH	SS 24h	21.73	21.87	0.143	3.84250	0.585	3.53576	0.31947
H08	SYBR	Unkn-16	Chl I.E protein	SS 24h	21.72	21.65	0.060	4.49016	0.652	4.67293	0.15953
H09	SYBR	Unkn-16	Chl I.E protein	SS 24h	21.62	21.65	0.060	4.74440	0.676	4.67293	0.15953
H10	SYBR	Unkn-16	Chl I.E protein	SS 24h	21.61	21.65	0.060	4.78424	0.680	4.67293	0.15953

Well	Fluor	Content	Target	Sample	Quantitation Cycle (Cq)	Cq Mean	Cq Std. Dev	Starting Quantity (SQ)	Log SQ	SQ Mean	SQ Std. Dev
A03	SYBR	Std-01	GAPDH		17.05	17.05	0.000	100.00000	2.000	100.00000	0.00000
A04	SYBR	Unkn-01	GAPDH	Water 0h	21.83	21.82	0.207	6.26842	0.797	6.32135	0.69535
A05	SYBR	Unkn-01	GAPDH	Water 0h	21.61	21.82	0.207	7.04166	0.848	6.32135	0.69535
A06	SYBR	Unkn-01	GAPDH	Water 0h	22.03	21.82	0.207	5.65398	0.752	6.32135	0.69535
A07	SYBR	Std-08	Chl. PS I		17.20	17.20	0.000	100.00000	2.000	100.00000	0.00000
A08	SYBR	Unkn-09	Chl. PS I	Water 0h	23.04	22.56	0.423	2.30809	0.363	3.12094	0.72971
A09	SYBR	Unkn-09	Chl. PS I	Water 0h	22.23	22.56	0.423	3.71954	0.570	3.12094	0.72971
A10	SYBR	Unkn-09	Chl. PS I	Water 0h	22.42	22.56	0.423	3.33519	0.523	3.12094	0.72971
B03	SYBR	Std-02	GAPDH		20.09	20.09	0.000	10.00000	1.000	10.00000	0.00000
B04	SYBR	Unkn-02	GAPDH	Water 2h	21.76	21.54	0.192	6.50262	0.813	7.33799	0.72351
B05	SYBR	Unkn-02	GAPDH	Water 2h	21.43	21.54	0.192	7.74673	0.889	7.33799	0.72351
B06	SYBR	Unkn-02	GAPDH	Water 2h	21.43	21.54	0.192	7.76464	0.890	7.33799	0.72351
B07	SYBR	Std-09	Chl. PS I		19.47	19.47	0.000	10.00000	1.000	10.00000	0.00000
B08	SYBR	Unkn-10	Chl. PS I	Water 2h	21.85	21.81	0.034	4.65525	0.668	4.76500	0.09512
B09	SYBR	Unkn-10	Chl. PS I	Water 2h	21.79	21.81	0.034	4.81619	0.683	4.76500	0.09512
B10	SYBR	Unkn-10	Chl. PS I	Water 2h	21.79	21.81	0.034	4.82357	0.683	4.76500	0.09512

C03	SYBR	Std-03	GAPDH		25.72	25.72	0.000	1.00000	0.000	1.00000	0.00000
C04	SYBR	Unkn-03	GAPDH	Water 4h	21.34	21.22	0.141	8.12834	0.910	8.68863	0.65611
C05	SYBR	Unkn-03	GAPDH	Water 4h	21.07	21.22	0.141	9.41040	0.974	8.68863	0.65611
C06	SYBR	Unkn-03	GAPDH	Water 4h	21.25	21.22	0.141	8.52715	0.931	8.68863	0.65611
C07	SYBR	Std-10	Chl. PS I		25.00	25.00	0.000	1.00000	0.000	1.00000	0.00000
C08	SYBR	Unkn-11	Chl. PS I	Water 4h	21.42	21.53	0.163	6.00363	0.778	5.63042	0.52652
C09	SYBR	Unkn-11	Chl. PS I	Water 4h	21.46	21.53	0.163	5.85946	0.768	5.63042	0.52652
