INVOLVEMENT OF SALICYLIC ACID IN THE RESISTANCE RESPONSES OF DIFFERENT WHEAT CULTIVARS TO TWO RUSSIAN WHEAT APHID BIOTYPES

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“Nothing in life is to be feared, it is only to be understood.”

-Marie Curie

"Imagination is more important than knowledge. For knowledge is limited, whereas imagination embraces the entire world, stimulating progress, giving birth to evolution.”

-*Albert Einstein*

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DECLARATION

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LIST OF ABBREVIATIONS

AOS	Allene oxide synthase
APX	Ascorbate peroxidase
ARC-SGI	Agricultural Research Council-Small Grain Institute
AtSGT1	*Arabidopsis thaliana* SA glucosyltransferase1
Avr	Avirulence
BA2H	Benzoic acid-2-hydroxylase
C4H	Cinnamate 4- hydroxylase
CA	Cinnamic acid
CAT	Catalase
CC	Coiled-coil
cDNA-AFLP	cDNA-amplified fragment length polymorphism
CP	Crossing point
Cq	Quantification cycle
DEPC	Diethyl pyrocarbonate
Dn	Diuraphis noxia
DTT	Dichlorodiphenyltrichloroethane
E	Efficiency
EDTA	Ethylenediaminetetraacetic acid
ET	Ethylene
EtBr	Ethidium bromide
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

GTP	Guanosine triphosphate
G-proteins	GTP-binding proteins
GPX	Glutathione peroxidase
h.p.i	Hours post infestation
H_2O_2	Hydrogen peroxide
HCl	Hydrogen chloride
HPLC	High performance liquid chromatography
HPOD	Hydroperoxy octadecanoic acid
HR	Hypersensitive reaction
IAA	Indoleacetic acid
ICS	Isochorismate synthase
IPL	Isochorismate pyruvate lyase
IPM	Integrated Pest Management
JA	Jasmonic acid
LOX	Lipoxygenase
LRR	Leucine-rich repeat
LZ	Leucine zipper
MAMP	Microbe-associated molecular pattern
MAPK	Mitogen-activated protein kinase
MES	Methyl esterase
MeSA	Methyl salicylate
MeSAG	Methyl salicylate O-β-glucoside
$MgCl_2$	Magnesium chloride
MOPS	3-(N-morpholino)-propanesulfonic acid
NaCl	Sodium chloride
NaOH	Sodium hydroxide

NBS	Nucleotide binding site
NBS-LRR	Nucleotide binding site-leucine rich repeat
NO	Nitric oxide
NPR1	Non-expressor of PR1
O^{2-}	Superoxide anion
O_3	Ozone
OH^-	Hydroxyl radical
OsBSMT1	*Oryza sativa* Salicylic acid / benzoic acid carboxyl methyltransferase 1
PAL	Phenylalanine ammonia lyase
PAMP	Pathogen-associatecd molecular pattern
PCD	Programmed cell death
PR	Pathogenesis-related
PTI	PAMP-triggered immunity
PVP	Polyvinylpyrrolidone
R gene	Resistance gene
ROS	Reactive oxygen species
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
RWA	Russian wheat aphid
SA	Salicylic acid
SABP	Salicylic acid binding protein
SAG	Salicylic acid 2-O-β-glucoside
SAGT	Salicylic acid UDP-glucosyl transferase
SAMT	Salicylic acid methyl- transferase
SAR	Systemic acquired resistance
SGE	Salicylic acid glucose ester
SOD	Superoxide dismutase

TCA	Trichloroacetic acid
TIR	Toll and interleukin-1 receptor
TMV	Tobacco mosaic virus
TOGT	Tobacco glucosyltransferase
Tris-HCl	Tris(hydroxymethyl)aminomethane
Tween20	Polyoxyethylenesorbitanmonolaurat
USSR	Union of Soviet Socialist Republics
UV	Ultra violet

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INTRODUCTION

Wheat (*Triticum aestivum* L.) is undoubtedly one of the major cereal food crops in the world. In 2009, the production of wheat in South Africa was 1.958 million tonnes, which is much higher than sorghum (0.276 million tonnes) and barley (0.216 million tonnes) and only lower than maize (12.05 million tonnes) (FAOSTAT, http://faostat.fao.org/). In addition, to its high basic caloric value, wheat has a high protein content (~13%), that is higher than other main cereal crops in the world, such as maize and rice. For this reason, wheat is an important source of plant protein in the human diet (FAO, 1998).

Agricultural development is a crucial event in human history. It is believed that domestication of wheat progenitors (einkorn wheat and emmer wheat) and other crops, such as barley, lentil and pea took place in the Fertile Crescent about 10,000 years ago. It is situated in southeastern Turkey and northern Syria today (Simcha *et al*., 2000). However, concomitant with the development of agriculture are the concerns about the prevention of plant diseases and pests. Current research indicates that the worldwide crop loss potential, due to pests and pathogens, is about 33% (Oerke and Dehne, 2004).

