An investigation into possible sugar signaling events during the infection of wheat with *Puccinia triticina*

by

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Abbreviations.

ABA	Abscisic acid
AP	Ascorbate peroxidase
Asp	Aspartic acid
ATP	Adenosine triphosphate
Avr	Avirulence
BA	Benzoic acid
BA2-H	Benzoic acid 2-hydoxylase
bO	Bacterio-opsin
BSA	Bovine serum albumin
CC	Coiled coil
CHS	Chalcone synthase
DDRT-PCR	Differential Display Reverse Transcription Polymerase Chain Reaction
DEPC	Diethylpyrocarbonate
DIN	Dark inducible
dpm	Disintegrations per minute
dCTP	Deoxycytosine triphosphate
dNTPs	Deoxynucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra-acetic acid
ET	Ethylene
GDP	Guanosine diphosphate

Glc	Glucose
Glu	Glutamic acid
Gly	Glycine
GTP	Guanosine triphosphate
H_2O_2	Hydrogen peroxide
hpi	Hours post infection
HR	Hypersensitive response
HXK	Hexokinase
HXT	Hexose transporter
IR	Infected resistant
IS	Infected susceptible
JA	Jasmonic acid
LRR	Leucine rich repeat
MAPK	Mitogen activated protein kinase
MAPKK	Mitogen activated protein kinase kinase
MAPKKK	Mitogen activated protein kinase kinase kinase
MST	Monosaccharide transporter
NAD^+	Nicotinamide adenine dinucleotide ion
NADH	Reduced nicotinamide adenine dinucleotide
NBS	Nucleotide binding site
NPR1	Natriuretic peptide receptor 1
PAL	Phenylalanine ammonia lyase
PK	Protein kinase

PMSF	Phenylmethylsulfonyl fluoride
PR	Pathogenesis related
Pro	Proline
PVP	Polyvinylpyrrolidone
R	Resistance
ROS	Reactive oxygen species
RT	Reverse transcription
RT-PCR	Reverse transcription PCR
SA	Salicylic acid
SAR	Systemic acquired resistance
SDS	Sodium dodecylsulfate
SOD	Superoxide dismutase
TaCwi01	Triticum Aestivum Cell wall invertase
TaMst01	Triticum Aestivum Monosaccharide transporter
Thr	Threonine
TIR	N-terminal Toll and Interleukin-1 receptor
TMV	Tobacco mosaic virus
Tris	2-amino-2-(hydroxymethyl) aminomethane
Tween^{Tm} 20	Polyoxyethylene sorbitan monolaurate
Tyr	Tyrosine

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Chapter 1:
Introduction

1. Introduction

Wheat (*Triticum aestivum* L.) is one of the most economically important crops. It is planted all over the world, including South Africa, where it was introduced by Jan van Riebeeck in 1652 (Van Niekerk, 2001). Today, wheat forms part of the daily diet of most people in the world. There is therefore an ever increasing demand for wheat. Wheat was the first domesticated crop and together with rice and maize, provides more than 60% of the human daily intake of calories and proteins (Gill *et al*., 2004). Wheat currently occupies 17% of the total crop area (210 million hectares in 2002) and it is predicted that in order to meet human demand in 2050, crop production must increase annually by 2% on the same area of cultivated land (Van Niekerk, 2001).

Apart from raising yield potential, wheat breeding has contributed by reducing yield losses due to diseases (Dubin and Rajaram S., 1996). Increased disease resistance can potentially increase yield which benefit farmers using these cultivars (Byerlee and Moya, 1993). Leaf rust, caused by *Puccinia triticina* L., is an important disease of wheat in most environments (Samborski, 1985). In field trails on different cultivars, Sayre *et al*. (1998) reported mean wheat yield losses ranging between 7.7 and 31.2% due to infection with leaf rust. Based on these statistics, in order to meet the demand of an annual 2% growth in wheat production, it is more viable to counteract losses due to pathogens than to breed wheat with improved yields.

It is therefore important to get a better understanding of plant defense in order to improve yield. While a lot is known about the plant defense response, more research is needed to fully understand it. The drive to increase knowledge needs to be accelerated.

When a plant is confronted with a pathogen, there are two possible outcomes. The first is where the pathogen grows and reproduces on the plant, in which case the plant is said to be susceptible. The other is where the pathogen fails to grow and reproduce, in which case the plant is said to be resistant (Vogel *et al.*, 2002).

Any plant-pathogen interaction can be divided into different phases. Normally, pathogens cannot infect a plant because of the basal defense mechanisms that are active, preventing the pathogen from recognizing the plant as a possible energy source (Johal *et al*., 1995). This is called "Basic incompatibility". When a mutation occurs that allows the pathogen to infect the plant, a situation of "Basic compatibility" is found. When basic compatibility is bridged and the plant again detects the pathogen, a plant defense system can be activated (Johal *et al*., 1995). If the plant succeeds in overcoming infection, the third step in the pathogen-plant interaction is reached which is called "Host incompatibility" or "Specific resistance" (Johal *et al*., 1995)

For the activation of a specific resistance response, two conditions must be met. Firstly, there must be a functional avirulence (*Avr)* gene present within the pathogen (Flor, 1971). Secondly, a resistance (*R*) gene must be present in the plant. The signal represented by the *Avr* gene must be recognized by R protein activity, either directly or indirectly leading to the activation of the defense response (Tang *et al*., 1996).

This genetic interaction between the *R* and *Avr* genes is called "gene-for-gene" resistance (Flor, 1971; Keen, 1990). Downstream of this interaction is an effective signaling system which activates the defense response. By activating the defense response, a plant is able to withstand the infection, thus confining the pathogen more effectively than synthetic fungicides and herbicides (Hammond-Kosack and Jones, 1996).

The downstream signaling system entails the activation of pre-existing protein kinases, phosphatases and other key proteins (Staskawicz *et al*., 1995; Bent, 1996). These proteins activate the expression of defense genes that prevent the pathogen from growing. The growth of biotrophic pathogens is arrested by killing plant tissue at the infection site (Farmer and Ryan, 1992; Baker and Orlandi, 1995; Boller, 1995). This is called the hypersensitive response (HR) (Greenberg, 1997).

A novel signaling system was described in plant-pathogen interactions (Herbers *et al*., 1996; Roitsch, 1999; Fotopoulos *et al*., 2003) where sugar molecules and the proteins that interact with them, form the steps of a signaling module (Rolland *et al*., 2002).
Glucose (Glc) was the first sugar molecule that was shown to play a part in signaling, with monosaccharide transporters and hexokinases playing key roles (Lalonde *et al*., 1999; Roitsch, 1999; Rolland *et al*., 2002; Rolland and Sheen, 2005). Glucose is produced from sucrose when invertase cleaves sucrose into glucose and fructose (Kingston-Smith *et al.*, 1998). Sucrose is the transportable sugar in plants and not glucose. Thus for glucose to move it needs to be transported by a monosaccharide transporter (Roitsch, 1999).

In a previous study, (JJ Appelgryn, unpublished results) two genes that are putatively involved in the defense response of wheat, were cloned. Both were found to be differentially expressed upon *P. triticina* infection of resistant Thatcher+*Lr34* wheat.

The aim of this study was therefore to confirm the identity of these genes and to postulate a possible role for the two encoded proteins in the defense response of wheat upon infection with leaf rust. In addition, the presence of a sugar signaling event during the wheat leaf rust interaction was investigated.

Chapter 2:
Literature review

2: Literature review

2.1 Introduction

During the evolution and development of plants and plant pathogens, a battle for survival started for both organisms. Since plants form the largest group of autotrophic organisms, they form the bottom of all food chains and are therefore the primary target for plant pathogens. Pathogens use plants exclusively during their life cycle for reproduction. Because of this, plants continually evolve to prevent the pathogen from infecting it thereby limiting the reproduction of the pathogen. The pathogen on the other hand must co-evolve to overcome the defensive barriers of the plant to ensure its own survival (Vogel *et al.*, 2002).

Humans use plants as a major food source and started breeding programs to improve plants to produce more food. However, during these breeding programs, a gain in crop yield is sometimes achieved with the simultaneous loss in natural defense systems (Dubin and Rajaram., 1996).

With an ever growing human population and breeding programs reaching the limit regarding high crop yield, the scientific approach shifted to the prevention of crop losses due to pathogens. The new aim of research was to understand the natural defense system of plants which is evident in wild type plants but not in the majority of modern day crops (Johal *et al*., 1995).

Two possible outcomes arise when a plant is infected with a pathogen. During the first, the pathogen grows and reproduces on the plant, in which case the plant is said to be susceptible, while in the second, the pathogen fails to grow and reproduce and the plant is said to be resistant (Vogel *et al.*, 2002).

Plants have developed numerous defense mechanisms to protect themselves against pathogens (Yang *et al*., 1997; Caldo *et al*., 2004). These include the strengthening of mechanical barriers, oxidative burst, formation of anti-pathogenic compounds, HR, as well as other inducible defense responses (Yang *et al*., 1997).

While resistance in plants is the rule, susceptibility is the rare exception. In their review, Johal *et al.* (1995) summarized the plant defense reaction with a schematic representation (Fig. 2.1).

Normally, pathogens cannot infect a plant because of the basal defense mechanisms that are active, preventing the pathogen from recognizing the plant as a possible energy source. This is called "Basic incompatibility" and occurs when the pathogen and plant cannot form any kind of interaction. This can be divided into two different lines of defense, namely passive and active defense (Johal *et al*., 1995).

The first is passive defense where no energy is required or no induction of defense mechanisms occurs to prevent pathogen infection. Passive defense includes the cell wall and cuticle that are present as physical barriers for the pathogen to overcome. Secondly, the active part of basic incompatibility includes cell wall cross linking, the synthesis of pathogenesis related (PR) proteins and systemic acquired resistance (SAR) (Johal *et al*., 1995).

When a mutation occurs during evolution which allows the plant to form an interaction with the pathogen with the latter colonizing the plant, a situation of "Basic compatibility" arise. This step is reached when the pathogen has the ability to overcome the structural and chemical barriers which characterize basic incompatibility. This is found when biotrophic pathogens are able to either suppress the plant HR or are able to avoid being detected by the plants defense system. For necrotrophic pathogens which are able to live on dead matter the HR as well as any other defense responses that the plant directs against it will be ineffective (Johal *et al*., 1995).

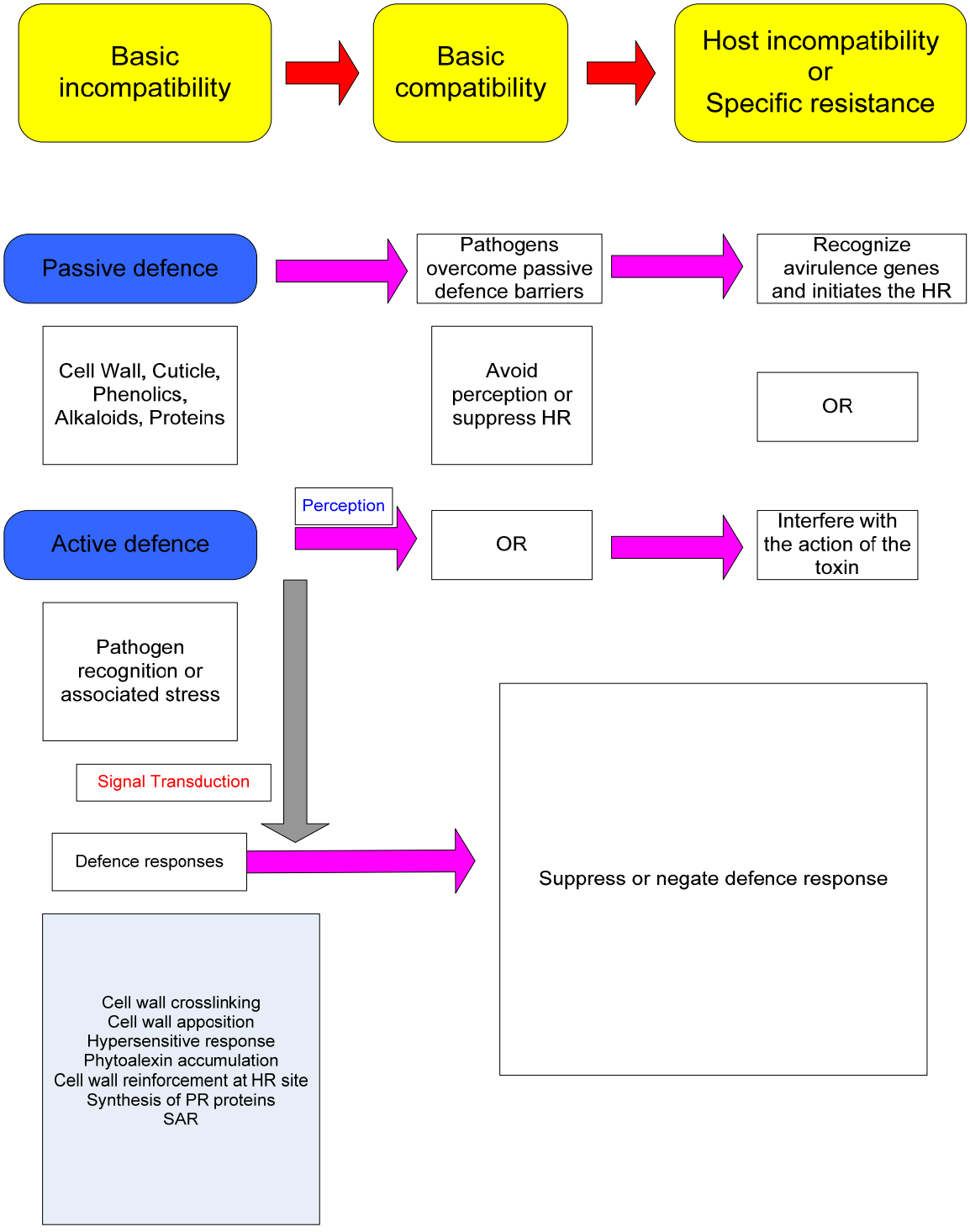

Figure 2.1. A schematic representation detailing mechanisms underlying resistance and disease development in plant–pathogen interactions (Johal *et al*., 1995).

Should basic compatibility not be complete and the plant manages to detect the attacking pathogen, a plant defense system can be activated. If the plant succeeds in defending itself by overcoming the infection, the third step in the pathogen – plant interaction is reached which is called "Host incompatibility" or "Specific resistance". The gene-for-gene interaction is the starting point for most incompatible interactions between the pathogen and the plant. The presence of the pathogen is detected by the plant allowing the plant to initiate its defense responses. In many cases the HR, which is a very effective way of arresting biotrophic pathogen growth, is activated within the resistant plant. This activation depends on complex signal transduction pathways within the plant cell (Johal *et al*., 1995).

The reason for this complexity is that for every pathogen a unique detection system exists within resistant plants that depend on the presence of a resistance protein being present. Despite this unique detection system, downstream signaling events often overlap, leading to a very complex, but very precise activation of the plant defense reaction (Tang *et al*., 1996).

Plant defense signaling is also not restricted to certain parts within the cell. Signaling takes place between different cellular organelles, from the outside to the inside of the cell, between cells in the same location, cells in different locations and even between different plants (Hammond-Kosack and Jones, 1996).

In this literature review, all of the above mentioned aspects of the plant defense response will be elaborated on.

2.2 The gene-for-gene interaction

During the incompatible plant-pathogen interaction, a rapid defense response is initiated by the plant (Greenberg, 1997). This response can effectively prevent pathogens from growing and reproducing on the plant. For the activation of this response, two conditions

must be met. Firstly, there must be a gene present within the pathogen which is called the *Avr* gene (Flor, 1971). Secondly, a gene called the *R* gene must be present in the plant. During these interactions, the signal represented by the *Avr* gene is recognized by *R* gene activity, either directly or indirectly.

This genetic interaction between the *R* and *Avr* genes is called "gene-for-gene" resistance (Flor, 1971; Keen, 1990). To understand the molecular basis of disease resistance, knowledge about the mechanisms of *Avr* signal perception and signal transduction by the host is needed. Most of the knowledge to date was gained in studies on plant pathogenic bacteria (Dangl and Jones, 2001).

Resistance to pathogens that is controlled by means of a gene-for-gene interaction is an active process (Greenberg, 1997). Many different defense responses are activated during such an interaction. These include the induced transcription of many defense related genes and synthesis of many different defense related proteins. This induced expression also often takes place in susceptible plants during pathogen infection, but the timing and abundance thereof differs from that of a resistant plant (Glazebrook and Ausubel, 1994; Greenberg, 1997).

A significant effort by several laboratories in the past years has resulted in the identification of many *R* genes from model and crop species (Hammond-Kosack and Jones, 1997; Dangl and Jones, 2001). Although these genes provide resistance to different types of pathogens, the encoded proteins share several structural similarities.

The most common features of these proteins include leucine-rich repeats (LRR), nucleotide binding sites (NBS) and in some cases also a serine/threonine protein kinase domain, an N-terminal Toll and Interleukin-1 receptor (TIR) or coiled coil (CC) domain (Dangl and Jones, 2001; Dodds *et al*., 2004).

It was originally speculated that resistance proteins that were predicted to be cytoplasmic, were involved in the recognition of pathogenic *Avr* determinants whose activity requires

a location inside the plant cell (Hammond-Kosack and Parker, 2003; Böhnert *et al*., 2004). However, after a series of unsuccessful attempts to demonstrate any direct interaction between R proteins and their corresponding Avr factors, this direct interaction is now considered to happen rarely. Some R proteins have a membrane-spanning region and an extracellular LRR domain (Fritig *et al.*, 1998). This suggests that the interaction between the R protein and Avr determinant can also occur outside the cell.

The tomato *Pto-AvrPto* gene-for-gene interaction is one of the best characterized and will be discussed as an example (Tang *et al*., 1996; Dangl and Jones, 2001). The *Pto* gene encodes a serine/threonine protein kinase that confers resistance to *Pseudomonas syringae* pv. *tomato* (Tang *et al*., 1996). Pto itself does not contain LRRs but requires the presence of the LRR containing Protein Research foundation (Prf) protein (Dangl and Jones, 2001). The direct interaction between Pto and AvrPto was observed using the yeast two-hybrid system (Scofield *et al.*, 1996; Tang *et al.*, 1996). This was the first direct proof of a physical interaction between the R and Avr proteins. The search for other plant proteins interacting with Pto led to the isolation of several genes potentially involved in the Pto signalling pathway (Fritig *et al.*, 1998).

A model describing the *Pto-AvrPto* gene-for-gene interaction was proposed by Zhou *et al.* (1997). Two of the major outcomes of *R* gene mediated signalling pathways are the activation of the HR and induced defense gene expression. Several reports have suggested that the HR and defense gene expression activation result from two distinct pathways that are activated by the *Pto-AvrPto* interaction (Zhou *et al.*, 1997). These suggestions were supported by reports from Jakobek and Lindgren (1993), Cameron *et al.* (1994) and Cao *et al.* (1994). Their observations are consistent with the notion that the signal perceived by an *R* gene product is transduced via two separate pathways, namely the HR pathway and the defense gene activation pathway (Zhou *et al.*, 1997.)