C10	SYBR	Unkn-11	Chl. PS I	Water 4h	21.72	21.53	0.163	5.02817	0.701	5.63042	0.52652
D04	SYBR	Unkn-04	GAPDH	Water 24h	20.98	20.94	0.258	9.84701	0.993	10.15745	1.41146
D05	SYBR	Unkn-04	GAPDH	Water 24h	20.66	20.94	0.258	11.69829	1.068	10.15745	1.41146
D06	SYBR	Unkn-04	GAPDH	Water 24h	21.17	20.94	0.258	8.92704	0.951	10.15745	1.41146
D08	SYBR	Unkn-12	Chl. PS I	Water 24h	21.05	21.38	0.316	7.46895	0.873	6.21283	1.17051
D09	SYBR	Unkn-12	Chl. PS I	Water 24h	21.42	21.38	0.316	6.01687	0.779	6.21283	1.17051
D10	SYBR	Unkn-12	Chl. PS I	Water 24h	21.68	21.38	0.316	5.15267	0.712	6.21283	1.17051
E04	SYBR	Unkn-05	GAPDH	SS 0h	19.81	19.77	0.062	18.40089	1.265	18.70743	0.62364
E05	SYBR	Unkn-05	GAPDH	SS 0h	19.70	19.77	0.062	19.42502	1.288	18.70743	0.62364
E06	SYBR	Unkn-05	GAPDH	SS 0h	19.82	19.77	0.062	18.29639	1.262	18.70743	0.62364
E08	SYBR	Unkn-13	Chl. PS I	SS 0h	20.45	20.70	0.215	10.64951	1.027	9.25174	1.21481
E09	SYBR	Unkn-13	Chl. PS I	SS 0h	20.80	20.70	0.215	8.65497	0.937	9.25174	1.21481
E10	SYBR	Unkn-13	Chl. PS	SS 0h	20.84	20.70	0.215	8.45073	0.927	9.25174	1.21481

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F04	SYBR	Unkn-06	GAPDH	SS 2h	21.99	21.81	0.158	5.77249	0.761	6.36996	0.52109
F05	SYBR	Unkn-06	GAPDH	SS 2h	21.70	21.81	0.158	6.73042	0.828	6.36996	0.52109
F06	SYBR	Unkn-06	GAPDH	SS 2h	21.73	21.81	0.158	6.60697	0.820	6.36996	0.52109
F08	SYBR	Unkn-14	Chl. PS I	SS 2h	22.43	22.53	0.092	3.30543	0.519	3.11184	0.17089
F09	SYBR	Unkn-14	Chl. PS I	SS 2h	22.60	22.53	0.092	2.98194	0.474	3.11184	0.17089
F10	SYBR	Unkn-14	Chl. PS I	SS 2h	22.57	22.53	0.092	3.04814	0.484	3.11184	0.17089
G04	SYBR	Unkn-07	GAPDH	SS 4h	20.08	19.87	0.195	15.92187	1.202	17.84917	1.82523
G05	SYBR	Unkn-07	GAPDH	SS 4h	19.84	19.87	0.195	18.07418	1.257	17.84917	1.82523
G06	SYBR	Unkn-07	GAPDH	SS 4h	19.69	19.87	0.195	19.55146	1.291	17.84917	1.82523
G08	SYBR	Unkn-15	Chl. PS I	SS 4h	20.68	21.05	0.330	9.27107	0.967	7.56858	1.51871
G09	SYBR	Unkn-15	Chl. PS I	SS 4h	21.32	21.05	0.330	6.35313	0.803	7.56858	1.51871
G10	SYBR	Unkn-15	Chl. PS I	SS 4h	21.14	21.05	0.330	7.08155	0.850	7.56858	1.51871
H04	SYBR	Unkn-08	GAPDH	SS 24h	22.15	22.23	0.141	5.28386	0.723	5.09681	0.37393
H05	SYBR	Unkn-08	GAPDH	SS 24h	22.13	22.23	0.141	5.34030	0.728	5.09681	0.37393
H06	SYBR	Unkn-08	GAPDH	SS 24h	22.39	22.23	0.141	4.66627	0.669	5.09681	0.37393
H08	SYBR	Unkn-16	Chl. PS I	SS 24h	22.41	22.68	0.296	3.33868	0.524	2.87534	0.49087