1.1. Russian wheat aphid

The Russian wheat aphid [*Diuraphis noxia* (Kurdjumov), RWA] is a tiny (less than 2 mm long), pale green coloured aphid with a spindle shaped body. Viewed from the side, the terminal segment of the abdomen has a supracaudal structure that appears as a double tail (Stoetzel, 1987). The aphids have a pierce and suck mechanism to feed on the

phloem of its host plant, by utilizing specialized stylet-like mouthparts to probe intercellulary through epidermal and mesophyll cell layers in order to locate the phloem in the vascular bundle, from where they obtain their nutrients (Pollard, 1973). The typical phenotypic symptoms of RWA feeding on susceptible cultivars are longitudinal leaf chlorosis (white, yellow and purple to reddish-purple) and leaf rolling, which causes a prostrate growth habit and interfere with self-pollination and grain-filling (Cabrera *et al.*, 1995; van der Westhuizen *et al.*, 1998a; Walters *et al.*, 1980).

The original habitat of the RWA is southern Russia and the surrounding countries of the Mediterranean, such as Iran and Afghanistan (Hewitt *et al.*, 1984). The sporadic outbreaks of the RWA have occurred in the former Union of Soviet Socialist Republics (USSR) since 1912 [Kurdjumov 1913, as quoted by Kovalev *et al.* (1991)]. Nowadays, the RWA is widely distributed and is a serious insect pest of cereal crops throughout the world, including South Africa (since 1978), Mexico (since 1980), USA (since 1986) and Canada (since 1988) (Gilchrist *et al.*, 1984; Jones *et al.*, 1989; Miller *et al.*, 1994; Walters *et al.*, 1980).

In 1978 the occurrence of the RWA in South Africa was reported for the first time. It caused a dramatic decrease in yield of cereal crops (Walters, 1984). RWA infestation causes approximately 60% to 90% of crop losses in field experiments (du Toit and Walter, 1984). In 1992, the first resistant wheat cultivars were released in South Africa after the discovery of host plant resistance in bread wheat (Marasas *et al.*, 1997). In 2001, it was estimated that between 70% and 85% of the cultivated wheat was resistant. The safety and economical benefits of host resistance have hugely contributed to this increased use of resistant cultivars (Tolmay, 2001; van Niekerk, 2001). However, the presence of a resistance breaking biotype of RWA in South Africa was confirmed in December 2005

(Tolmay *et al*., 2006). All cultivars marketed as resistant during the 2005 season were damaged by the new biotype of RWA.

The United States experienced a similar situation. Only one RWA biotype occurred in 1986. In 2003, a new biotype, which has overcome existing resistance, appeared in Colorado (Shufran *et al*., 2007). Haley *et al.* (2004) identified and classified the original RWA biotype as RWA1 and new biotype as RWA2 according to the differential response of wheat plants containing different resistance genes of RWA resistance. A genetic study of RWA nuclear and mitochondrial DNA showed variation among biotypes from the United States and South Africa (Lapitan *et al*., 2007; Shufran *et al*., 2007). Hence, the performance of this recently identified RWA biotype in South Africa (RWASA2) was compared with that of the original RWA biotype (RWASA1) (Tolmay *et al*., 2007). In South Africa, the presence of RWASA2 was also confirmed by examining the damage ratings in different wheat cultivars after infestation with different RWA colonies (Tolmay *et al*., 2007). Jimoh *et al.* (2011) demonstrated that RWASA2 causes more severe damage than RWASA1. In addition, the reproductive rate of RWASA2 is higher than that of RWASA1. In the United States, eight wheat cultivars were used at two constant temperatures, and the plants were evaluated for overall damage and leaf rolling. There were no differences in symptoms induced by RWA2 compared with RWA1. The ratings of damage and leaf rolling were higher for RWA2 than for RWA1 for all susceptible cultivars and temperature treatments. RWA2 also induced plant injury more rapidly than RWA1 (Jyoti *et al*., 2006).

1.2. Measures and strategies to control RWA

1.2.1. Agricultural practice

In the study of Kriel *et al.* (1986), the greatest yield loss was associated with infestation of wheat during the flag leaf and second leaf stage. If damage caused by the RWA could be controlled at a young stage of the plant's development, it could considerably prevent extensive damage. Other studies also showed that the planting date could impact the degree of damage caused by the RWA (Butts, 1992). In South Africa, the Agricultural Research Council - Small Grain Institute (ARC-SGI) suggested that only winter and intermediate type cereals should be planted after May and not later than July. This could prevent the RWA immigrating from the early season to the later season, and restrict infestation of the young plants. Due to the fact that the young leaves would suffer severe RWA infestation, it is also suggested not to plant the spring type cereals (du Toit, 1983).