During the *Pto-AvrPto* interaction, the two signalling pathways are likely to split directly after the Pto-AvrPto protein interaction (Fig. 2.2a) (Zhou *et al.*, 1997). In this model, the AvrPto protein is secreted directly into the plant cell by *Pseudomonas* where it physically

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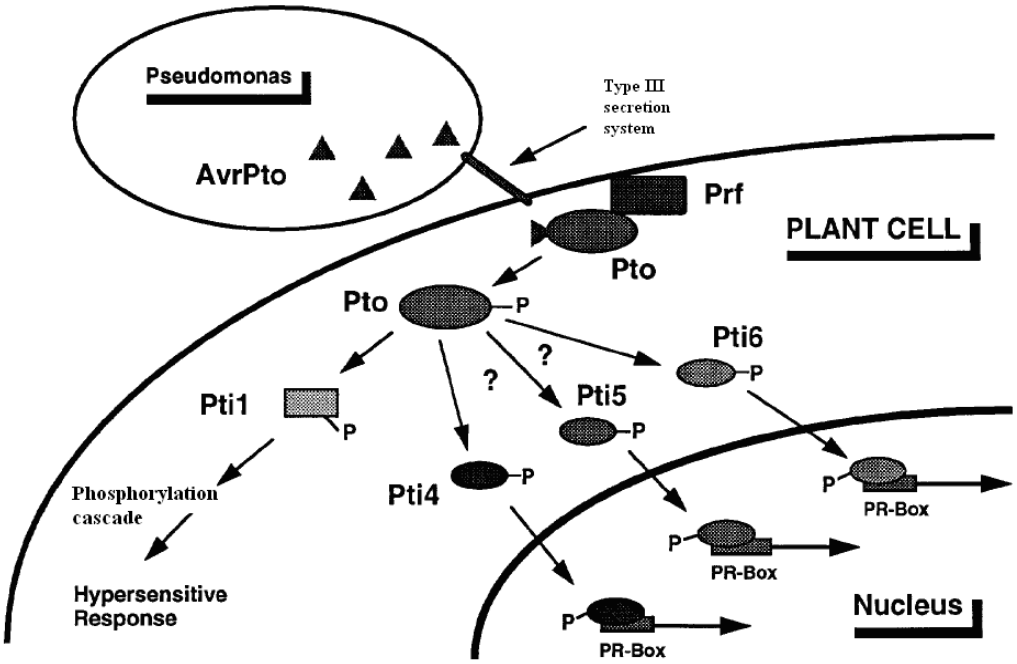

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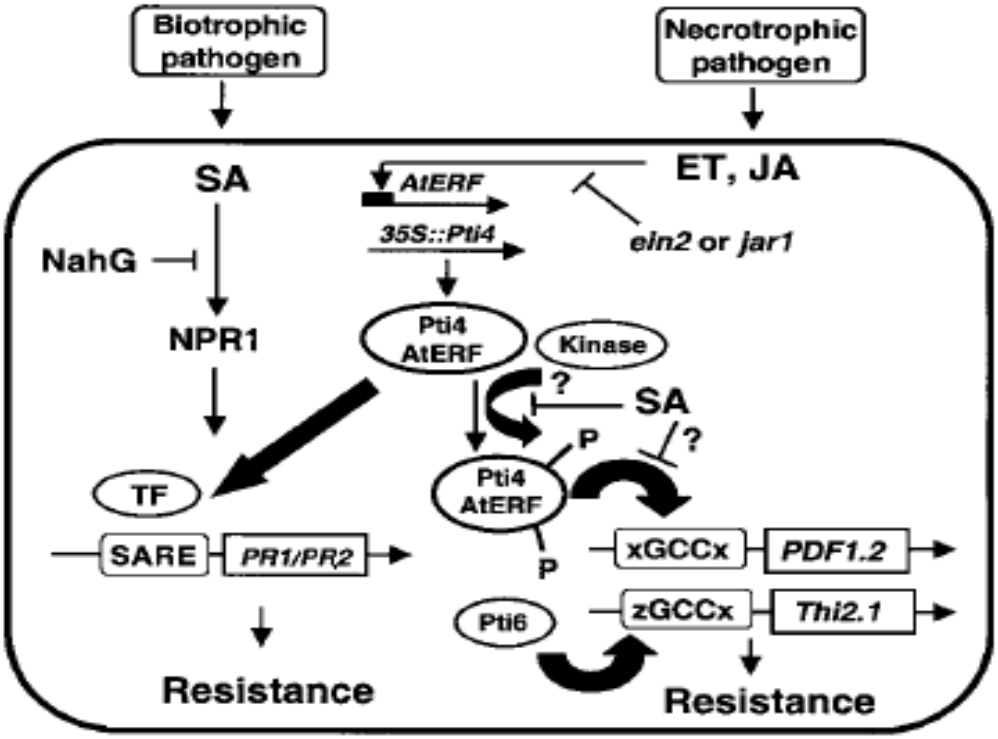


Figure 2.2 Activation of the defense response of tomatoes mediated by the *R-Avr* interaction.

(a) The proposed model for Pto-mediated signal transduction (Zhou *et al*., 1997).

(b) The improved model for Pti4 function (Gu *et al.*, 2002).

interacts with the cytoplasmic Pto kinase (Scofield *et al.*, 1996; Tang *et al.*, 1996). This interaction which may also involve Prf, activates the Pto kinase which leads to the autophosphorylation of the protein itself (Thara *et al*., 1999; Gu *et al*., 2000; Gu *et al*., 2002). The Pto kinase in turn then phosphorylates downstream target proteins (Gu *et al*., 2002) thereby activating them to play a unique role in the resistance response. Downstream proteins include Pti1 that is thought to mediate the HR signaling pathway, while three transcription factors (Pti4, 5, 6) are proposed to mediate induced defense gene expression (Zhou *et al*., 1997).

This suggestion was confirmed by Gu *et al.* (2002) when they determined that the nuclear localization of Pti4 is independent of Pto. A new model was then proposed to show the role of Pti4 in the signaling pathway (Fig 2.2b).

First, the pathogen attack and/or the associated increase in ethylene (ET) activate the induced expression of *Pti4* (Gu *et al*., 2000; Gu *et al*., 2002). When Pti4 becomes available, Pto kinase phosphorylates the protein, which could facilitate its localization DNA binding and/or interaction with other transcription factors (Gu *et al*., 2002). This phosphorylation of Pti4 is not regulated by ET or jasmonic acid (JA), but most probably by salicylic acid (SA) (Gu *et al*., 2002). Being a transcriptional activator, Pti4 seems to regulate the SA dependent expression of *PDF1.2*.

Gu *et al*. (2002) proposed that Pti4 could play an important role in linking the SA and ET/JA signaling pathways. SA regulates Pti4 phosphorylation through attenuation. When unphosphorylated, Pti4 could regulate the induced expression of *PR1*, but when phosphorylated, it could regulate the expression of *PDF1.2*. Therefore, by regulating the activity of the Pto kinase, the phosphorylation levels of Pti4 could be regulated and the appropriate signaling pathway, be it SA or ET/JA. Lower SA levels could lead to both phosphorylated and unphosphorylated Pti4, and the concomitant activation of both SA and ET/JA dependent gene expression. Pti4 could therefore act as a very precise and convenient switch between the two different signal transduction pathways.

2.3 Defense related signaling

Signal transduction events following various plant-pathogen interactions are very complex and differ between the different interactions. Hammond-Kosack and Jones (1996) gave a general overview of signal transduction (Fig. 2.3).

When plants are infected, receptor proteins intercept pathogen-derived or interaction-dependent signals. Immediately downstream of these recognition events, the activation of pre-existing protein kinases, phosphatases and other key proteins follow (Staskawicz *et al*., 1995; Bent, 1996). One class of protein kinases that is involved in the signal transduction events, is mitogen activated protein kinases (MAPK). MAPKs are general signal transducing proteins within cells. Zhang and Klessig (2001) concluded that there are 20 different MAPKs within the *Arabidopsis* genome while other plants probably have similar numbers.

The basic assembly of a MAPK cascade is a three protein kinase module conserved in all eukaryotes (Zhang and Klessig, 2001). MAPK is the final component of the cascade and is activated by phosphorylation of threonine (Thr) and tyrosine (Tyr) residues in a tripeptide motif, Thr-X-Tyr, with X being either glutamic acid (Glu), glycine (Gly), proline (Pro) or aspartic acid (Asp). This motif is located in the activation loop between subdomains VII and VIII of the kinase catalytic domain. The phosphorylation of MAPK is mediated by a MAPK kinase (MAPKK) which is in turn activated by a MAPKK kinase (MAPKKK). There are multiple members of each of the three different MAP kinases in a cell and it is this trait that contributes to the specificity of the unique transmitted signal (Zhang and Klessig, 2001).

Other rapidly induced events that have been detected in infected plants include protein phosphorylation and dephosphorylation, changes in Ca^{2+} concentration, ion fluxes, increased inositol triphosphate and diacylglycerol levels and alterations in the ratio of proteins with bound guanosine triphosphate (GTP) or guanosine diphosphate (GDP) (Dixon *et al*., 1994; Low and Merida, 1995; Ward *et al*., 1995).

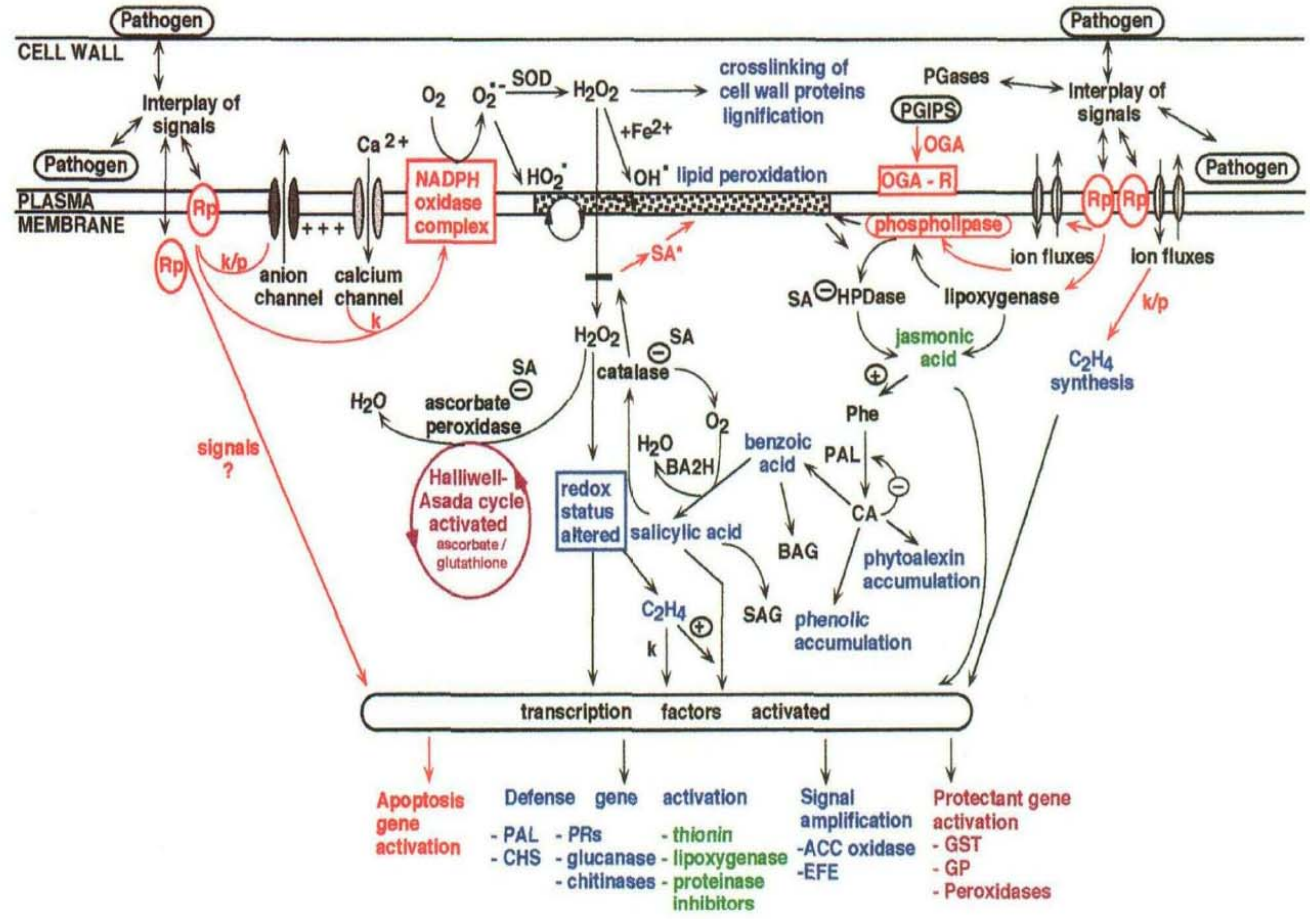


Figure 2.3. Complexity of signaling events controlling activation of defense responses (Hammond-Kosack and Jones, 1996). (+) indicates positive and (-) negative interactions. Components and arrows indicated in red are only postulated to be present in plant cells, whereas those in blue indicate known plant defense responses; green indicates plant defense responses also activated by JA and purple indicates plant protection mechanisms.

The extremely rapid induction of the oxidative burst and/or ethylene biosynthesis (Baker and Orlandi, 1995; Boller, 1995) suggests that induced gene expression is not required for these responses (Hammond-Kosack and Jones, 1996).

Cross-linking of cell wall proteins and callose deposition also do not appear to involve gene expression (Hammond-Kosack and Jones, 1996). In contrast, rapid increases in phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) activities correlate well with gene activation (Logemann *et al*., 1995).

Elevated SA levels probably occur when plants increase pre-existing benzoic acid 2-hydoxylase (BA2-H) activity to convert stored benzoic acid (BA) to SA but also by *de novo* PAL and BA2-H protein synthesis (Léon *et al*., 1995; Mauch-Mani and Slusarenko, 1996).

Induced expression of various housekeeping genes is also likely to accompany the defense response to ensure that adequate pools of precursor compounds are maintained (Kawalleck *et al*., 1992). Furthermore, in young plant tissues, histone and cell-cycle-regulated gene expression may be repressed either to redirect all available cellular resources to defense-related metabolism (Logemann *et al*., 1995) or to preclude cell death (Hammond-Kosack and Jones, 1996).

Once the earliest defense responses have been activated, the complexity of the biochemical pathways within the responding cell is likely to increase enormously as new signal molecules are generated (Fig. 2.3). This hierarchy of signaling events probably provides the overall framework to coordinately induce the diverse array of defense responses in various cellular compartments. Considerable amplification of specific defense responses then occurs, via either positive feedback or signal cross-talk (Hammond-Kosack and Jones, 1996).

At some stage during the incompatible plant-pathogen interaction, damage is inflicted upon both the responding host cell and the pathogen (Hammond-Kosack and Jones,

1996). As a result, the formation of additional signal molecules called elicitors occurs at the host-pathogen interface, probably in a less controlled manner. The particular microbial species and its mode of pathogenesis are likely to influence the diversity of second-generation elicitors that are produced (Hammond-Kosack and Jones, 1996).

These new signals include chitin fragments, lipid peroxides, arachidonic acid, cell wall oligosaccharide fragments and a localized change in cellular redox state (Fig 2.3) (Farmer and Ryan, 1992; Baker and Orlandi, 1995; Boller, 1995).

As a consequence, a second wave of signal perception and transduction events occurs that activates additional defense responses, amplify or repress the original response or induce cell death. The activation of specific plant cellular protection mechanisms is likely to accompany the defense response. These mechanisms include the upregulation of the cytoplasmic Halliwell-Asada cycle (Fig. 2.3) that minimizes the consequences of oxidative stress where hydrogen peroxide (H_2O_2) is reduced to H_2O by ascorbate peroxidase (AP) (Zhang and Kirkham, 1996).

This is followed by the regeneration of reduced ascorbate, which is a product of AP. The reaction is catalyzed by either the reduced nicotinamide adenine dinucleotide (NAD(P)H)-dependent monodehydroascorbate reductase or reduced glutathione-dependent dehydroascorbate reductase coupled with glutathione reductase (Zhang and Kirkham, 1996).

Furthermore, increased transcription of specific superoxide dismutase (SOD) and catalase genes may occur to ensure that maximal enzymatic activity is maintained within the appropriate cellular compartments to prevent plant cellular damage or death (Bowler *et al*., 1994). Thus, mutations in genes which control the activation of signal pathways for cellular protection genes could be responsible for the uncontrolled spreading of lesions in response to avirulent pathogens (Dangl *et al*., 1996).

In the initially attacked cells, the rapid defense response may ultimately lead to cell death, whereas in the surrounding cells, induced defense reactions may be more transcription dependent (Hammond-Kosack and Jones, 1996). The magnitude and type of signals perceived by neighboring cells depend on the relative rates of signal production, diffusion and reactivity towards macromolecules (Hammond-Kosack and Jones, 1996).

Also, as plasmodesmata are filled with callose due to deposition, cellular protection mechanisms become less overloaded and cell wall architecture is modified by the cross-linking and lignification events, both symplastic and apoplastic routes for signal molecules are eventually blocked. This eventually results in the progressive shut down of defense signaling pathways once the invading microbe has been successfully contained (Hammond-Kosack and Jones, 1996).

2.4 The Hypersensitive Response

Plant disease resistance includes numerous biological and biochemical changes, but it is often not clear whether these changes are necessary to limit pathogen growth and reproduction (Greenberg, 1997; Greenberg and Yao, 2004). Greenberg (1997) subdivided cell death during pathogen attack into two different categories, namely cell death taking place during susceptible interactions and secondly, cell death during resistant interactions. The latter was further divided into cell death occurring during HR and cell death occurring during SAR.

The HR is one of the most powerful mechanisms by which pathogen attack can be overcome. The induction of the HR is controlled by means of the previously described gene-for-gene interactions during a plant pathogen interaction (Melchers and Stuiver, 2000).

To characterize the sub-type of cell death taking place during a pathogen attack as an HR, a few conditions must be met. Firstly, the plant must initiate active protein synthesis (Keen *et al*., 1981; Croft *et al*., 1990). Secondly, the pathogenic elicitors of the HR

require that the plants metabolism must be active (Strobel *et al.*, 1996). The third requirement is an overproduction of any single component of the signal transduction pathway (Greenberg, 1997). Finally, cell death must be genetically controlled (Dietrich *et al*., 1994; Greenberg *et al*., 1994).

The classical HR is defined as the death of host cells within a few hours of pathogen contact, but can be phenotypically quite diverse, ranging from HR in a single cell to spreading necrotic areas accompanying limited pathogen colonization (Holub *et al*., 1994). The appearance of the HR can be environmentally dependent and can in particular be attenuated at high humidity (Klement, 1982; Hammond-Kosack and Jones, 1996).

The HR has been proposed to play a causal role in disease resistance (Heath, 1980). During interactions with obligate biotrophic pathogens that form intimate haustorial associations with host cells, plant cell death would deprive the pathogen of access to further nutrients. In interactions involving hemibiotrophic and necrotrophic pathogens, the role of the HR is thus less clear because these pathogens can obtain nutrients from dead plant cells (Greenberg, 1997).

However, cellular decompartmentalization may lead to the release of harmful preformed substances that are stored in the vacuole (Osbourn, 1996). Alternatively, the levels of induced phytoalexins which usually are rapidly turned over in plant cells, may accumulate to inhibitory concentrations because they are no longer metabolized. The HR may therefore cause pathogen arrest but may also occur as a consequence of the activation of other defense responses (Greenberg, 1997).

The HR correlates well with the oxidative burst, membrane damage, ion fluxes, endonuclease activation, DNA cleavage and gene expression (Greenberg, 1997). It is not clear which of the above mentioned prerequisites may be involved specifically in the regulation or execution of the HR (Greenberg, 1997).

Several laboratories have tried to determine the role of each individual change that occurs during the resistance response. In particular, much emphasis has been placed on understanding the role and regulation of the oxidative burst and its relationship to the HR. In some systems, it is possible to detect superoxide in the apoplasm of cells undergoing an HR (Doke, 1983; Auh and Murphy, 1995; Greenberg, 1997), whereas in other systems only H_2O_2 accumulates to detectable levels (Levine *et al*., 1994).

The oxidative burst precedes cell death, which makes it a candidate as a source of signaling molecules initiating the HR (Greenberg, 1997). In animal cells, oxidative stress activates apoptosis by two independent signaling mechanisms (Santana *et al.*, 1996), making the oxidative burst in plant cells a prime candidate for the HR switch. Glazener *et al.* (1996) used bacterial mutants to determine whether the oxidative burst is responsible for the activation of the HR. The bacterial mutants were defective in eliciting the HR in tobacco because of a mutation in the *hrp* locus.

It was found that the production of reactive oxygen species (ROS) in the form of H_2O_2 using this *hrp*-strain did not differ from that elicited by a wild-type bacterial strain (Glazener *et al.*, 1996). However, plant cell death did not occur when using the *hrp*-strain, thereby uncoupling the oxidative burst from the HR. However, Levine *et al.* (1994) found that the oxidative burst manifesting as H_2O_2 production in a soybean suspension culture system might play a role in activating the HR. When H_2O_2 production was enhanced by inhibiting catalase during an HR elicited by avirulent bacteria, the occurrence of cell death was greatly increased (Greenberg, 1997).