H09	SYBR	Unkn-16	Chl. PS I	SS 24h	22.64	22.68	0.296	2.92642	0.466	2.87534	0.49087
H10	SYBR	Unkn-16	Chl. PS I	SS 24h	23.00	22.68	0.296	2.36093	0.373	2.87534	0.49087

Well	Fluor	Content	Target	Sample	Quantitation Cycle (Cq)	Cq Mean	Cq Std. Dev	Starting Quantity (SQ)	Log SQ	SQ Mean	SQ Std. Dev
A03	SYBR	Std-01	GAPDH		16.27	16.27	0.000	100.00000	2.000	100.00000	0.00000
A04	SYBR	Unkn-01	GAPDH	Water 0h	20.97	20.81	0.262	2.71073	0.433	3.11540	0.65125
A05	SYBR	Unkn-01	GAPDH	Water 0h	20.50	20.81	0.262	3.86665	0.587	3.11540	0.65125
A06	SYBR	Unkn-01	GAPDH	Water 0h	20.94	20.81	0.262	2.76880	0.442	3.11540	0.65125
A07	SYBR	Std-08	PR3		22.07	22.07	0.000	100.00000	2.000	100.00000	0.00000
A08	SYBR	Unkn-09	PR3	Water 0h	28.13	27.96	0.165	1.37214	0.137	1.54634	0.17893
A09	SYBR	Unkn-09	PR3	Water 0h	27.80	27.96	0.165	1.72966	0.238	1.54634	0.17893
A10	SYBR	Unkn-09	PR3	Water 0h	27.97	27.96	0.165	1.53721	0.187	1.54634	0.17893
B03	SYBR	Std-02	GAPDH		19.15	19.15	0.000	10.00000	1.000	10.00000	0.00000
B04	SYBR	Unkn-02	GAPDH	Water 2h	20.72	20.72	0.097	3.27846	0.516	3.29591	0.24272
B05	SYBR	Unkn-02	GAPDH	Water 2h	20.81	20.72	0.097	3.06238	0.486	3.29591	0.24272
B06	SYBR	Unkn-02	GAPDH	Water 2h	20.62	20.72	0.097	3.54689	0.550	3.29591	0.24272
B07	SYBR	Std-09	PR3		25.42	25.42	0.000	10.00000	1.000	10.00000	0.00000
B08	SYBR	Unkn-10	PR3	Water 2h	26.35	26.87	0.541	4.78274	0.680	3.46575	1.27352
B09	SYBR	Unkn-10	PR3	Water 2h	26.84	26.87	0.541	3.37385	0.528	3.46575	1.27352
B10	SYBR	Unkn-10	PR3	Water 2h	27.43	26.87	0.541	2.24068	0.350	3.46575	1.27352
C03	SYBR	Std-03	GAPDH		22.36	22.36	0.000	1.00000	0.000	1.00000	0.00000
C04	SYBR	Unkn-03	GAPDH	Water 4h	20.39	20.37	0.049	4.21482	0.625	4.29276	0.16091
C05	SYBR	Unkn-03	GAPDH	Water 4h	20.31	20.37	0.049	4.47780	0.651	4.29276	0.16091
C06	SYBR	Unkn-03	GAPDH	Water 4h	20.40	20.37	0.049	4.18566	0.622	4.29276	0.16091
C07	SYBR	Std-10	PR3		28.14	28.14	0.000	1.00000	0.000	1.00000	0.00000
C08	SYBR	Unkn-11	PR3	Water 4h	26.49	26.43	0.058	4.32152	0.636	4.50785	0.18312



C09	SYBR	Unkn-11	PR3	Water 4h	26.38	26.43	0.058	4.68758	0.671	4.50785	0.18312
C10	SYBR	Unkn-11	PR3	Water 4h	26.43	26.43	0.058	4.51445	0.655	4.50785	0.18312
D03	SYBR	Std-04	GAPDH		25.29	25.29	0.000	0.10000	-1.000	0.10000	0.00000
D04	SYBR	Unkn-04	GAPDH	Water 24h	19.93	21.92	3.549	5.96593	0.776	4.18762	3.58760