When the oxidative burst was blocked by an inhibitor of NADPH oxidase or kinase activity was blocked by the alkaloid K252A, cell death of plant cells infiltrated with avirulent *Pseudomonas* was decreased by a factor of two (Greenberg, 1997). This was despite the fact that K252A completely blocked the oxidative burst when cultures were treated with the Pmg elicitor (Levine *et al*., 1994). The fact that K252A completely blocked the oxidative burst but not cell death, suggested that H_2O_2 alone may not be sufficient to activate cell death. However, since the oxidative burst was not monitored

after bacterial inoculation in the presence of K252A, it is possible that K252A completely inhibited the oxidative burst by Pmg but not by avirulent bacteria. It was thus suggested that residual ROS may remain when soybean cells are treated with K252A and avirulent bacteria (Levine *et al.*, 1994). Results of both Levine *et al.* (1994) and Glazener *et al.* (1996) indicated that H_2O_2 may not be sufficient to account for cell death observed during the resistance response. One possibility is that if the H_2O_2 concentration is high enough, additional plant defense signals are not necessary. It has been suggested that since catalase may be inhibited by SA (Chen *et al.*, 1993), a rise in H_2O_2 at the site of lesion formation might contribute to the coordinated activation of cell death (Léon *et al.*, 1995).

Another component that could trigger cell death, is superoxide. Infiltration of SOD into tobacco leaves infected with the tobacco mosaic virus (TMV) compromised the development of the HR (Doke and Ohashi, 1988). It was shown by Jabs *et al.* (1996) that the *lsd1* mutant of *Arabidopsis*, which showed an apparent HR after shifting uninfected plants from short-day to long-day growth conditions, accumulated superoxide in the apoplasm of leaf tissue. These observations point to a possible function for superoxide in regulating the initiation and/or extent of cell death during the HR.

Because both plants and animals show apoptosis in response to oxidative signals, it will be interesting to determine whether there is any similarity in the mechanism of apoptotic activation in these highly divergent systems.

Other potential signals for HR induction are the flux and exchange of ions. During the early resistance response, there is an efflux of K^+ (Mittler *et al*., 1995). A hint that ion fluxes do play an important role in regulating the HR came from tobacco plants that constitutively expressed the bacterio-opsin (bO) protein, a bacterial proton pump from *Halobacterium halobium* that requires rhodopsin for active proton pumping in bacteria (Mittler *et al*., 1995; Greenberg, 1997). These transgenic plants showed an apparent spontaneous HR accompanied by visible DNA degradation (Mittler *et al.*, 1995). These plants expressed many defense responses normally seen during a plant resistance

response. In addition, bO-expressing tobacco showed elevated levels of DNA endonucleases that were activated during a TMV-induced resistance response on tobacco.

Calcium fluxes may play a role in the execution of the HR. When Ca^{2+} ion channels are blocked, cell death of soybean suspension cells in response to avirulent bacteria or H_2O_2, was reduced (Levine *et al.*, 1994). Treatment of such plant cells with a Ca^{2+} ionophore induced programmed cell death. In the case of soybean cell suspension cultures, Ca^{2+} fluxes were not associated with the expression of defense related genes that were induced by H_2O_2 (Levine *et al.*, 1994).

Calcium was required for the activation of DNA endonucleases that are associated with the resistance response, leading to DNA breakage. Conversely, DNA breakdown may occur after the cell has committed to a cell death program and may facilitate recycling of cellular constituents (Mittler and Lam, 1995).

Since membrane properties change during the resistance response, it is possible that lipid-based signals could be responsible for regulating the HR. Because of the differential activation and/or localization of lipoxygenase and phospholipase D respectively during the resistance response, it has been suggested that the products of these particular enzymatic reactions might act as signal molecules in the activation of the HR (Croft *et al.*, 1990; Young *et al.*, 1996).

2.5 Systemic Acquired Resistance

Systemic acquired resistance refers to a specific defense response that plays an important role in the ability of plants to defend themselves against pathogens (Ryals *et al.*, 1996). After necrotic lesion formation either due to the HR or as a symptom of disease, the SAR pathway is activated. This activation results in the development of a broad-spectrum, systemic resistance (Hunt and Ryals, 1996; Neuenschwander *et al.*, 1996). Systemic acquired resistance can be distinguished from other disease resistance responses by both the spectrum of pathogen protection and the associated changes in gene expression (Ryals

et al., 1996). In tobacco, SAR activation results in a significant reduction of disease symptoms caused by fungi (Vernooij *et al*., 1995).

Associated with SAR is the expression of a set of genes called *SAR* genes (Ward *et al*., 1991). Not all defense related genes are expressed during SAR and the particular spectrum of gene expression therefore distinguishes the SAR response from other resistance responses in plants (Ryals *et al.*, 1996).

The SAR signal transduction pathway appears to function as a potentiator or modulator of other disease resistance mechanisms. When SAR is activated, a normally compatible plant-pathogen interaction can be converted into an incompatible one (Uknes *et al*., 1992; Mauch-Mani and Slusarenko, 1996). When the SAR pathway is breached, a normally incompatible interaction becomes compatible (Delaney *et al*., 1994; Mauch-Mani and Slusarenko, 1996).

In many cases, this resistance is expressed locally at the site of pathogen attack and systemically in uninfected parts of the plant (Mauch-Mani and Métraux, 1998). SAR implies the production of one or several translocatable signals by the plant that is involved in the activation of resistance mechanisms in uninfected parts of the plant (Mauch-Mani and Métraux, 1998).

Evidence has accumulated that SA is a possible signal for SAR (Klessig and Malamy, 1994; Ryals *et al.*, 1996; Sticher *et al.*, 1997; Yang *et al.*, 1997; Mauch-Mani and Métraux, 1998). In 2004, Gozzo summarized the mechanism of SAR induction and described it as follows.

When cell suspensions are inoculated with avirulent pathogens, they promptly respond by forming partially reduced forms of oxygen which result in the accumulation of H_2O_2 (Gozzo, 2004). This compound has a number of effects, some of which may counter pathogen attack and at the same time, may be harmful to the plant itself. To avoid self damage, plant metabolism increases the synthesis of strategic antioxidants such as

ascorbic acid, glutathione and related enzymes to replace the oxidizing potential of H_2O_2 with milder oxidants (Zhang and Kirkham, 1996). In the gene-for-gene plant resistance, the oxidative burst is followed by a rapid cell death around the site of attempted invasion, producing the necrotic lesions typical of the HR. This local HR triggers a notable accumulation of SA in the neighboring cells. Salicylic Acid gradually accumulates in distant tissues while a growing expression of PR proteins takes place in local and systemic tissues (Shulaev *et al.*, 1997).

These PR proteins are specific to plants and include glucanases, chitinases and peroxidases, some of which may play a role in restricting the development of fungal or bacterial pathogens via hydrolytic action on their cell walls (Klessig and Malamy, 1994; Hunt and Ryals, 1996; Kombrink and Somssich, 1997; Nandi *et al*., 2004). However, most of the defense reactions depend on the phenylpropanoid pathway and culminate with cell wall lignification. The phenylpropanoid pathway is initiated by the enzymatic conversion of phenylalanine to cinnamic acid (Gozzo, 2004). This conversion is catalyzed by the enzyme PAL (Koukol and Conn, 1961). These defense reactions do not become evident until after attack from a challenging, even unrelated, pathogen (Gozzo, 2004).

The central role of SA as a signal transducer of SAR was demonstrated in transgenic plants lacking SA formation. These plants failed to express SAR (Gaffney *et al.*, 1993). Salicylic acid may interact with iron-based enzymes, either as a chelator of the metal ion or through binding to related proteins (Gozzo, 2004). The formation of phenolic free-radicals, resulting from the interaction with catalase or ascorbate peroxidase, has been proposed to be involved in the induction of SAR (Durner and Klessig, 1995). Although the precise action of how SAR provides resistance is not fully known, the following model was suggested (Fig 2.4).

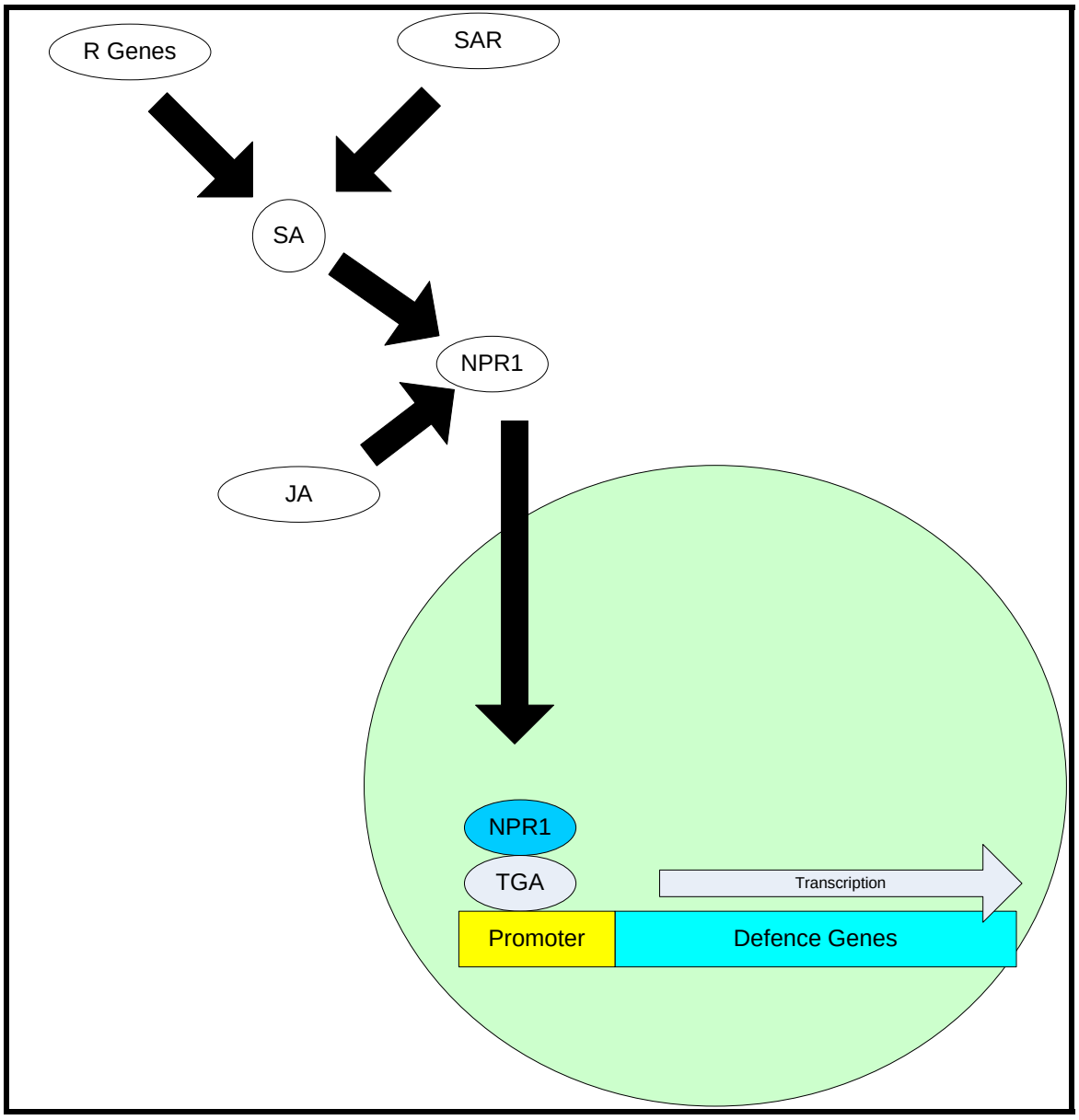


Figure 2.4. Regulation of defense gene expression by NPR1 (McDowell and Woffenden, 2003).

Salicylic acid accumulation causes the translocation of natriuretic peptide receptor 1 (NPR1) into the nucleus (Eckardt, 2003; McDowell and Woffenden, 2003). NPR1 interacts with members of the TGA family of transcription factors. This enhances the binding of these factors to the SA response elements in the promoters of PR genes. Numerous PR genes as well as other genes functioning in the SAR pathway are then transcribed (Després *et al.,* 2000; Kinkema *et al*., 2000; Zhou *et al.*, 2000; Subramaniam *et al.*, 2001; Fan and Dong, 2002). It is difficult to predict which mechanism is used by NPR1 to translocate to the nucleus (Ryals *et al*., 1996). Furthermore, the *in vivo* interaction of NPR1 with TGA proteins is dependent on induction by SA (Fig 2.4), even though TGA proteins are expressed constitutively in the nucleus (Eckardt, 2003).

NPR1 proteins are localized in both the nucleus and the cytoplasm of unstimulated tissue (Després *et al*., 2003). In other words, SA is thought to stimulate the enhanced nuclear translocation of NPR1 where the activated protein interacts with TGA factors. This interaction was confirmed by Després *et al*. (2003) and the redox changes influenced by SA in the NPR1 and TGA factor interaction enhance the DNA binding activity of the TGA factors (Eckardt, 2003).

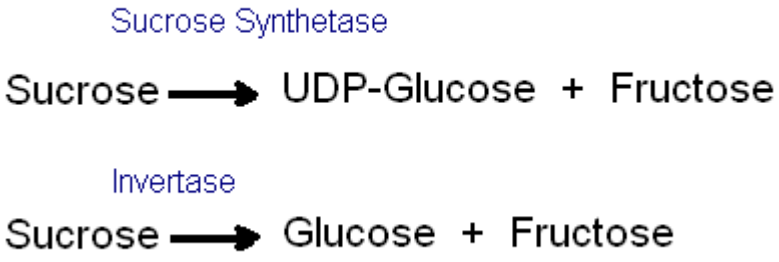
2.6 Sugar Signaling as a Possible Pathogen Defense Activator

Sugar production through photosynthesis is a vital process in plants and the sugar status modulates and coordinates internal regulators and environmental signals that govern growth and development (Koch, 1996; Sheen, 1999; Smeekens, 2000). Although the regulatory effect of sugars on photosynthetic activity and plant metabolism has long been recognized (Rolland *et al*., 2002), the concept of sugars as central signaling molecules is relatively new. Recently, research to reveal the molecular mechanisms underlying sugar sensing and signaling in plants, has started. This includes the demonstration of hexokinase (HXK) as a Glc sensor that modulates gene expression and multiple plant hormone-signaling pathways (Sheen,1999; Smeekens, 2000).

Key enzymes that could be involved in sugar signaling include, invertase, monosaccharide transporters (MST) and HXK (Fotopoulos *et al*., 2003).

2.6.1 Invertase enzymes

In most plants, carbon assimilated in leaf mesophyll cells (source cells) is transported to the heterotrophic organs (sink organs) as sucrose (Lalonde *et al*., 2004). Utilization of sucrose as a source of carbon and energy requires cleavage by either invertase or sucrose synthase. The chemical reactions that follow illustrate the difference in action between the two enzymes.



Invertase, which hydrolyzes sucrose into glucose and fructose, exists in several isoforms with different biochemical characteristics and distinct subcellular localizations (Tang *et al.*, 1999). Invertases with acidic pH optima (acid invertases) are ionically bound to the cell wall (cell wall invertase) or accumulate as soluble proteins in the vacuole (vacuolar invertase). Invertases with neutral or slightly alkaline pH optima (neutral and alkaline invertases) are thought to be cytoplasmic proteins (Tang *et al.*, 1999).

Several different functions have been proposed for invertases. These include the cleavage of sucrose to provide growing tissues with hexoses as a source of energy and carbon (Tang *et al.*, 1999), the generation of a sucrose concentration gradient between source and sink tissues to aid sucrose transport (Eschrich, 1980), the regulation of cell turgor for cell expansion (Meyer and Boyer, 1981; Wyse *et al*., 1986; Perry *et al*., 1987)

and the control of sugar composition in storage organs such as fruits (Klann *et al*., 1993). Some of the invertases are possibly also involved in the responses of plants to environmental factors such as wounding and infection (Sturm and Chrispeels, 1990; Benhamou *et al*., 1991). The specific roles of invertase isoforms in the different subcellular compartments are largely unknown.

Expression of yeast invertase in the cytosol, vacuole or apoplast of transgenic tobacco led to stunted plant growth and reduced root formation (Sonnewald *et al*., 1991). Soluble sugars and starch analysis indicated that sucrose distribution was impaired in all cases. Thus, invertase expression at the wrong place and time had extreme consequences for the physiology and development of plants. In developing tomato fruit (Ohyama *et al*., 1995; Klann *et al*., 1996) and mature potato tubers (Zrenner *et al*., 1996), downregulation of vacuolar invertase activity by gene suppression or an antisense mRNA approach altered the hexose-to-sucrose ratio without major effects on plant development (Tang *et al.*, 1999).

The lack of invertase activity in a natural mutant of maize (Miller and Chourey, 1992) caused an early degeneration and withdrawal of maternal cells from the endosperm, thereby interrupting the transport of photoassimilates into the developing kernel.
As a result, seeds had only one-fifth the normal weight (Tang *et al.*, 1999).

2.6.2 Monosaccharide transporters

Sucrose must flow into the apoplasm to serve as an alternative path for sugars to be imported into cells via cell wall invertases. Invertase enzymes secreted into the periplasmic space hydrolyze this sucrose which is subsequently taken up into the cells by MST. This alternative path occurs during pathogen infection and in certain sink tissues such as pollen (Sherson *et al.*, 2003). The hexose uptake route not only provides a means to enhance sink supply by steepening the sugar gradients, but plays a role in controlling cell division and storage (Borisjuk *et al*., 1998).

Ruan *et al*. (1997) showed that the difference in sugar content between tomato varieties was independent of sugar export rates from leaves, but correlated well with hexose uptake activity in fruits. This finding suggested that hexose transport limited sugar content. Monosaccharide transporter activities have been identified in a variety of plant species (Maynard and Lucas, 1982; Gogarten and Bentrup, 1989).

In contrast to the hexose transporters (HXT) of yeast which function as uniporters, *Chlorella* and *Arabidopsis* HXTs belonging to Clade I sugar transporting proteins function as H^+-cotransporters (Aoshima *et al.*, 1993; Sauer, 1997; Lalonde *et al.*, 2004). Despite this difference in the transport mechanism, yeast and plant transporter genes are homologous and encode proteins composed of 12 membrane-spanning domains as part of the glycoside-pentoside-hexuronide cation symporter family (Lalonde *et al.*, 2004).

Lalonde *et al.* (2004) compared the transporters from four completely sequenced eukaryotic genomes, namely *Saccharomyces cerevisiae*, *Homo sapiens, Arabidopsis thaliana* and *Oryza sativa* and found that MSTs can be distinguished according to phylogeny, substrate spectrum, transport mechanism and cell specificity. They reported that thirteen clusters were identified in the MST superfamily, with 66 and 22 putative MSTs in the *Arabidopsis* and rice genomes, respectively. The availability of many transporters mediating monosaccharide transport may not be unexpected, if one considers the complex requirements for all types of different sugar transport ways.

2.6.3 Hexokinase enzymes

The first enzyme functioning in the glycolytic pathway is HXK (Rolland and Sheen, 2005). Hexokinase catalyzes the phosphorylation of Glc to glucose-6-phosphate (Wilson, 2003).

Plant HXK has been shown to be involved in sugar sensing and signaling (Jang *et al.*, 1997; Xiao *et al.*, 2000) and is proposed to be a dual-function enzyme with both catalytic

and regulatory functions (Jang and Sheen, 1994; Jang *et al.*, 1997; Perata *et al.*, 1997; Umemura *et al.*, 1998; Pego *et al.*, 1999). Although additional evidence is needed to further elucidate how HXK functions as a sugar sensor, it has been shown that HXK-dependent signaling functions can be uncoupled from HXK-dependent metabolism (Jang *et al.*, 1997; Moore and Sheen, 1999). Although a HXK-dependent sugar signal transduction pathway has been shown, HXK-independent sugar signaling pathways also exist in plants (Jang and Sheen, 1997; Lalonde *et al.*, 1999; Roitsch, 1999; Sheen, 1999; Smeekens, 2000).

2.6.4 A proposed sugar signaling system in plants

Rolland *et al.* (2002) proposed the following sugar signaling system for plants. It is mentioned that abiotic and biotic stress stimuli such as drought, salinity, wounding and infection by viruses, bacteria and fungi, can modulate source-sink activities. Since extracellular invertase, a key enzyme for hydrolyzing sucrose (Sturm, 1999), is regulated by stress stimuli and hormones, it has been proposed to be a central modulator of assimilate partitioning, thereby integrating sugar, stress and hormonal signals (Roitsch, 1999). The latter was proven in *Arabidopsis* upon powdery mildew infection, where the expression of a MST and a cell wall invertase gene was increased in other cells away from the infection site (Fotopoulos *et al.*, 2003). This indicated that sugar signals were activated during pathogen infection. Although stress may alter sugar levels, experiments with protein kinase (PK) inhibitors suggested that sugars and stress-related stimuli may independently activate different signaling pathways (Ehness *et al*., 1997; Roitsch, 1999).

It is interesting to note that sugars regulate the expression of wound inducible proteinase inhibitor II and lipoxygenase genes (Johnson and Ryan, 1990; Sadka *et al*., 1994), PR genes (Herbers *et al*., 1996; Xiao *et al*., 2000) and dark-inducible (DIN) genes (Fujiki *et al*., 2001). Some of the DIN genes are also inducibly expressed by sugar starvation, pathogens and senescence (Quirino *et al*., 2000; Fujiki *et al*., 2001; Ho *et al*., 2001). This suggested that a response to metabolic stress could be the underlying mechanism of activating the sugar signaling system. In addition, many jasmonate-, abscisic acid

(ABA)- and stress-inducible genes are co-regulated by sugars (Reinbothe *et al*., 1994; Sadka *et al*., 1994).

Further studies will be required to reveal the genetic and molecular basis of sensing and signaling pathways connecting sugar and stress in plants. Interestingly, an ancient regulatory system controlling metabolism, stress resistance and ageing appears to be conserved from yeast to mice (Kenyon, 2001).

In yeast, Glc sensing and signaling pathways play a central role in survival (Ashrafi *et al*., 2000; Lin *et al*., 2000; Fabrizio *et al*., 2001). The delayed senescence and increased stress resistance observed in *Arabidopsis* HXK antisense plants (Xiao *et al*., 2000) similarly connect plant sugar metabolism and sensing with the control of stress resistance and ageing (Fotopoulos *et al.*, 2003).

2.6.5 Yeast as a model of sugar signaling

Lalonde *et al.* (1999) described sugar signaling in yeast. *Saccharomyces cerevisiae* contains a large spectrum of 200 integral membrane proteins, many of which are clearly involved in transmembrane solute transport. For example, yeast contains 20 permeases for amino acid transport (André, 1995; Nelissen *et al*., 1997) and 20 permeases for sugar transport (André, 1995; Boles and Hollenberg, 1997). This number of transport systems suggests that complex regulatory networks are absolutely necessary to control the uptake of nutrients in response to a rapidly changing external environment.

As shown in Fig. 2.5, yeast has developed a two-pronged regulatory system to ensure coordination between the supply of sugars from the environment and the enzymatic machinery of cells. First, the extracellular concentration of sugars is sensed and sugar transport activity regulated to ensure that optimum sugar transport takes place, since too much sugar inside the cell could alter the osmotic potential. Secondly, the sugar transport activity determines the flow of sugars into the cell. The latter subsequently generates intracellular signals for further regulatory processes (Lalonde *et al*., 1999).

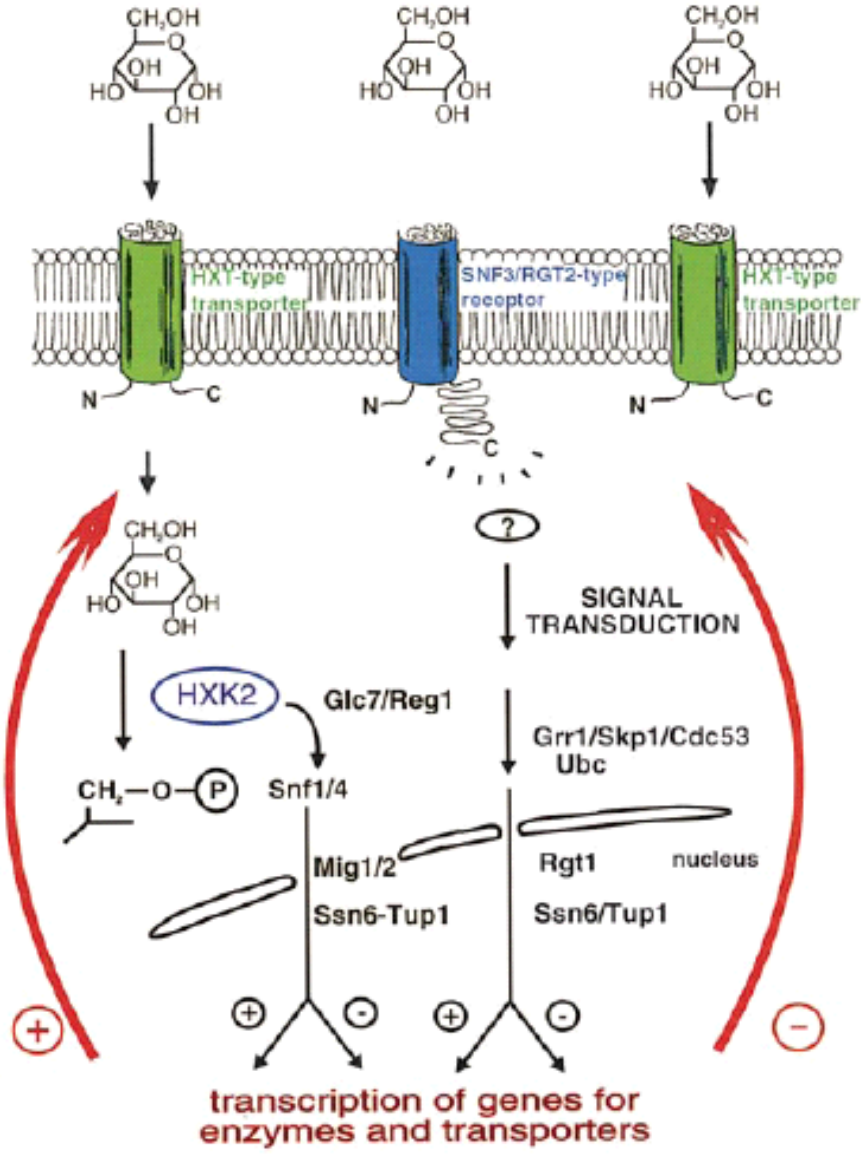


Figure 2.5. Sugar signal transduction system in *S. cerevisiae* (Lalonde *et al*., 1999).

In *S. cerevisiae*, multiple transport systems for glucose are regulated at the transcriptional level in response to the external concentration of glucose. For example, HXT2 and HXT7 (Fig. 2.5) serve as high-affinity glucose transporters and are induced by low levels of glucose but repressed at high levels, whereas HXT1 functions as a low-affinity transporter and is induced only by high concentrations of glucose (Boles and Hollenberg, 1997; Özcan *et al*., 1998). Consequently, sensors of extracellular glucose that respond not only to the kind of carbon source in the medium, but also to its concentration, are required (Lalonde *et al*., 1999).

Once inside the cell, glucose is phosphorylated by three different kinases namely HXK1, HXK2, and glucokinase 1(GLK1) (Lalonde *et al.*, 1999). After phosphorylation, it is converted through the glycolytic pathway mainly into ethanol. In contrast to the uptake of glucose, the regulation of the intracellular glucose concentration, phosphorylation and flow through of glycolysis must be controlled by intracellular signals (Boles and Hollenberg, 1997).

Furthermore, the expression of genes needed for the utilization of alternative carbon sources like sucrose or galactose and genes involved in gluconeogenesis, must be shut off in the presence of sufficient amounts of glucose, the preferred carbon source. This is achieved through a mechanism known as glucose (or carbon) catabolite repression (Ronne, 1995; Gancedo, 1998).

The glucose signal that triggers induction of hexose transporter genes is generated by the hexose sensors SNF3 and RGT2 (Fig. 2.5) (Lalonde *et al*., 1999). On the other hand, the signal that triggers glucose repression is somehow connected to the kinase activity of HXK2 (Ma *et al*., 1989; Rose *et al*., 1991).

In principle, there are two possibilities for sensory proteins to detect signaling molecules. Firstly, sensors might act as receptors, binding the triggering molecule (e.g., glucose) and transducing the signal via other proteins. Secondly, sensors might behave like enzymes

or transporters and undergo structural changes so as to monitor the presence or absence of the triggering compound directly (Lalonde *et al*., 1999).

Genes that take part in yeast sugar signaling, have been reported to also play a role in plant defense activation (Fotopoulos *et* al., 2003). It is thus possible that sugar signaling itself may be one of the initial signals responsible for the activation of a defense response. Although sugar signaling was already reported during certain plant-pathogen interactions such as in the case of *Arabidopsis* and *Erisyphe cichoracearum*, there is the possibility that it can take part in other plant-pathogen interactions as well (Fotopoulos *et al*., 2003).

2.7 Wheat and leaf rust interaction

Leaf rust caused by *P. triticina* Eriks. is an important disease of wheat in many wheat production regions. There are about 50 listed wheat resistance genes that give resistance against leaf rust (McIntosh *et al*., 1998; Singh and Huerta-Espino, 2003). The average life of race-specific resistance genes has been about three years in Mexico when they were commercially used (Singh and Dubin, 1997).

In contrast to the above mentioned, the slow rusting resistance *Lr34* gene has been associated with durable leaf rust resistance (Roelfs, 1988; Dyck, 1991; Singh and Rajaram, 1991). The *Lr34* gene was first described by Dyck *et al*. (1966) in the wheat cultivar Frontana. The gene was later located on chromosome 7D (Dyck, 1987). Researchers have studied the expression and effects of the *Lr34* gene since Thatcher near-isogenic lines became available.

The *Lr34* gene is difficult to detect in seedlings or adult plants based on low infection type, given that under most conditions mostly high infection types are seen (Dyck, 1987; Drijepondt and Pretorius, 1989; Singh and Gupta, 1992; Rubiales and Niks, 1995; Singh and Huerta-Espino, 2003). Under low temperature and light conditions it may be possible

to detect *Lr34* (Dyck, 1987; Singh and Gupta, 1992). Therefore, to determine its effects, slow rusting components must be evaluated in the greenhouse or disease progress assessed in the field. The *Lr34* gene is reported to lengthen the latent period and reduce infection frequency and size of uredinia (Drijepondt and Pretorius, 1989; Rubiales and Niks, 1995). The resistance conferred by the *Lr34* gene is associated with a reduced rate of haustorium formation in the early stages of infection due to a reduced rate of intercellular hyphal development (Rubiales and Niks, 1995).

The *Lr34* gene is associated with leaf tip necrosis in adult plants (Dyck, 1991; Singh, 1992) and is known to enhance the expression of several other race-specific genes (German and Kolmer, 1992). The *Lr34* gene also interacts in an additive manner with other slow rusting genes (Singh and Rajaram, 1991; Singh and Huerta-Espino, 2003).

Chapter 3:
Materials and Methods

3. Materials and Methods

3.1 Materials.

3.1.1 Plant materials

In the study, susceptible Thatcher, as well as resistant Thatcher+*Lr34* cultivars were used for all analyses. The leaf rust strain *Puccinia triticina* Eriks. UVPrt9 was used for all infections.

3.1.2 Other materials.

Hybond N^+ membranes, the Gfx^{TM} Micro Plasmid Prep Kit, Gfx^{TM} PCR DNA and Gel Band Purification Kit and [α- ^{32}P]- deoxycytosine triphosphate (dCTP) were obtained from GE Healthcare. The HexaLabel^{TM} DNA Labeling Kit and restriction enzymes were from Fermentas and the RobustT II RT-PCR Kit from Finnzymes. Scintillation fluid used was Ultima Gold XR obtained from Packard. All other chemicals were of highest quality and purity. All restriction enzymes used were obtained from Roche and the Taq-polymerase was obtained from Promega.

3.2 Methods

3.2.1 Cultivation of wheat plants

Seed of all wheat cultivars was planted in a soil mixture of 33% sand and 67% clay. The seeds were germinated at 25^{o}C in a glasshouse with a 16 h light/8 h dark regime and received water every second day. Plants were fertilized 14 days after germination with multifeed water soluble fertilizer.

3.2.2 Infection of wheat with leaf rust

Adult Thatcher and Thatcher+*Lr34* plants were sprayed with freshly harvested *P. triticina* spores that were suspended in distilled water containing a drop of polyoxyethylene sorbitan monolaurate (Tween^{Tm} 20). Plants were left to dry and placed in a dark dew-simulation cabinet at 20ºC for 16 h to allow for spore germination. Plants were then moved to the glasshouse. For expression analysis, infected plants were harvested at 3 h intervals for a total of 36 h starting immediately after inoculation. Harvested plants were immediately frozen in liquid nitrogen to stop all cellular processes. The tissue was ground to a fine powder in liquid nitrogen and stored at -70ºC.

3.2.3 Southern Blot analysis

3.2.3.1 Total DNA extraction

The two isolated cDNA fragments obtained from JJ Appelgryn (Unpublished results) were sequenced by Inqaba Biotech using BigDye Terminator Technology.

Total genomic DNA was extracted from uninfected wheat leaves using a method for monocots described by Sambrook *et al*. (1989). The ground tissue was resuspended in 2 volumes extraction buffer (25 mM NaCl, 5 mM 2-amino-2-(hydroxymethyl)-aminomethane (Tris) pH 8, 2.5 mM ethylene diamine tetra-acetic acid (EDTA), 1.2 mM sodium-meta-bisulfate), mixed and incubated for 30 min at 65^{o}C. An equal volume of chloroform/isoamylalcohol (24/1) was added to the tube followed by vigorous mixing.

Samples were centrifuged at 12 000 *g* for 15 min and the upper phase collected. DNA was precipitated overnight with 2 volumes 95% (v/v) ethanol at -20^{o}C. The precipitated DNA was scooped out and transferred to clean tubes. The DNA was washed several times with 70% (v/v) ethanol where after the pellet was air dried and finally dissolved in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

3.2.3.2 Enzyme digestion and agarose gel electrophoresis

Twenty five microgram genomic DNA from both cultivars was digested using 20 U *Hind*III enzyme in the presence of 5 mM Tris-HCl pH 7.5, 1 mM $MgCl_2$, 10 mM NaCl and 0.1 mM dithiothreitol (DTT)) for 2 h at 37°C. All samples were separated on a 0.8% (w/v) agarose gel (Sambrook *et al*., 1989) using 0.5 X TAE [20 mM Tris-HCl pH 8.0, 0.28% (v/v) glacial acetic acid, 0.5 mM EDTA] as running buffer at 100 V for 1 h. After separation, the gel was photographed using the Geldoc XR system (Biorad).

3.2.3.3 Transfer of the DNA onto the membrane

The digested genomic DNA was transferred to a Hybond N^+ nylon membrane according to Chomczynski (1992). The DNA was first denatured in 3 M NaCl and 0.4 M NaOH for 30 min and then transferred onto the Hybond membrane using a transfer buffer (1.5 M NaCl, 0.4 M NaOH) for 2 h through capillary action. After transfer, the membrane was neutralized in 0.2 M NaH_2PO_4 and 0.2 M Na_2HPO_4 pH 6.8. The membrane was dried for 15 min at 70°C.

3.2.3.4 Preparation of the probe

Plasmids containing the two different cloned cDNA fragments were used as templates to amplify the cDNA fragments using the gene specific primers (Table 3.1). Each reaction contained 1 ng plasmid, 1 U Taq DNA polymerase, 10 mM Tris-HCl pH 9.0, 50 mM KCl, 2 mM $MgCl_2$, 0.1% (v/v) Triton X-100, 0.2 mM deoxynucleotide-triphosphates (dNTPs) and 25 µM of each primer. The amplification regime was a first denaturation step at 94ºC for 2 min followed by 30 cycles of a 94ºC denaturation step for 30 sec, an annealing step for 1 min at the primer specific annealing temperature (Table 3.1) and a 72ºC elongation step for 2 min, followed by a final elongation step of 10 min at 72ºC. Products were separated on a 1% (w/v) agarose gel at 100 V for 1 h (3.2.3.2). Amplified fragments were excised from the gel and purified with the Gfx™ PCR DNA and Gel Band Purification Kit.

Table 3.1 Nucleotide sequences of primers used in this study

Primer	Primer sequence	Annealing temperature	Primer function
Bovis 26	5'-CAA CTT TCG ATG GTA GGA TAG-3'	58ºC	18S rRNA forward primer
Bovis 27	5'-CTC GTT AAG GGA TTT AGA TTG-3'	58ºC	18S rRNA reverse primer
Bovis 61	5'-CTC TTC ATC TGC CTC TAT GTG-3'	45ºC	MST forward primer
Bovis 62	5'-CTA CTA CTT CTA CTA CGT ACG T-3'	45ºC	MST reverse primer
Bovis 121	5'-TCG TTA GCT ACG TCG ACA AT-3'	48ºC	HXK forward primer
Bovis 122	5'-ACC ATG CCA TTT GGG AAT-3'	48ºC	HXK reverse primer
Bovis M9f	5'-GGC AAG CAG CTG CTG CAG T-3'	53ºC	Invertase forward primer
Bovis M9r	5'-GGC ACC ATT CAT CAT GGG-3'	53ºC	Invertase reverse primer

One hundred nanogram of the respective amplified DNA fragments was diluted with water to a final volume of 40 µl and denatured at 95°C for 10 min. The fragment was radio-actively labeled using 10 µCi [α-^{32}P]-dCTP according to the manufacturers instructions using the HexaLabel™ DNA Labeling Kit. The radioactive labeled DNA was denatured at 95ºC for 5 min, where after it was cooled on ice and added to the hybridization tube.

3.2.3.5 Hybridization

The membrane was prehybridized for 2 h at 42°C in prehybridization solution [0.75 M NaCl, 0.075 M Na-citrate, 0.02% (w/v) sodium dodecylsulfate (SDS), 0.1% (w/v) lauroyl sarcosine, 1% (w/v) polyvinylpyrrolidone (PVP), 1% (w/v) ficoll, 1% (w/v) bovine serum albumin (BSA)]. The denatured probe was added to the hybridization solution and the membrane hybridized overnight at 42°C. After hybridization, the excess probe was removed by washing the membrane twice for 5 min with washing buffer 1 [0.3 M NaCl, 0.03 M Na-citrate, 0.1% (w/v) SDS] at room temperature and twice for 15 min with washing buffer 2 (0.075 M NaCl, 7.5 mM Na-citrate, 0.1% (w/v) SDS) at 65ºC. After washing, membranes were exposed to a Kodak Imaging screen for 48 h and scanned using the Personal Molecular Imager® FX from Biorad.

3.2.4 RNA Expression analysis

3.2.4.1 RNA extraction

RNase free solutions were prepared by adding 0.1% (v/v) diethylpyrocarbonate (DEPC), leaving it overnight at room temperature and autoclaving it the following morning to destroy the DEPC.

RNA was extracted from treated wheat leaves as described by Chomczynski (1992). Approximately 0.1 g ground tissue was vortexed in 0.8 ml extraction buffer [38% (v/v) buffer saturated phenol pH 4.3, 0.8 M guanidine thiocyanide, 0.8 M ammonium thiocyanide, 0.1 M sodium acetate pH 5.0, 5% (v/v) glycerine].

After a 5 min incubation at room temperature, 0.2 volume chloroform was added, mixed and centrifuged for 15 min at 12 000 *g* at 4°C. The upper phase was transferred to a clean eppendorf and RNA was precipitated by adding 1 volume precipitation buffer (1.2 M NaCl, 0.8 M sodium citrate, 3 M sodium acetate pH 5.2) and 1 volume isopropanol for 10 min. Precipitated RNA was recovered at 12 000 *g* for 10 min at 4ºC, the pellet washed twice with 75% (v/v) EtOH and the RNA finally dissolved in DEPC-treated water. The concentration of the RNA was spectrophotometrically determined (Sambrook *et al*., 1989).

3.2.4.2 Expression analysis using reverse transcription-PCR (RT-PCR).

The expression of MST and HXK genes was followed with RT-PCR using the RobusT II RT-PCR kit according to manufacturer's specifications.