D05	SYBR	Unkn-04	GAPDH	Water 24h	19.81	21.92	3.549	6.53869	0.815	4.18762	3.58760
D06	SYBR	Unkn-04	GAPDH	Water 24h	26.02	21.92	3.549	0.05823	-1.235	4.18762	3.58760
D07	SYBR	Std-11	PR3		32.12	32.12	0.000	0.10000	-1.000	0.10000	0.00000
D08	SYBR	Unkn-12	PR3	Water 24h	26.37	26.55	0.174	4.70644	0.673	4.15498	0.51121
D09	SYBR	Unkn-12	PR3	Water 24h	26.58	26.55	0.174	4.06161	0.609	4.15498	0.51121
D10	SYBR	Unkn-12	PR3	Water 24h	26.71	26.55	0.174	3.69689	0.568	4.15498	0.51121
E04	SYBR	Unkn-05	GAPDH	SS 0h	19.00	18.93	0.210	12.14857	1.085	12.88201	2.11438
E05	SYBR	Unkn-05	GAPDH	SS 0h	18.70	18.93	0.210	15.26545	1.184	12.88201	2.11438
E06	SYBR	Unkn-05	GAPDH	SS 0h	19.10	18.93	0.210	11.23201	1.050	12.88201	2.11438
E08	SYBR	Unkn-13	PR3	SS 0h	25.43	25.51	0.116	9.09634	0.959	8.60620	0.68299
E09	SYBR	Unkn-13	PR3	SS 0h	25.46	25.51	0.116	8.89619	0.949	8.60620	0.68299
E10	SYBR	Unkn-13	PR3	SS 0h	25.64	25.51	0.116	7.82606	0.894	8.60620	0.68299
F04	SYBR	Unkn-06	GAPDH	SS 2h	20.78	20.68	0.146	3.13880	0.497	3.39852	0.38692
F05	SYBR	Unkn-06	GAPDH	SS 2h	20.75	20.68	0.146	3.21354	0.507	3.39852	0.38692
F06	SYBR	Unkn-06	GAPDH	SS 2h	20.51	20.68	0.146	3.84321	0.585	3.39852	0.38692
F08	SYBR	Unkn-14	PR3	SS 2h	25.48	25.72	0.274	8.76684	0.943	7.48864	1.39113
F09	SYBR	Unkn-14	PR3	SS 2h	25.67	25.72	0.274	7.69208	0.886	7.48864	1.39113
F10	SYBR	Unkn-14	PR3	SS 2h	26.02	25.72	0.274	6.00699	0.779	7.48864	1.39113
G04	SYBR	Unkn-07	GAPDH	SS 4h	20.78	20.72	0.159	3.14285	0.497	3.28590	0.40729
G05	SYBR	Unkn-07	GAPDH	SS 4h	20.55	20.72	0.159	3.74542	0.574	3.28590	0.40729
G06	SYBR	Unkn-07	GAPDH	SS 4h	20.85	20.72	0.159	2.96944	0.473	3.28590	0.40729

G08	SYBR	Unkn-15	PR3	SS 4h	25.34	25.79	0.401	9.68336	0.986	7.24931	2.13906
G09	SYBR	Unkn-15	PR3	SS 4h	26.10	25.79	0.401	5.66880	0.753	7.24931	2.13906
G10	SYBR	Unkn-15	PR3	SS 4h	25.93	25.79	0.401	6.39577	0.806	7.24931	2.13906
H04	SYBR	Unkn-08	GAPDH	SS 24h	21.22	20.80	0.521	2.23915	0.350	3.24992	1.36239
H05	SYBR	Unkn-08	GAPDH	SS 24h	20.97	20.80	0.521	2.71133	0.433	3.24992	1.36239
H06	SYBR	Unkn-08	GAPDH	SS 24h	20.22	20.80	0.521	4.79926	0.681	3.24992	1.36239
H08	SYBR	Unkn-16	PR3	SS 24h	27.87	28.11	0.215	1.64766	0.217	1.40113	0.21748
H09	SYBR	Unkn-16	PR3	SS 24h	28.18	28.11	0.215	1.31926	0.120	1.40113	0.21748
H10	SYBR	Unkn-16	PR3	SS 24h	28.28	28.11	0.215	1.23646	0.092	1.40113	0.21748