Each RT-PCR reaction consisted of 10 ng total RNA, 25 pmol of the respective gene primers (Table 3.1), 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1 x Robust buffer mix and 0.4 µl of each Robust enzyme mix. The amplification regime was a 42ºC reverse transcription (RT) step followed by 30 cycles of a 94ºC denaturation step for 30 sec, an annealing step for 1 min and a 72ºC elongation step for 2 min, followed by a final elongation step of 10 min at 72ºC. The reactions were allowed to run to completion.

In order to standardize the RNA concentration, a control RT-PCR was performed for 18S rRNA using Bovis 26 and 27 as primers (Table 3.1). To determine the expression profiles of the MST and HXK genes, gene specific primers were used (Table 3.1). Bovis 61 and 62 were used for MST and Bovis 121 and 122 for HXK amplification respectively. The primer annealing temperatures used were 58ºC for 18S rRNA, 45ºC for MST and 48ºC for HXK.

To visualize the amplified cDNA, it was separated on a 1% (w/v) agarose gel as described (3.2.3.2). Quantification of the yield was done using the Gel Doc XR system, where the cDNA band intensity was measured for each sample. The sample value was divided by the corresponding 18S rRNA value to standardize the yield. This value was further divided by the value of time 0 to express every sample relative to time 0.

3.2.4.3 Expression analysis using a modified Northern blot.

A total of 20 µg extracted RNA (3.2.4.1) of each sample was resuspended in 50% (v/v) formamide, heated at 65ºC and transferred onto a Hybond N^+ nylon membrane using a Slot Blot system (Hoefer). After washing each well twice with 2X SSC (0.3 M NaCl, 0.03 M sodium citrate pH 7.0), the membrane was dried for 15 min at 70ºC.

Preparation of the invertase probe (3.2.3.4) as well as hybridization (3.2.3.5) were done as described previously. The expression was quantified using the Biorad Personal FX system and results given relative to time 0.

3.2.5 Enzyme assays

3.2.5.1 Invertase activity.

Invertase activity was determined according to the method described by Tang *et al.* (1999).

Two gram ground tissue was vortexed in 5 volumes ice-cold buffer [25 mM sodium acetate, 0.5% (v/v) β-mercaptoethanol, 10 mM lysine, 1 mM EDTA pH 5.0, 0.1 mM phenylmethylsulfonyl fluoride (PMSF)] and centrifuged for 30 min at 12 000 *g* at 4ºC. After the cleared supernatant that was used for acid invertase activity determination was removed, the pellet was washed with ice-cold water and left overnight in 1 M NaCl at 4ºC for extraction of cell wall proteins. After recentrifugation, the cleared supernatant was used to determine cell wall invertase activity.

Protein concentration was determined by the method of Bradford (1976) using Biorad protein stain with BSA as standard on a microtiter plate reader (Bio-Rad Model 3550) according to the method described by Rybutt and Parish (1982).

Activity of acid soluble invertases was determined using 100 µg total protein at 37ºC with 50 mM sucrose as substrate in a 10.5 mM citric acid, 29 mM phosphate buffer pH 5.4. Cell wall

invertase activity was determined using 100 µg total protein at 37ºC on 50 mM sucrose as substrate in a 13.5 mM citric acid, 26.5 mM phosphate (pH 4.6) buffer.

All reactions were stopped after 1 h by adding alkaline copper reagent [1% (w/v) $CuSO_4$, 1 mM NaOH, 0.02% (w/v) Na_2CO_3, 0.005% (w/v) sodiumtartrate, 0.005% (w/v) SDS]. Reducing sugars were measured using the method of Somogyi (1952). Equal volume of Somogyi's reagent [40 mM $(NH_4)_6Mo_7O_{24}$, 0.4 M H_2SO_4, 20 mM Na_2AsO_4] was added to the reaction tube and boiled for 10 min. The same volume of Nelson's reagent (0.2 M Na_2CO_3, 0.2 M $NaHCO_3$, 40 mM $KNaC_4H_4O_6$, 1 M Na_2SO_4, 25 mM $CuSO_4$) was added and the absorbance measured at 540 nm.

3.2.5.2 Monosaccharide transporter activity

The MST assay was done as described by Fotopoulos *et al.* (2003).

Disks were cut from infected and uninfected leaves and placed into equilibration buffer (20 mM phosphate buffer pH 5.8, 1 mM $CaCl_2$) for 1 h. After equilibration, the leaf disks were placed into Glc buffer (20 mM phosphate buffer pH 5.8, 1 mM $CaCl_2$, 500 µm Glc, 1 µm [^{14}C]-Glc) and shaken for 1.5 h at 25ºC. Disks were blotted dry on filter paper and washed twice with ice-cold equilibration buffer.

A CO_2 binding step was added to the assay. This was to verify whether the [^{14}C]-Glc that was taken up by the leaf disk was used during respiration. Filter paper dipped in 20% (w/v) KOH was placed in a separate compartment inside the reaction tube to bind released CO_2 while glucose uptake was taking place.

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The leaf disks and filter papers were placed in 80% (v/v) ethanol and incubated in a waterbath at 80ºC for 1 h. The contents of each reaction were placed into scintillation vials containing 5 ml Ultima Gold XR scintillation fluid and liquid scintillation counting was done and results obtained in disintegrations per minute.

3.2.5.3 Hexokinase activity

The hexokinase assay was done according to Fox *et al.* (1998).

A tube with 0.2 g powdered tissue of each sample was vortexed for 30 s in extraction buffer [50 mM Tris-HCl pH 8.75, 1 mM $MgCl_2$, 10% (v/v) glycerol, 100 μM PMSF, 0.05% (v/v) β-mercaptoethanol] in a 1:1 ratio.

The mixtures were centrifuged for 15 min at 13 000 *g* at 4ºC and the supernatant transferred to a new tube. It was then dialyzed against buffer [50 mM Tris-HCl pH 8.75, 1 mM $MgCl_2$, 10% (v/v) glycerol, 0.05% (v/v) β-mercaptoethanol] for 60 min with 2 changes of the buffer.

Activity in the extract was spectrophotometrically assayed at 340 nm at 30ºC in a 1 ml reaction volume containing 50 mM Tris-HCl pH 8.75, 10 mM $MgCl_2$, 110 mM Glc, 200 μM nicotinamide adenine dinucleotide (NAD^+), 500 μM adenosine triphosphate (ATP), 2 U glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and 33 μl of the crude extract (50 – 100 μg of protein). With a pathlength of 1 cm, the amount of reduced nicotinamide adenine dinucleotide (NADH) formed is equal to the absorbance divided by the extinction coefficient of NADH (6.22) (Stevens and Stevens, 1979). Protein concentration was determined as described in 3.2.5.1.

Chapter 4:
Results

4. Results

During a previous study, two gene fragments were cloned from leaf rust infected wheat using Differential display (DD) RT-PCR (JJ Appelgryn, unpublished results). Since both gene fragments showed putative differential expression upon infection with leaf rust, the role of these gene fragments and their encoded protein products were investigated in wheat infected with leaf rust in order to formulate a possible function for the encoded proteins during the infection process. Both fragments were first sequenced to determine their identity.

4.1 Cell wall invertase

4.1.1 Sequence analysis

The first cDNA fragment was 832 bp in length with the 3'-untranslated region being 168 bp (Fig. 4.1a). When translated to a polypeptide sequence, the longest open reading frame was 220 amino acids in length (Fig. 4.1b).

The obtained polypeptide sequence was used to do a blast search to search for genes that share homology with the cloned cDNA fragment. Genes that showed the highest homology are given in Table 4.1. The sequences encoded cell wall invertases from various plants. All five resulting homologs had a significant E-value very close to zero, confirming the identity of the cloned gene as a cell wall invertase. The isolated gene fragment was named *TaCwi01* (*T. aestivum* cell wall invertase 01). The polypeptide sequences obtained from the blast result were further aligned with the cloned fragments predicted polypeptide sequence to indicate the high level of homology (Fig. 4.2).

The encoded polypeptide was further analyzed for conserved domains within the polypeptide sequence (Table 4.2). Included were a number of phosphorylation sites which would allow for regulation through protein phosphorylation, two myristylation sites which play a part in protein trafficking where myristyl transferases carry the protein to its location over the

a:

```
5′-GAAGAATTCT CGAGCGGCAA GCAGCTGCTG CAGTGGCCGG TGGAGGAGCT -50
   GGATCAGCTG AGGGGCAAGG CTGTGAGCGT GGGTGACAAG TCGTCAAGCC -100
   TGGCCAGCAC TTTGAGGTCA CTGGCCTACA GTCCTATCAG TCTGACGTGG -150
   AGGTGAGCTT CGAGGTGCCG AGCCTGGATA AGGCGGAGCC GTTCGATCCG -200
   GCCTACGCCA ACGACGCGCA GAAGCTGTGC GGGATGAAGA ACGCCGACGT -250
   CAAGGGCGGG GTGGGGCCCT TCGGCCTCTG GGTCCTGGCC TCTTCCAACC -300
   TGGCCGAGAA GACCGCCGTG TTCTTCAGAG TCTTCAAGGA TGGGCATGGC -350
   AAGCCTCTCG TCCTCATGTG CAGTGACCCC ACCAAGTCAT CTCTCACCCC -400
   AGGTCTATAC AAGCCGACTT TTGCCGGGTT TGTCGACACC GACATCTCAT -450
   CCGGGAAGAT CTCTTTGAGA AGCTTAATCG ACCGTTCGGT GGCTGAGAGC -500
   TTCGGCGCCG GAGGGAAGAC CTGCATCCTA TCGAGAGTGT ACCCATCCAT -550
   GGCGATCGGG ACAGACGCGC ATCTTTACGT GTTTAACAAC GGGGACACCG -600
   ACATCAAGGT GTCCAAACTA ACGGCCTGGG AGATGAAAAA ACCCATGATG -650
   AATGGTGCCT AAGCAGTATA TACTGTATAT GTTTTCTCGA GAGTTTAAGT -700
   TTCTGTTGAA TGACATTTCG ACATCGATGT ACACCACGCA ACTGATTGCT -750
   CGCTTAATGA GACCTCTTTG AGTAGAAGGG TTAGAGAAAT TTGTTGACGA -800
   TTATAGTTCC TGCCCATGTG TACGATTAAT C – 3′                -831
```

b:

```
EEFSSGKQLL QWPVEELDQL RGKAVSVGDK VVKPGQHFEV TGLQSYQSDV -50
EVSFEVPSLD KAEPFDPAYA NDAQKLCGMK NADVKGGVGP FGLWVLASSN -100
LAEKTAVFFR VFKDGHGKPL VLMCSDPTKS SLTPGLYKPT FAGFVDTDIS -150
SGKISLRSLI DRSVAESFGA GGKTCILSRV YPSMAIGTDA HLYVFNNGDT -200
DIKVSKLTAW EMKKPMMNGA                                  -220
```

Figure 4.1 Sequence analysis of a cloned putative cell wall invertase cDNA fragment. In (a) the nucleotide sequence of *TaCwi01* is indicated. The coding region is shadowed in grey with the stop codon indicated in black, while in (b) the longest open reading frame is shown.

Table 4.1 Blast analysis results of TaCwi01

Genbank Accession nr.	**Name of sequence**	**Organism**	**E-value**
AAZ29515	Cell wall invertase	*Lolium perenne*	$9e^{-113}$
ABB77250	Cell wall invertase BObetaFRUCT2	*Dendrocalamopsis oldhamii*	$2e^{-106}$
AAP59437	Cell wall invertase	*Saccharum* hybrid cultivar	$2e^{-104}$
NP_001047034	Os02g0534400	*Oryza sativa* (japonica cultivar-group)	$2e^{-95}$
AAC28320	Invertase	*Zea mays*	$1e^{-89}$

```
Saccharum           YDRRKDRYYPDNPNGDYH-RLRYNYGNFYASKTFYDPANRRRVLLGWANKSDSVPDDKAK 297
Zea                 YDGGKDRYYPDDPAGDYHHRRRYDYGNYYASKTFYDPVERRRVLLGWANESDSVPDDKAK 352
Oryza               YNKVTERYVPDNPAGDYH-RLRYDYGNFYASKTFFDPVKHRRILLGWANESDSVTYDKAK 344
TaCwi01             ------------------------------------------------------------E 1
Lolium              YDNVKERYVPDNPTGDVYQRLQYDYGNFYASKTFFDPVKQRRILLGWANESDSVAHDKAK 351
Dendrocalamopsis    YNNKTERYVPDDPNGD-YHRLRYDYGNFYASKTFFDPAKRRRVLLGWANESDSVPDDKAK 344
                                                                             :

Saccharum           GWAGIHAIPRKIWLDPSGKQLLQWPIEEVEKLRGKPVSVGSKLVKPGEHFEVTGVATYQA 357
Zea                 GWAGIHAIPRKIWLDPTGKQLLQWPIHEVEKLRGKAVSVDAKLVKPGDHFEVTGIATYQA 412
Oryza               GWAGIHAIPRKVWLDPSGKQLLQWPIEELETLRGKSVSVFDKVVKPGEHFQVTGLGTYQA 404
TaCwi01             EFS-------------SGKQLLQWPVEELDQLRGKAVSVGDKVVKPGQHFEVTGLQSYQS 48
Lolium              GWAGIQAIPRKIWLDPSGKQLVQWPVEELEKLRGKPVNVGDKVVKPGQHFEVTGLQSYQS 411
Dendrocalamopsis    GWAGIHAIPRKIWLDPSGKQLLQWPIEEVEQLRGKPVSVGDKVVKSGEHFEVTGLGSYQA 404
                     ::             :****:***:.*:: ****.*.*  *:**.*:**:***: :**:

Saccharum           DVEVTFEVS------SLEKAEAFDPAYGNDAQKLCGVKGADVKGGVGPFGLWVLASAD 409
Zea                 DVEVSFELELEAGTSLLEKAEAFDPAYDDDAQKLCGVKGADARGGVGPFGLWVLASAD 470
Oryza               DVEVSLEVS------GLEKAEALDPAFGDDAERLCGAKGADVRGGV-VFGLWVLASAG 455
TaCwi01             DVEVSFEVP------SLDKAEPFDPAYANDAQKLCGMKNADVKGGVGPFGLWVLASSN 100
Lolium              DVEVSFEVS------SLDKAEPFDPAYSNDAQKLCGIKGADVKGGVGPFGLWVLSSAD 463
Dendrocalamopsis    DVEVSFEVS------SLDKAEPFDPAFSNDAQKLCGIKGADEKGGVGPFGLWVLASAN 456
                    ****::*:        *:***.:***: :**::*** *.** :***  ******:*:.

Saccharum           LQEKTAVFFRVFK-DGYG-KPKVLMCTDPTKSSLSPDLYKPTFAGFVDIDISSGKIALR 466
Zea                 LQERTAVFFRVFR-DGHG-KPKVLMCTDPTKSSLSPDLYKPTFAGFVDADISSGKITLR 527
Oryza               LEEKTAVFFRVFKPAGHGAKPVVLMCTDPTKSSLSPDLYKPTFAGFVDTDISSGKISLR 514
TaCwi01             LAEKTAVFFRVFK-DGHG-KPLVLMCSDPTKSSLTPGLYKPTFAGFVDTDISSGKISLR 157
Lolium              LAEKTAVFFRVFK-DGYG-KPIVLMCSDPTKSSLTPDLYKPTFAGFVDTDISSGKISLR 520
Dendrocalamopsis    LEEKTAVFFRVFK-DGYG-KHVVLMCTDPTKSSLSPDLYKPTFAGFVDTDISSGKISLR 513
                    * *:********:  *:* *  ****:*******:*.********** *******:**

Saccharum           SLIDRSVVESFGAGGKTCILSRVYPSIAVGKDAHLYVFNNGEVDVKVSSLTAWEMKKPL 525
Zea                 SLIDRSVVESFGAGGKTCILSRVYPSIAVGKDAHLYVFNNGEVDVTVSGLTAWEMKKPL 586
Oryza               SLIDRSVVESFGAGGKTCILSRVYPSMAIGDKAHLYVFNNGEADIKISHLKAWEMKKPL 573
TaCwi01             SLIDRSVAESFGAGGKTCILSRVYPSMAIGTDAHLYVFNNGDTDIKVSKLTAWEMKKPM 216
Lolium              SLIDRSVVESFGAGGKTCILSRVYPSMALGKDAHLHVFNNGETDIKVSKLTAWEMKRPL 579
Dendrocalamopsis    SLIDRSVVESFGARGKTCILSRVYPSIAIGQNAHLYVFNNGEADIKVSHLTAWEMKKPL 572
                    *******.***** ************:*:* .***:*****:.*:.:* *.*****:*:

Saccharum           MNGA- 529
Zea                 MNGA- 590
Oryza               MNGA- 577
TaCwi01             MNGA- 220
Lolium              MNGA- 583
Dendrocalamopsis    MNGA- 576
                    ****
```

Figure 4.2 Amino acid alignment of TaCwi01 with cell wall invertases from different plants obtained from Genbank. (*) indicate an identical amino acid, (:) a conserved substitution and (.) a semi-conserved substitution.

Table 4.2 Motif search analysis of TaCwi01

Motif	Location	Identity
CK2_PHOSPHO_SITE	26 – 29	Casein kinase II phosphorylation site.
CK2_PHOSPHO_SITE	48 – 51	Casein kinase II phosphorylation site.
CK2_PHOSPHO_SITE	129 – 134	Casein kinase II phosphorylation site.
CK2_PHOSPHO_SITE	158 – 161	Casein kinase II phosphorylation site.
CK2_PHOSPHO_SITE	208 – 211	Casein kinase II phosphorylation site.
MYRISTYL	78 – 83	N-myristoylation site.
MYRISTYL	172 – 176	N-myristoylation site.
PKC_PHOSPHO_SITE	5 – 7	Protein kinase C phosphorylation site.
PKC_PHOSPHO_SITE	151 – 153	Protein kinase C phosphorylation site.
PKC_PHOSPHO_SITE	155 – 157	Protein kinase C phosphorylation site.
Glyco_hydro_32	1 – 172	Glycosyl hydrolase family 32.
SCPU	126 – 144	Spore Coat Protein U domain.

plasma membrane and one Glyco_hydro_32 site which gives the protein hydrolase activity to cleave sucrose. Finally, a SPCU motif was present. This motif is a spore coat protein U domain with currently unknown function.

4.1.2 Genomic presence of *TaCwi01* in wheat

To confirm that the cloned *TaCwi01* fragment did not originate from a contaminating biological source, it had to be proven that it is present within the genome of the subject organism, in this case wheat. It was initially decided to confirm this with a Southern blot. However, after several attempts, no hybridization could be found between the *TaCwi01* probe and the Thatcher or Thatcher+*Lr34* genomic DNA. This could have been due to practical problems, because of the size of the wheat genome.

To circumvent the problem, it was decided to PCR amplify a portion of the *TaCwi01* gene from 100 ng Thatcher or Thatcher+*Lr34* genomic DNA using two *TaCwi01* specific primers that were designed using the cloned cDNA sequence. A PCR product 875 bp in length was amplified from both Thatcher as well as Thatcher+*Lr34* genomic DNA (Fig. 4.3). Since this was the expected size, it was concluded that the cloned cDNA fragment was indeed present within the genomes of the two cultivars.

4.1.3 *TaCwi01* gene expression analysis

For expression analysis, total RNA was extracted from infected leaf tissue. The extracted total RNA from leaf rust infected Thatcher (IS) and Thatcher+*Lr34* (IR) was separated on an agarose gel to confirm the quality and concentration (Fig. 4.4). The quality of RNA was good and concentrations of all samples similar, allowing us to proceed with the expression analysis of *TaCwi01* upon infection. To confirm the differential transcription of *TaCwi01*, RT-PCR was initially done using the designed primers used for genomic analysis. After several unsuccessful attempts to amplify the *TaCwi01* fragment, it was concluded that the gene was expressed at low levels in wheat making it difficult to detect using RT-PCR.

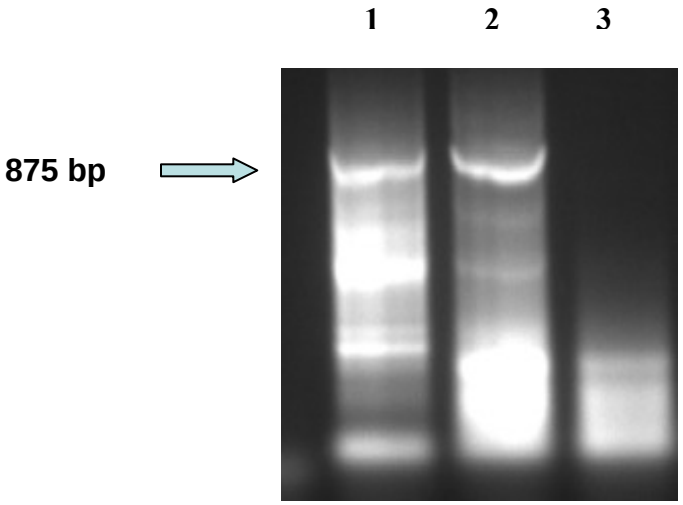


Figure 4.3 PCR amplification of *TaCwi01* from genomic wheat DNA. In lane 1, the amplification from Thatcher is indicated, in lane 2 from Thatcher+*Lr34* while in lane 3 the negative control is shown.

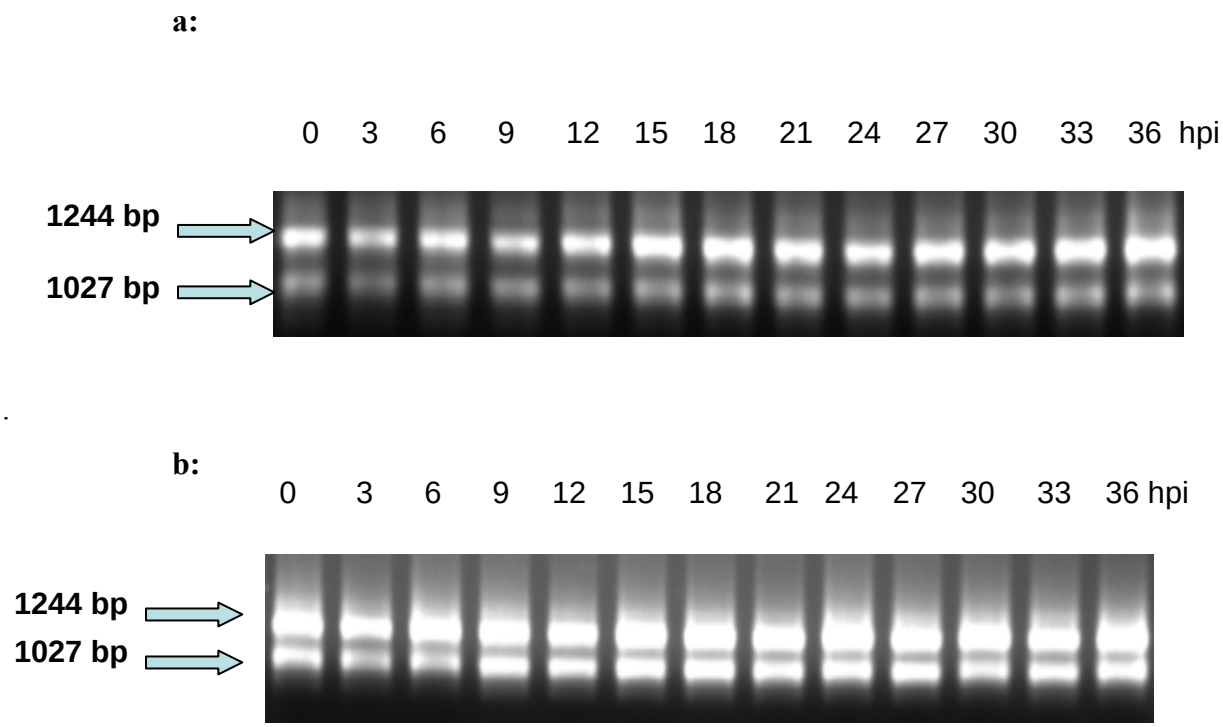


Figure 4.4 Extraction of total RNA from leaf rust infected wheat. In (a), RNA extracted from infected susceptible (IS) plants is shown, while RNA extracted from infected resistant (IR) plants is given in (b). The times of harvest in hours post infection (hpi) is shown at the top.

It was thus decided to revert to a modified Northern blot using a large amount of RNA as target material.

The total RNA was probed with *TaCwi01* and the hybridization value of each time interval was expressed relative to that of time 0. Expression of *TaCwi01* in the IS plants showed a 1.7 fold increase in expression at 3 hpi (Fig. 4.5). From 12-33 hpi, the expression in the IS plants was switched off completely where after expression was again switched on at 36 hpi. In contrast, expression in the IR plants was inhibited directly after infection with the expression remaining below that of time 0 for the duration of the study except for a transient peak at 21 hpi.

4.1.4 Invertase enzyme activity

To investigate the role of invertases during pathogen infection, it was decided to do an enzyme activity assay. Since two types of invertase proteins are present in plant cells, activity determination was done for both acid and cell wall invertase enzymes respectively.

The substrate for invertase, sucrose, is a reducing sugar that can be hydrolyzed by invertase into glucose and fructose. The amount of monosaccharides formed can then be determined spectrophotometrically. By setting up a standard curve with equal known concentrations of glucose and fructose in a detection reaction, the concentration of unknown samples was determined from the curve (Fig. 4.6).

For the acid invertases, the IS plants showed a gradual decrease in activity over the time study (Fig. 4.7a). No significant increase or decrease in acid invertase activity was seen for IR plants. It is interesting to note that the inherent invertase activity for the IS plants was twice that of IR plants at the start of the time trial.

In IS plants, cell wall invertase activity remained constant with two significant increases at 3 and 27 hpi. (Fig. 4.7b). In IR plants, the activity was immediately inhibited and only showed a transient increase in activity at 24 hpi. In contrast to acid invertases, the cell wall

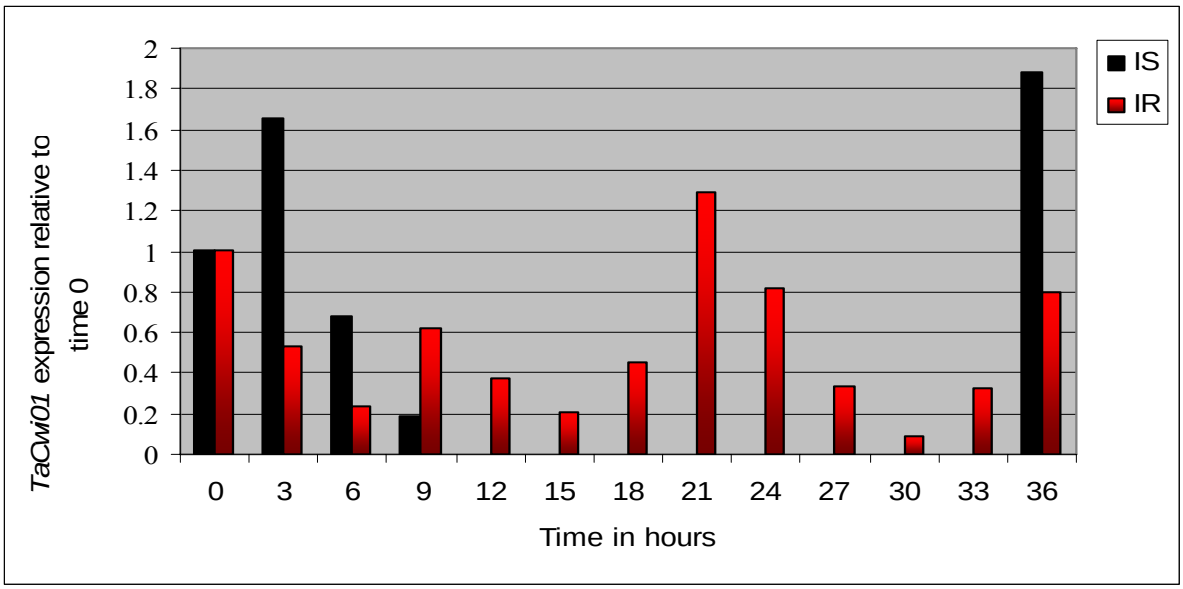


Figure 4.5. Expression analysis of *TaCwi01* in leaf rust infected wheat. The obtained value for each time interval was expressed relative to that of time 0. IR = infected resistant and IS = infected susceptible.

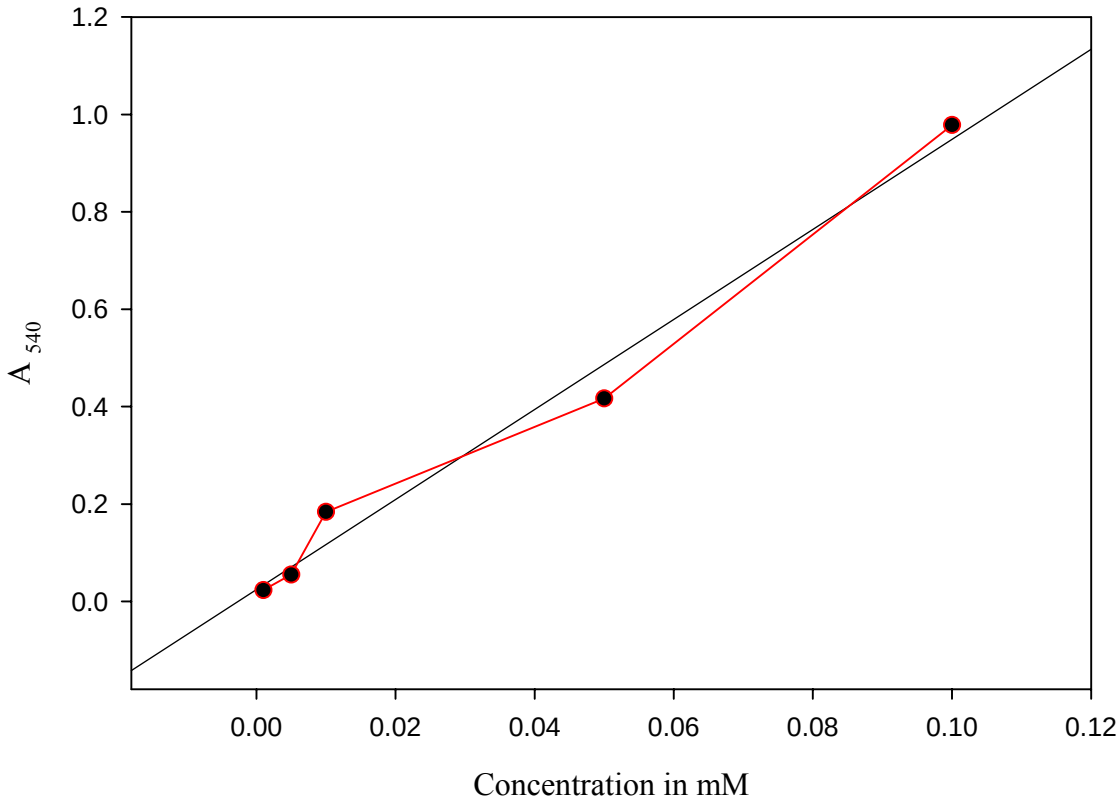
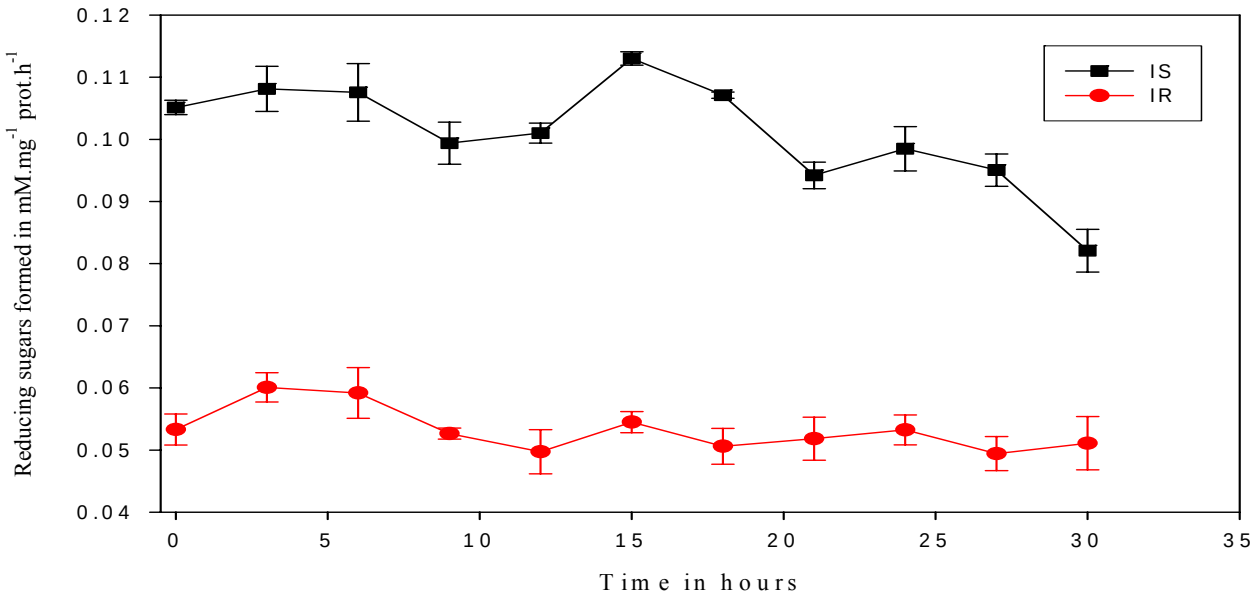


Figure 4.6 Standard curve for invertase activity. Absorbance was measured for known combined glucose and fructose concentrations with a regression coefficient (r^2) at 0.9828730203.

a:



b:

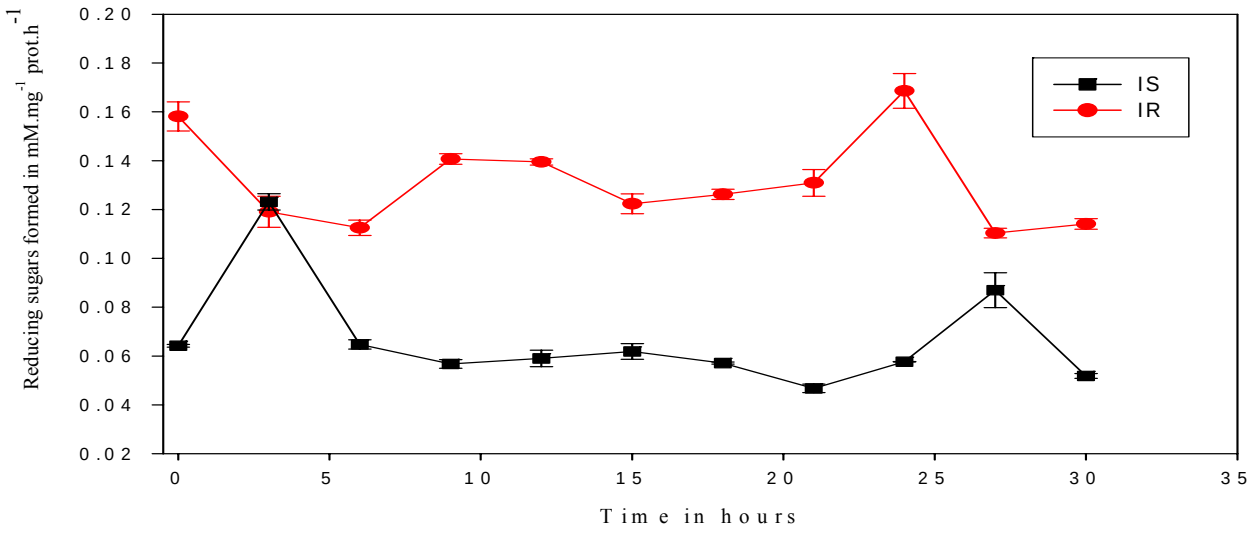


Figure 4.7 Invertase enzyme activity following leaf rust infection of wheat. In (a), acid invertase activity and in (b) cell wall invertase activity is indicated. Error bars indicate standard deviation and n = 3. in IS plants are indicated in black squares and in IR plants in red circles.

invertase activity was three times higher in IR plants compared to IS plants at the start of the time trial.

4.2 Monosaccharide transporter

4.2.1 Sequence analysis

The sequence of the second cloned cDNA fragment was 650 bp in length with a 220 bp 3'-untranslated region and poly-A region present (Fig 4.8a). When translated into a polypeptide sequence, the longest open reading frame was 139 amino acids in length (Fig. 4.8b). The encoded polypeptide sequence was used to do a blast search to search for genes that share homology. In Table 4.3 the genes that showed the highest level of homology are given. All genes encoded monosaccharide transporters with a significant E-value close to zero, confirming the identity of the isolated gene fragment. The cDNA fragment was named *T. aestivum* monosaccharide transporter (*TaMst01)* according to results obtained from the blast search. Since the four *O. sativa* fragments obtained from the Blast search represented the same gene, a representative of them together with the *Zea mays* gene were aligned with the polypeptide sequence of TaMst01. The very high level of homology again became evident, confirming the identity of the isolated gene fragment (Fig. 4.9).

TaMst01 was also analyzed for conserved domains within the polypeptide sequence (Table 4.4). Included was an N-glycosylation site, two myristylation sites for protein trafficking via myristyl transferases, a MFS domain which classify it as a major facilitator superfamily which accommodates membrane transport and the Sugar_tr which characterizes sugar transporters.

4.2.2 Genomic presence of *TaMst01* in wheat

A Southern blot was done to confirm that *TaMst01* originated from wheat (Fig. 4.10). Hybridization revealed that the probe only hybridized with the Thatcher DNA (Fig. 4.10b). Despite numerous attempts, no hybridization was found in the Thatcher+*Lr34* plants. To

a:

```
GTGGGGGAGA TGCCCAAGGG GTACGCGGCG GCGGTGGTGC TCTTCATCTG  -50
CCTCTATGTG GCCGGGTTCG CGTGGTCGTG GGGGCCCCTG GGGTGGCTGG -100
TGCCCAGCGA GATCTTCCCG CTGGAGATCA GGCCGGCGGG GCAGAGCATC -150
AACGTGTCGG TGAACATGCT CTTCACCTTC GTCATCGCGC AGGCGTTCCT -200
CACCATGCTC TGCCACATGA AGTTCGGCCT CTTCTACTTC TTCGCCGGCT -250
GGGTGGTGAT CATGACCGTC TTCATCGCGC TCTTCCTGCC GGAGACCAAG -300
AACGTGCCCA TCGAGGAGAT GGTGCTCGTC TGGAAGGGAC ACTGGTTCTG -350
GCGCAGGTAC ATCGGAGACG CTGACGTCCA CGTCGGCGCC AACAACGGCA -400
AGGGCGCCGC CATCGCATAG ATTCCTTCCT TTCCTAGCTC CGTCTCCCTC -450
GTGTACATTA ATTGCTTTTC TTCCTCTCTT CCTTGTTTGT CCTCGTAGAT -500
GCATGCTTAG GTCAAACCGT GTGTTTCTCT TCCGTATGTG CTTCAAGATT -550
CTTGCATTAA TTAAGATGGT ACCATCAATC ATCATTGTTC TCATACTACG -600
TACGTAGTAG AAGTAGTAGT ACTTTCACTT GAAAAAAAAA AAAAAAAAAA -650
```

b:

```
VGEMPKGYAA AVVLFICLYV AGFAWSWGPL GWLVPSEIFP LEIRPAGQSI -50
NVSVNMLFTF VIAQAFLTML CHMKFGLFYF FAGWVVIMTV FIALFLPETK -100
NVPIEEMVLV WKGHWFWRRY IGDADVHVGA NNGKGAAIA             -139
```

Figure 4.8 Sequence analysis of a cloned monosaccharide transporter cDNA fragment. In (a) the nucleotide sequence of *TaMst01* is indicated. The coding region is shadowed in grey with the stop codon indicated in black and the poly-A tail underlined while in (b) the longest open reading frame is shown.

Table 4.3 Blast analysis results of TaMst01

Genbank Accession Nr.	**Sequence**	**Organism**	**E-value**
NP_001058704	Os07g0106200	*Oryza sativa* (japonica cultivar-group)	$4e^{-67}$
BAB19864	Monosaccharide transporter 3	*Oryza sativa*	$4e^{-67}$
BAC10381	Putative monosaccharide transport protein MST1	*Oryza sativa* (japonica cultivar-group)	$4e^{-67}$
BAD31945	Putative glucose transport protein STP1	*Oryza sativa* (japonica cultivar-group)	$1e^{-66}$
AAT90503	Monosaccharide transport protein 1	*Zea mays*	$1e^{-62}$

```
O. sativa     VCQVVVGTLIAVKFGTSGIGDIPKGYAAVVVLFICMYVAGFAWSWGPLGWLVPSEIFPLE 276
TaMst01       ------------------VGEMPKGYAAAVVLFICLYVAGFAWSWGPLGWLVPSEIFPLE 42
Zea mays      VCQLIVGTLIAAKFGTSGTGDIAKGYAAVVVVFICAYVAGFAWSWGPLGWLVPSEIFPLE 418
                           *:::*****.**:*** ************************

O. sativa     IRPAGQSINVSVNMLFTFVIAQAFLTMLCHMKFGLFYFFAGWVVIMTVFIALFLPETKNV 336
TaMst01       IRPAGQSINVSVNMLFTFVIAQAFLTMLCHMKFGLFYFFAGWVVIMTVFIALFLPETKNV 102
Zea mays      IRPAGQSINVSVNMFFTFCIAQAFLTMLCHFKFGLFYFFAGWVVIMTVFIAFFLPETKNV 478
              **************:*** ***********:*******************:********

O. sativa     PIEEMVLVWKSHWFWRRFIGDHDVHVGANHVSNNKLQP------- 374
TaMst01       PIEEMVLVWKGHWFWRRYIGDADVHVGANNGKGAAIA-------- 139
Zea mays      PIEEMVLVWKSHWFWKRFIADEDVHVGIGNNHPAKNGSDTKGADA 523
              **********.****:*:*:.* ***** .:
```

Figure 4.9 Alignment of TaMst01 with plant monosaccharide transporter polypeptides obtained from Genbank. (*) indicate an identical amino acid, (:) a conserved substitution and (.) a semi-conserved substitution.

Table 4.4 Motif search analysis of TaMst01

Motif	Location	Identity
ASN_GLYCOSYLATION	51 - 54	N-glycosylation site
MYRISTYL	47 - 52	N-myristoylation site
MYRISTYL	129 - 134	N-myristoylation site
MFS	1 - 100	Major facilitator superfamily profile
Sugar_tr	2 - 111	Sugar (and other) transporter

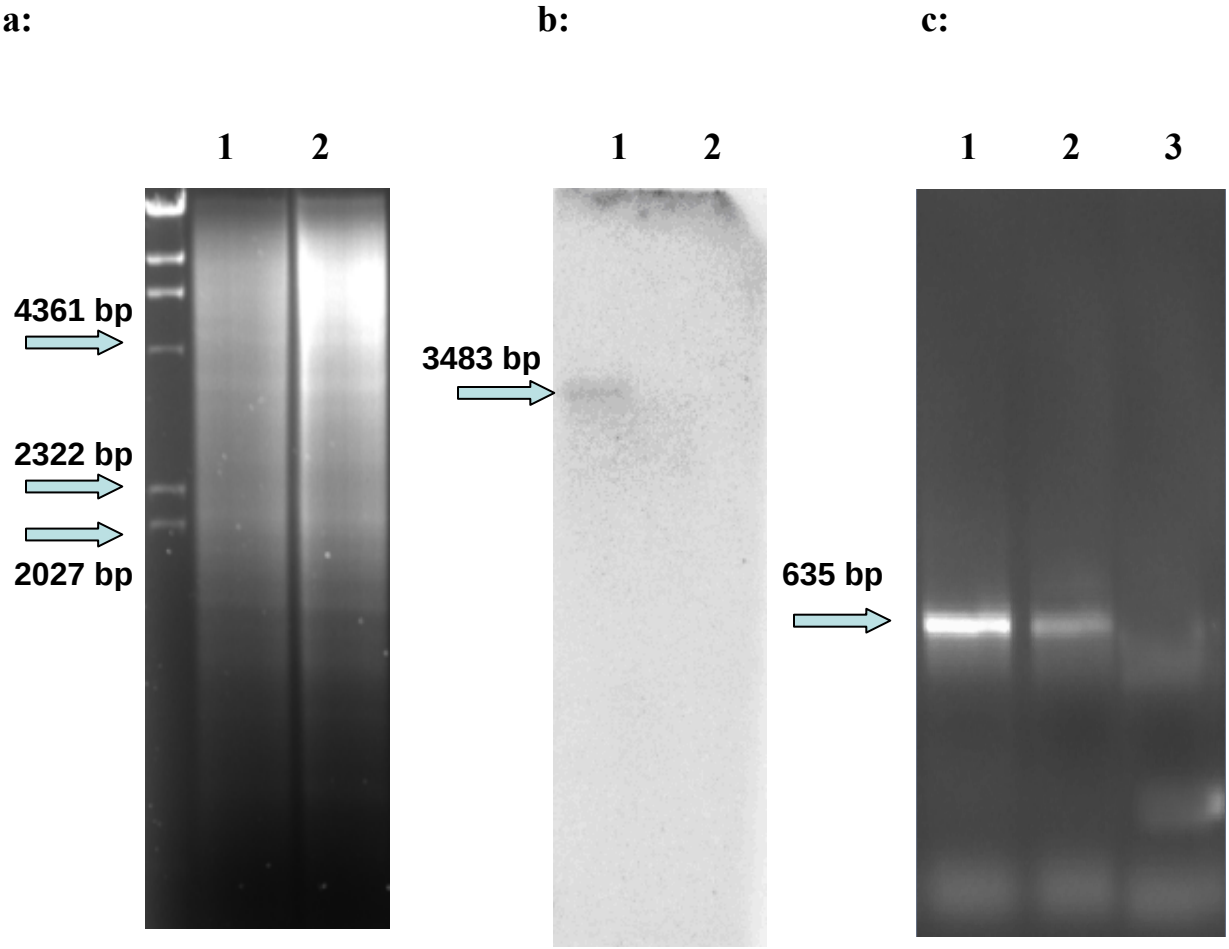


Figure 4.10. Genomic presence of *TaMst01* in the wheat genome. In (a) the digested genomic DNA separated on an agarose gel is indicated, while in (b) the resulting hybridization and in (c) the PCR amplification of *TaMst01* is shown. Lanes 1 and 2 contain digested genomic DNA from the Thatcher and Thatcher+*Lr34* cultivars respectively, and lane 3 is a negative control.

circumvent this problem, it was decided to PCR amplify a fragment of *TaMst01* from 100 ng genomic DNA using two *TaMst01* specific primers designed from the cloned cDNA sequence (Fig. 4.10c). A 635 bp PCR product was amplified from Thatcher as well as Thatcher+*Lr34* plants, confirming the presence of *TaMst01* in Thatcher+*Lr34* as well as thatcher.

4.2.3 *TaMst01* gene expression analysis

To determine the expression pattern of *TaMst01*, RT-PCRs were performed on 10 ng total RNA and the resulting products separated on an agarose gel (Fig. 4.11).

Expression of *TaMst01* was strongly induced in the IS plants at 6 hpi and again from 27 until 33 hpi. Expression analysis of IR plants revealed a quick strong induction at 3 hpi and a later steady increase from 30 up to 36 hpi. The expression level of *TaMst01* in both IR and IS plants were higher throughout the time trial compared to time 0 hpi. To confirm the identity of the amplified fragments as being *TaMst01*, a Southern blot was done on RT-PCR products using *TaMst01* as probe. The resulting hybridization pattern confirmed that it was indeed *TaMst01* that was amplified (Fig 4.11c).

4.2.4 Monosaccharide transporter enzyme activity

The substrate used for monosaccharide transporter proteins was radio-active labeled glucose, where the amount of glucose transported into the cell was determined using scintillation counting (Fig. 4.12). The amount of $^{14}CO_2$ released was measured to determine whether the transported glucose was used in respiration and released as CO_2.

Infected suscebtible plants showed a decrease in MST activity at 9 hpi (Fig. 4.12a), followed by a slow increase in activity until 27 hpi where after a strong increase in activity occurred at 30 hpi. Infected resistant plants reacted similarly with the only change being a transient doubling

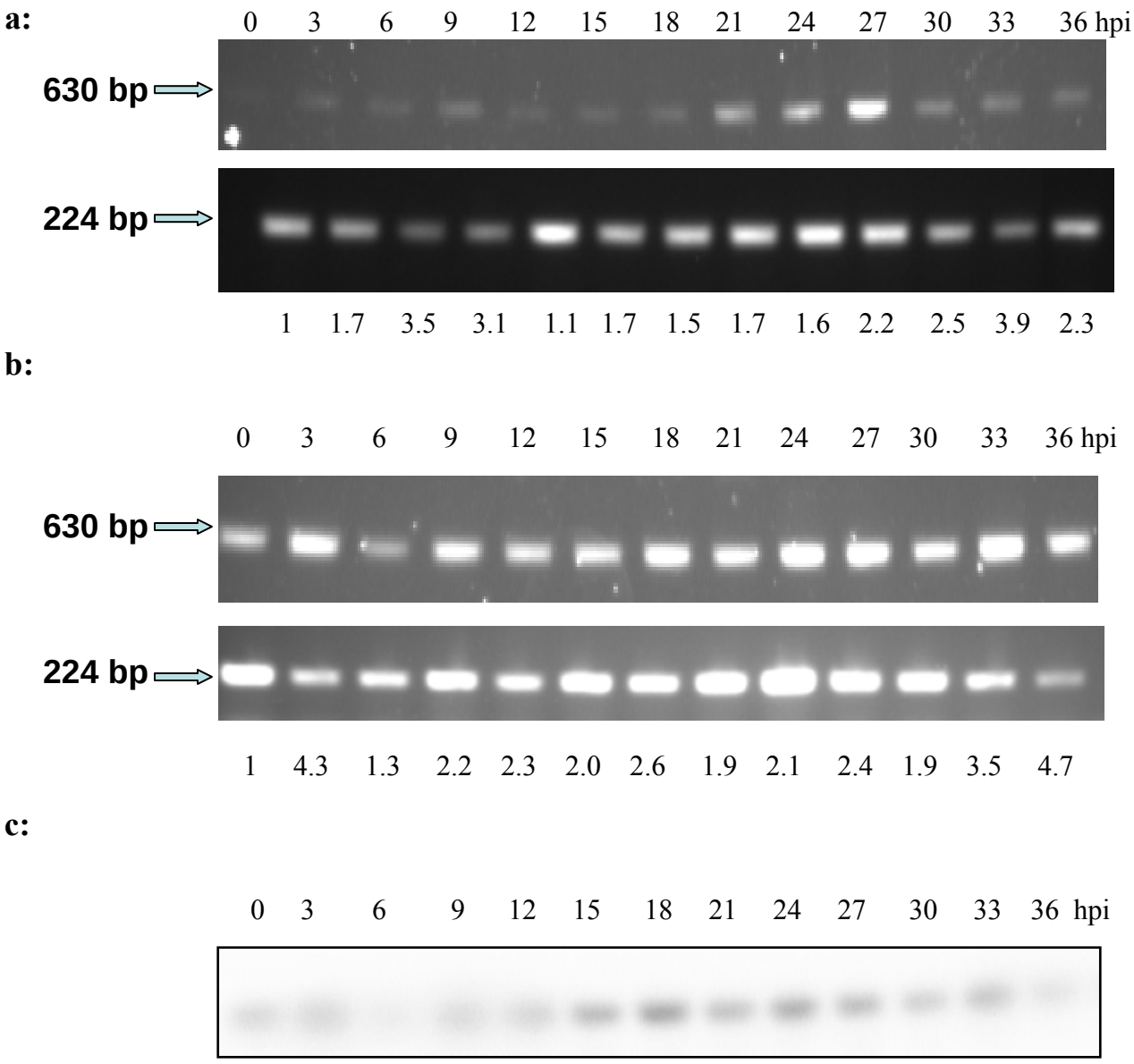
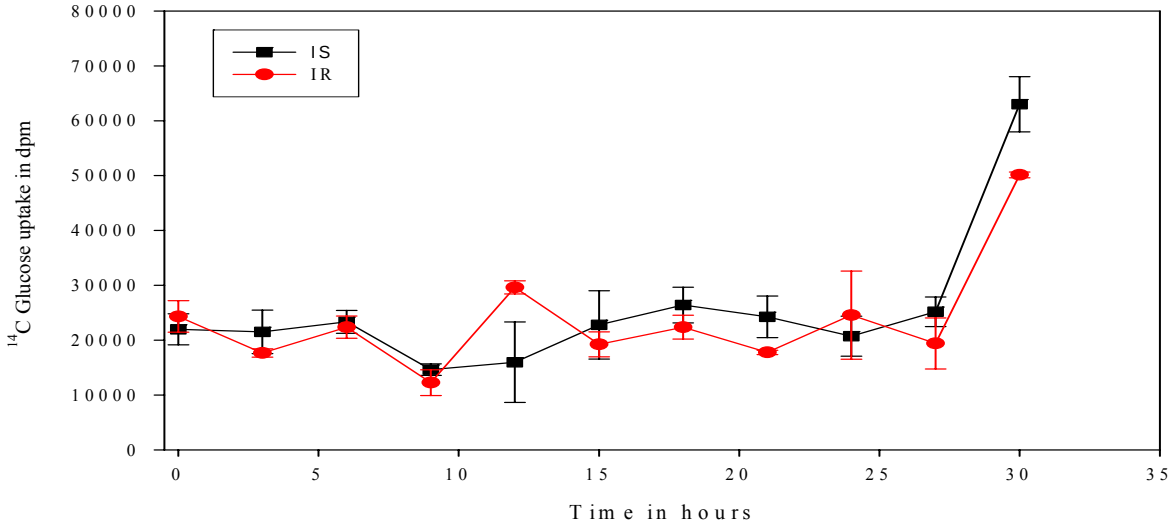


Figure 4.11 Expression analysis of *TaMst01*. Amplification of *TaMst01* from IS (a) and IR (b) plants is indicated. In both cases *TaMst01* expression is indicated in the top panel with the control 18S rRNA expression indicated in the bottom panel. The resulting hybridization of IR plants with *TaMst01* is shown in (c). Top legends indicate time in hours past infection (h.p.i.) while the bottom indicate expression relative to time 0.

a:



b:

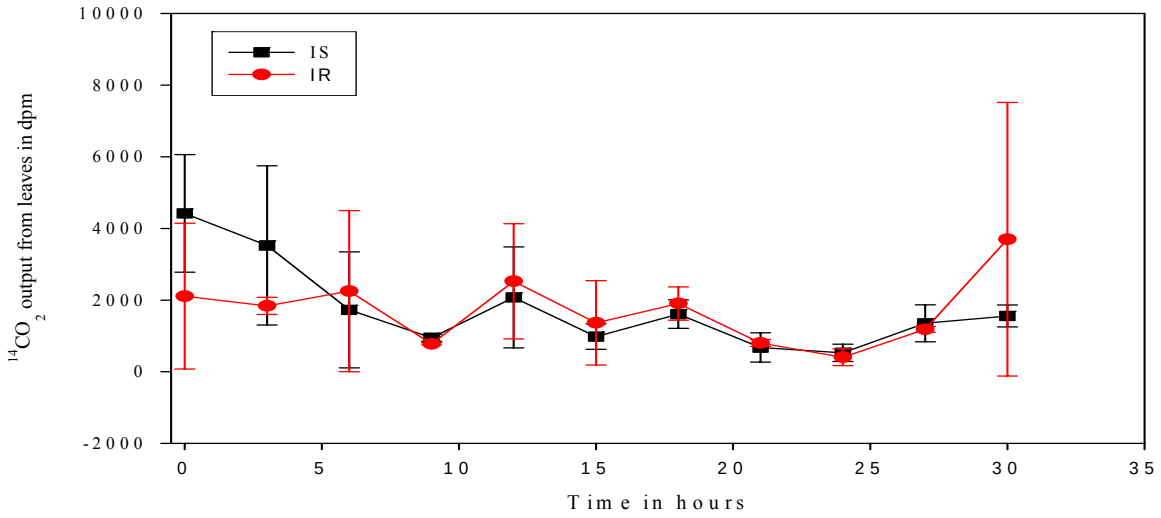


Figure 4.12 Monosaccharide transporter activity following leaf rust infection. In (a) the ^{14}C glucose uptake into leaf discs infected with leaf rust was measured over time, while in (b) the byproduct of respiration, $^{14}CO_2$ output was measured. DPM indicates disintegration per minute. Error bars indicate standard deviation and n = 3. in IS plants are indicated in black squares and in IR plants in red circles.

in activity at 12 hpi. Another significant increase in activity was found visible in IR plants from 27 to 30 hpi. Except for the increase at 12 hpi in IR plants, both IR and IS reacted similarly.

For the CO_2 assay there were no significant changes in the amount of CO_2 produced in both IR and IS plants. Although an increase in released CO_2 was visible in IR plants at 36 hpi, the standard deviation is too big to accept it as significant.

4.3 Hexokinase

4.3.1 Hexokinase gene expression analysis

For the expression analysis of hexokinase genes, a cloned HXK gene from wheat (Genbank accession number AY974231) was used for the development of two primers. Expression analysis was done using RT-PCR. Because the Hexokinase gene sequence is conserved and more than one HXK gene exists in wheat, more than one amplified fragment was detected. Due to low expression levels of these fragments, results are given in the form of a graph (Fig. 4.13). Since all four amplified fragments revealed identical expression patterns, only the result from the fragment with the expected size is given.

In IS plants (Fig. 4.13) there was an induction in gene expression at 6 to 9 hpi as well as from 30 to 33 hpi. Infected resistant plants had a similar profile with the exception that the induced expression levels were lower.

4.3.2 Hexokinase enzyme activity

An enzyme activity assay for HXK was done to confirm if any changes in enzyme activity were taking place after leaf rust infection. The enzyme activity was done spectrophotometrically and the results are given in Figure 4.14. Infected susceptible

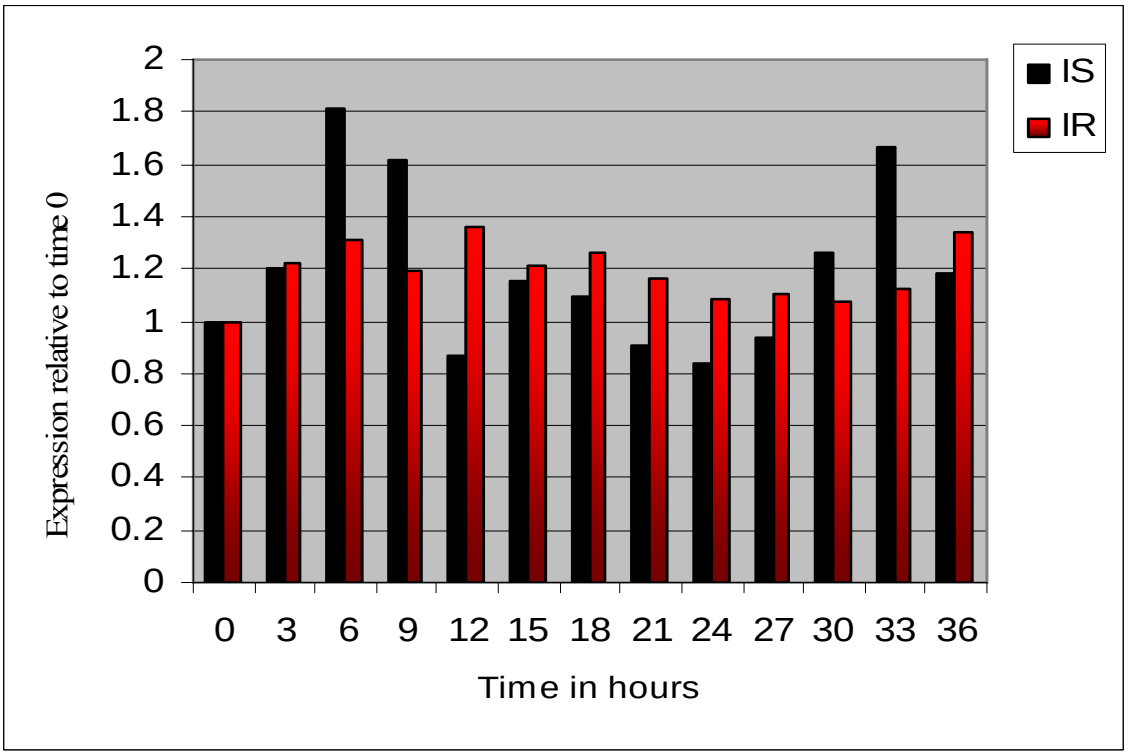


Figure 4.13 Gene expression analysis of hexokinase genes during leaf rust infection of wheat. The obtained value for each time interval was expressed relative to that of time 0.

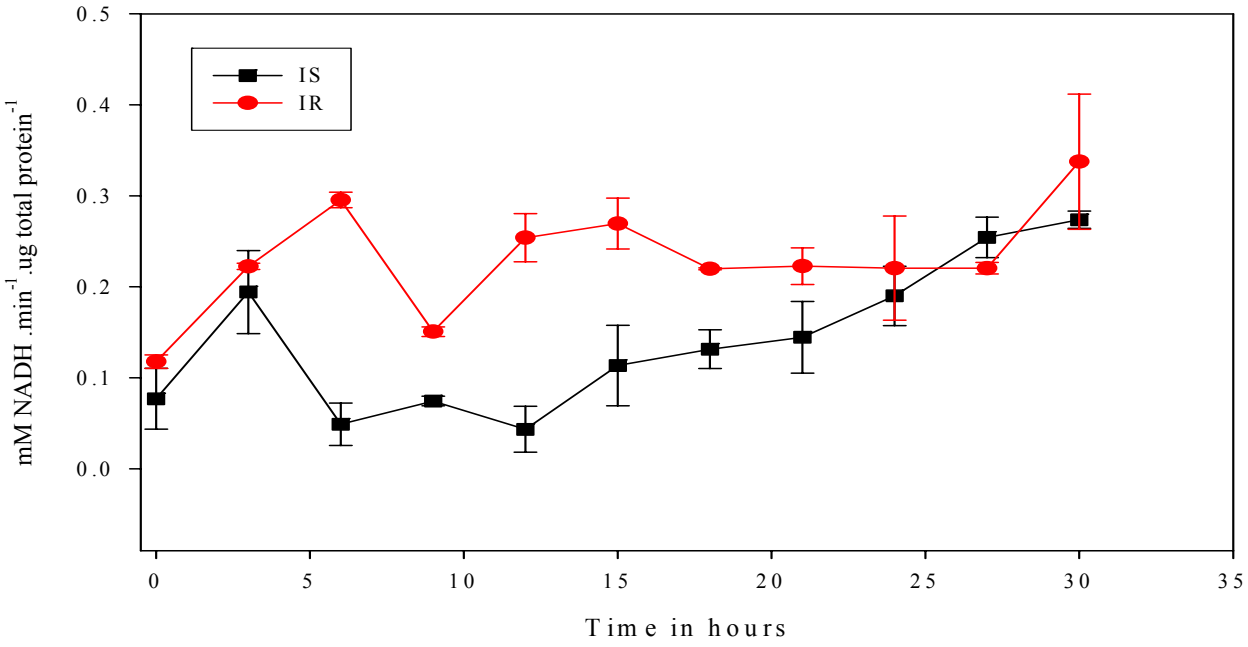


Figure 4.14 Hexokinase activity following infection with leaf rust. Activity in IS plants are indicated in black squares and in IR plants in red circles. Error bars indicate standard deviation and n = 3.

plants showed an initial doubling in activity at 3 hpi followed by an immediate decrease at 6 hpi (Fig.4.14). After that, the activity increased steadily over time to reach a maximum level at 30 hpi which was nearly three times that of time 0.

In IR plants, a 3 fold increase in activity was evident at 6 hpi (Fig. 4.14). The activity then remained relatively stable until 30 hpi where an increase in activity was again visible. The initial increase in hexokinase activity was therefore stronger and sustained over a longer period of time in IR plants opposed to that of IS plants.

Chapter 5:
Discussion

5. Discussion

Puccinia triticina causes leaf rust disease in wheat after successful infection (Sayre *et al*., 1998). Depending on the severity of infection, this disease can cause high yield loss. Traditional breeding programs have led to the development of several cultivars carrying different resistance loci that are resistant to leaf rust infection (Labuschagne *et al.*, 2002). Because of the economic importance of the disease, it is crucial to study the complex interaction between the fungus and the plant. It is especially important that genes and encoded proteins that play key roles during this interaction be identified. In a previous study, two differentially expressed cDNA fragments were cloned using DDRT-PCR (JJ Appelgryn, unpublished results). When sequenced, the fragments were identified as a cell wall invertase (*TaCwi01*) and a monosaccharide transporter gene (*TaMst01*) respectively.

Sugars are important signaling molecules in source/sink regulation, as well as during responses to biotic stress (Roitch, 1999). Previous reports suggested a role for invertases and monosaccharide transporters in plant defense as well as sugar signaling (Herbers *et al*., 1996; Lalonde *et al*., 1999; Roitsch, 1999; Xiao *et al.*, 2000; Rolland *et al*., 2002; Fotopoulos *et al*., 2003). In addition to these two proteins, hexokinase was also suggested to play a part in sugar signaling (Smeekens and Rook, 1997; Rolland *et al*., 2002). Since both an invertase and monosaccharide transporter gene were cloned from leaf rust infected wheat using DDRT-PCR, it implicated that sugar signaling could be involved in the activation of the wheat defense response upon leaf rust infection.

It was decided to investigate the role of the three mentioned genes and their encoded proteins during the interaction between *T. aestivum* and *P. triticina*. More specifically, the aim was to establish whether the three proteins could represent a sugar signaling module that could play a role in the activation in the defense response of the plant.

Amino acid analysis of the two encoded polypeptides revealed a number of interesting aspects. When a motif search was done for TaCwi01, a number of phosphorylation sites

were found, which suggested that the protein could be regulated by means of phosphorylation. In addition, the presence of two N-myristoylation sites indicated that the protein is translocated over membranes following translation. Based on this, the presence of a glycosyl hydrolase family 32 region and the high homology shared with cell wall invertases, indicated that the encoded polypeptide is an extracellular enzyme having invertase activity.

Since no cross hybridization was found between *TaCwi01* and the wheat genomic DNA, PCR amplification of a DNA fragment from both cultivars using two *TaCwi01* specific primers was used to circumvent the problem and, suggested that the gene originated from wheat.

The presence of a spore coat protein U domain in TaCwi01 as revealed by the motif search could indicate that this gene fragment originated from the fungus and not wheat. This domain is found in a bacterial family of spore coat proteins that are assembled on the spore surface after secretion across the spore membrane (Gollop *et al*., 1991). This particular domain currently has no known function. In future, a Southern blot using *P. triticina* genomic DNA must be done to confirm the presence or not of the gene in the fungal genome. This will pose quite a challenge, since *P. triticina* is an obligate biotroph, making culturing of the fungus on an artificial medium difficult. Recent advances in axenic culturing of the fungus (Bourassa *et al*., 2005) could however in future yield enough pure genomic DNA that could be used for Southern blot analysis.

Significant homology to plant monosaccharide transporters was found in the polypeptide sequence of TaMst01. Motifs present in the polypeptide indicated it to be membrane bound, because of the N-myristoylation and MFS motifs. The Sugar_tr domain confirmed that the encoded polypeptide was a sugar transport protein.

The Southern blot analysis showed hybridization between the *TaMst01* probe and Thatcher, but not Thatcher+*Lr34*, genomic DNA. This could indicate one of two possibilities. First, the gene is present in the Thatcher cultivar, but not in Thatcher+*Lr34*.

This however cannot be true since the original DDRT-PCR was done on RNA isolated from Thatcher+*Lr34* plants infected with leaf rust. The other possibility is that *TaMst01* from the resistant cultivar contains additional *Hind*III restriction palindromes. Digestion will fragmentize the gene into smaller segments, thereby weakening the hybridization signal making it very difficult to detect. The PCR amplification of a *TaMst01* gene fragment from Thatcher+*Lr34* genomic DNA did however support the possibility that *TaMst01* is a wheat gene present in both susceptible and resistant cultivars.

Once the identity of the fragments was confirmed, the expression of the genes, including that of hexokinases, in leaf rust infected wheat was studied. Furthermore, the enzymatic activity of invertases, monosaccharide transporters and hexokinases were determined to support any change in gene transcriptional activity.

Two types of invertases are present in plants, namely acid or soluble and cell wall invertases. Acid invertases are located inside the cell and cell wall invertases in the outer parts of the cell wall (Kingston-Smith *et al.*, 1998). Increased acid invertase activity has been reported for a number of plant-pathogen interactions (Ayres *et al.*, 1996; Hall and Williams, 2000). Wright *et al*. (1995) reported that acid invertase activity increased in wheat leaves infected with powdery mildew. They suggested that the increase in acid invertase activity was due to a signal coming from the pathogen. In their study, Herbers *et al*. (2000) found that when tobacco was infected with *Solanum tuberosum* virus Y, there was a decrease in acid invertase expression and enzyme activity.

Similarly, this study showed decreased acid invertase activity in IS plants, while the activity remained constant in IR plants. This suggests that in contrast to Wright *et al*. (1995), no hypothetical signal originating from the pathogen was produced during these two interactions that could activate the acid invertases.

In contrast, cell wall invertase expression and activity increased after infection of tobacco with *S. tuberosum* virus Y (Herbers *et al*., 2000). Similar increases in cell wall invertase

activity were evident during the *Arabidopsis*-white blister rust interaction (Chou *et al*., 2000) and the *Arabidopsis-Erysiphe cichoracearum* interaction (Fotopoulos *et al.*, 2003).

During the wheat - leaf rust interaction, increased cell wall invertase activity was detected in both IR and IS plants from 24 – 27 hpi. In addition, while the activity in IR plants was down regulated immediately after infection, a transient increase shortly after infection was shown in IS plants.

When cell wall invertase activity was compared to *TaCwi01* expression, there was a direct correlation between activity and expression. The early increase in IS activity was mirrored by an increase in expression, while the subsequent decrease in activity was accompanied by a total repression in expression. Infected resistant plants showed an immediate down regulation of both activity and expression, with a subsequent transient increase in expression (21 hpi) and activity (24 hpi).

Herbers *et al*. (2000) showed that during the potato virus Y and tobacco interaction, increased cell wall invertase expression was accompanied by an accumulation of hexose sugars. They suggested that sucrose is the only transport competent sugar in plants and that cell wall invertase activation was to stop this transport, hence the accumulation of glucose and fructose in the apoplastic region in plants. The fact that IR plants showed a complete early inhibition of cell wall invertase activity and expression may be due to the fact that the formation of monosaccharides is counteracted, which could then lead to a loss of energy and carbon when it is taken up by the pathogen.

Sutton *et al.* (1999) reported that powdery mildew can only take up glucose from the host and not sucrose. Thus, should *TaCwi01* be a pathogenic gene, the pathogen cell wall invertase could stop the flow of sucrose by producing monosaccharides from the sucrose in the plant tissue, which cannot be transported through the phloem in plants. The glucose can then be transported into the pathogen, which can only transport glucose over the cellular membrane.

Previous studies reported an increase in Glc uptake into leaf cells as was evident in *Arabidopsis* upon powdery mildew infection. This suggested that the pathogen infection site forms an additional sink (Clark and Hall, 1998; Sutton *et al.*, 1999; Fotopoulos *et al.*, 2003). It was proposed that this happened due to a nutrient demand from the host during the plant-pathogen interaction to either repair damage or to power the defense response. Furthermore, it was reported that the expression of the monosaccharide transporter gene *AtSTP4* increased fourfold in *Arabidopsis* upon *Alternaria brassicicola*, *Fusarium oxysporum* (Truernit *et al*., 1996) and *Erysiphe cichoracearum* infection (Hall and Williams, 2000). Truernit *et al*. (1996) showed the same increase in gene expression in *Arabidopsis* cell suspension cultures when treated with pathogenic elicitors. They concluded that AtSTP4 plays a part in supplying sugars to sink tissues due to pathogen infection.

While a similar fourfold induction in *TaMst01* gene expression was observed 3 hpi in the IR plants, IS plants reacted similarly at 6 hpi. Based on the results of Herbers (2003) that there was an accumulation of sugars in the apoplastic region, the possible reason for monosaccharide transporter gene activation is to transport the apoplastic monosaccharides into cells. Exactly how the signaling to activate this expression of the monosaccharide transporter gene takes place, is still largely unknown (Williams *et al.* 2000).

However, MST enzyme activity results revealed that the early expression profile of *TaMst01* did not affect MST activity. It was only in IR plants that a transient increase in MST activity occurred at 12 hpi. Later during the infection process, there was however significant increased enzyme activity in both IR and IS plants that could have been the result of increased gene expression over the duration of the experiment.

Once the monosaccharides were absorbed into the plant cell, the question arises of how the proposed sugar signal is integrated into the activation of the defense response. Studies with a variety of sugars, sugar analogues and metabolic intermediates suggested

that hexokinase, the first enzyme in glycolysis, can be a putative sugar sensor with a distinct regulatory function (Jang and Sheen, 1994; Rolland and Sheen, 2005).

There are six *HXK* and *HXK*-like genes in the *Arabidopsis* genome as well as three fructokinase and several fructokinase-like genes (Pego and Smeekens, 2000; Rolland *et al*., 2002). Mutational and functional analyses of *Arabidopsis HXK*s and *HXK*-like genes indicated their involvement in sugar sensing and signaling (Jang *et al*., 1997; Rolland *et al*., 2002). Although Jang *et al*. (1997) presented evidence that a role existed for hexokinase in sugar signaling and even proposed it being a sugar sensor, it is still a largely debated subject (Roitch, 1999). There was no correlation between the expression levels of *HXK* and the enzyme activity, indicating the possibility of regulation on translation.

The following conclusions can therefore be made from this study. A definite signaling event was evident during the latter stages of the study. The external cell wall invertase activity in both IR and IS plants was complemented by a similar increase in monosaccharide transporter activity. This indicates that putative monosaccharides produced outside the cell, were taken up into the cell. These sugars could then be used by the increased hexokinase activity as substrates, but not for respiration purposes since there was no increase in the released CO_2. This indicated that the hexokinases could use sugars in a signaling capacity which could play a role in the activation of defense responses. It was previously proposed that hexokinase could act as a MAPK (Rolland *et al.*, 2002). These results would therefore support the suggestion that hexokinase could act as a MAPK.

Other studies have shown that wheat responded within 15 hpi to the leaf rust with induced defense gene expression (JJ Appelgryn, unpublished results). Since the putative sugar signaling event took place long after this, the transmitted signal cannot represent the primary signal responsible for the activation of the plant defense response, but could rather act as part of the secondary defense response.

Besides the later activation, both cell wall invertase activity in IS plants and hexokinase enzyme activity in both IR and IS plants were induced soon after infection. This clearly indicated that the response of wheat was extremely rapid following inoculation, suggesting an early detection and signal transfer ability to warn the plant against infection. A sugar signaling event involving the three tested genes in this study could however not account for this early activation of the defense response, since the MST activity that forms a possible link between cell wall invertase and hexokinase remained unchanged.

That the increased activity of these two enzymes has a definite role during the infection process is beyond doubt. While a more general function for hexokinase can be envisaged based on the fact that there was no difference between IR and IS plants, the increased expression and activity of the cell wall invertase in IS but not IR plants, indicated a unique role for the enzyme. Based on the earlier suggestion that *TaCwi01* could represent a fungal gene, it can be foreseen that upon infection of the susceptible plants, the pathogenic *TaCwi01* gene is inducibly expressed. This induced expression is followed by increased activity in IS plants, where all available sucrose is broken up into monosaccharides that are available for uptake by the fungus. This mechanism could however not function in IR plants due to the fact that the growth and reproduction of the fungus is restricted. This hypothesis can however only be tested once *TaCwi01* has been extensively characterized.

Future research will focus on the further characterization of both *TaCwi01* and *TaMst01* and their unique roles within the defense response of wheat upon infection with leaf rust.

Chapter 6:
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Abstract.

Puccinia triticina (leaf rust) is an obligate biotrophic fungus. It is a major pathogen of *Triticum aestivum* (wheat) all over the world. In a previous study, two differentially expressed genes were cloned from resistant wheat infected with *P. triticina*. According to the sequence analysis, the first encoded a cell wall invertase (TaCwi01) and the other a monosaccharide transporter (TaMst01). These two genes and their encoded proteins appear to play a role during sugar signaling. Sugar signaling has already been described in several plant-pathogen interactions. In this study, the presence of sugar signaling as well as the roles of *TaCwi01*, *TaMst01* and a hexokinase gene in the leaf rust – wheat interaction were investigated.

Gene expression studies of all three genes showed both early as well as late changes in expression. *TaCwi01* gene expression showed an early induction in IS plants at 3 hpi followed by an immediate and complete inhibition until 36 hpi. Expression in IR plants was repressed for the duration of the study with a transient increase at 21 hpi. Enzyme activity analysis revealed a similar pattern of the expression with a significant activation in both IS and IR plants at 27 and 24 hpi respectively.

Results of *TaMst01* expression revealed both an early induction at 3 to 6 hpi and a late induction at 33 hpi in IS and IR plants. The later induction in expression of *TaMst01* was reflected in the significant increase in MST activity at 30 hpi. Hexokinase expression analysis showed a slight increase in expression from 0 to 6 hpi in both IS and IR plants. This was again associated with increased enzyme activity shortly after infection.

Furthermore it was shown that due to a SCPU domain in the polypeptide sequence of TaCwi01, it is possible that this gene could have originated from the pathogen. It was concluded that a putative sugar signaling took place during the infection of wheat with leaf rust and that all three genes played a definite role.

Opsomming

Puccinia triticina (blaarroes) is ‘n verpligte biotrofiese fungus wat die totale jaarlikse opbrengs van *Triticum aestivum* (koring) regoor die wereld bedreig. Tydens ‘n vorige studie is twee gene wat differensieël tot uiting kom uit weerstandbiedende koring na blaarroesinfeksie, gekloneer. Nadat die nukleotiedvolgorde bepaal is, is bevind dat hierdie gene vir ‘n selwandinvertase (TaCwi01) en ‘n monosakkarieddraerproteïen (TaMst01) kodeer. Beide hierdie gene en hul gekodeerde proteïene blyk ‘n rol tydens suikerseintransduksie te speel. Hierdie seinoordraging deur suiker is al tydens verskeie plant-patogeen interaksies aangetoon. Tydens hierdie studie is die rol van suikerseintransduksie sowel as die rol van die twee bogenoemde gene asook ‘n derde heksokinasegeen tydens die blaarroes – koring interaksie bepaal.

Al drie gene het beide ‘n vroeë sowel as ‘n latere verandering in geenuitdrukking getoon. Die uitdrukking van *TaCwi01* in IS plante het 3 ure na infeksie ‘n verhoging getoon, maar dit is direk daarna gevolg deur volkome inhibisie van uiting tot en met 36 ure na infeksie. Die uitdrukking van *TaCwi01* in IR plante is dadelik onderdruk behalwe vir ‘n tydelike verhoging in uitdrukking teen 21 ure na infeksie. In beide IS en IR plante het invertase aktiwiteit dieselfde tendens as die uitdrukkingsprofiel van die geen getoon.

TaMst01 geenuitdrukking resultate het beide ‘n vroeë en latere induksie getoon. In beide IS en IR plante het verhogings in geenuitdrukking van 3 tot 6 ure na infeksie asook later weer teen 33 ure na infeksie voorgekom. Die latere induksie is ook in die MST ensiemaktiwiteitanalise met ‘n groot verhoging in aktiwiteit gereflekteer. Heksokinase geenuiting het in beide IR en IS plante van 0 tot 6 ure na infeksie toegeneem. Dieselfde patroon is ook in die ensiemaktiwiteitsprofiel waargeneem.

Die voorkoms van ‘n SPCU domein in die TaCwi01 ensiem dui daarop dat dit dalk ‘n patogeengeen is. Die finale gevolgtrekking wat gemaak kan word is dat al drie bogenoemde gene wel ‘n rol tydens ‘n moontlike suikerseintransduksie gebeurtenis kan

speel en dat laasgenoemde tydens die infeksie van koring met blaarroes wel plaasgevind het.