The row spacing in crop planting regions would also impact RWA infestation. A higher density of crop plants would reduce RWA infestation (Walker, 1992). Another control measure deals with the volunteer wheat. Although the RWA prefers to feed on wheat and barley, it can also survive on other wild grass species, such as *Bromus wildenovii* and *Avena fatua,* over winter or summer, and emigrate to the crop host when they are cultivated (Hewitt *et al.*, 1984). The study of Clement *et al.* (1990) showed that there were no significant symptoms on those wild grass hosts. Maybe the good control of alternate host plants would relieve this problem.

1.2.2. Biological control

Natural enemies of the RWA include certain insect and fungal species which could contribute towards RWA control. There was a successful case in Chile. In 1987, the RWA invaded Chile, but did not cause serious economic losses. Studies between 1991 to 1992 showed that the imported parasitoids, which are natural enemies of the RWA, were adapted to the environment and efficiently controlled the RWA population (Starý, 1993). Biological control of the RWA is not feasible in South Africa, because of the developmental rate of the RWA. It is much higher than that of the natural enemies present in South Africa. Another problem is that the wheat agro-ecosystem, which is unstable, causes the RWA's natural enemy only being present for 3 to 4 months of the year. The reason for this is the drastic RWA population changes between agricultural seasons (Marasas *et al.*, 1997). Another complication might be that predators and parasitoids that attack the RWA are not at all effective at reaching them in the rolled leaves (Robinson, 1994). Entomopathogenic fungi can cause disease in insects and therefore play a role in the natural control of RWA. However, most fungi require substantial humidity to be effective, which makes them less effective in dry regions where the RWA is most prevalent (Feng *et al.*, 1991).

1.2.3. Chemical control

Chemical insecticides have been applied as an emergency RWA control measure in South Africa (Botha, 1984; Marasas *et al.*, 1997). At the onset of the problem in South Africa, insecticides registered for the control of other grain aphids were found to be ineffective against RWA (Marasas *et al.*, 1997). This might be due to the RWA's habit of feeding deep within the leaf whorl and rolled leaves. Chlorpyrifos has been effective due to its ability to vaporize and penetrate rolled leaves (Hill *et al.*, 1993; Robinson, 1994).

Although the application of systemic insecticides could rapidly control the RWA, the effectiveness of insecticides are not sustainable. They are expensive and harmful to the environment (du Toit and Walter, 1984; Marasas *et al.*, 1997). There is also the possibility, that in using insecticides the RWA might develop resistance to the specific insecticides used (Robinson, 1994). For controlling sporadic outbreaks of the RWA, insecticides are still being used (Hayes, 1998). Besides the disadvantages of using systemic insecticides, the bioinsecticides, which play an important role in Integrated Pest Management (IPM), might be another option. The biochemical insecticides are produced from naturally occurring substances (such as nicotine, alkaloids, rotenone and rotenoids), and certain biochemical compounds (such as insect pheromones, agonists, antagonists and plant hormones) (Regnault-Roger and Vincent, 2005). One indirect potential control measure, for example, is the "induction of crop plant resistance"; through application of salicylic acid (SA). SA could enhance plant defence responses to the RWA (El Modafar and El Boustani, 2005; Gerhardson, 2002). Methyl salicylate was registrated as a biopesticide by the United States Environmental Protection Agency in September, 2005 (EPA US, 2005).

1.2.4. Resistant host cultivars

It is generally accepted that the best measure of RWA control is via breeding and cultivation of resistant wheat cultivars. Genetic resistance to the RWA was first reported by Du Toit (1987) in two germplasm lines, PI137739 (*Dn1*) and PI262660 (*Dn2*). PI137739 is a hard white spring wheat from Iran, while PI262660 is a hard white winter wheat from Bulgaria (du Toit, 1987). Other *Diuraphis noxia* (*Dn*) resistance genes have been identified and described. In addition to *Dn1* and *Dn2,* which are single dominant genes, a recessive resistance gene, *Dn3*, was reported in goat grass (*Triticum tauschii*) (Nkongolo

et al., 1991). The *Dn4* gene is associated with PI372129, *Dn5* with PI294994, *Dn6* with PI243781, *Dn7* derived from rye, *Dn8* and *Dn9* from PI294994, and *Dnx* from PI220127 (du Toit *et al.*, 1995; Liu *et al.*, 2005).

In South Africa, research on resistant cultivars, bred in a backcross breeding program from PI137719 (*Dn1*), PI262660 (*Dn2*), PI294994 (*Dn5*), CItr2401 and Aus22498, shows early on that Tugela DN (containing *Dn1*) has a resistance ability when compared to the susceptible cultivar, Tugela (Marasas *et al.*, 1997). Two resistant cultivars, Tugela DN and Betta DN, were released for commercial production from the ARC-SGI breeding program (du Toit, 1992). In 1993, South Africa was the first country in the world to release the RWA resistant commercial wheat cultivars. Eight different cultivars have subsequently been released for the successful control of RWASA1 (Marasas *et al.*, 1997; Tolmay, 2001; van Niekerk, 2001). Most of the RWA resistant cultivars released containing *Dn1*, *Dn2* and *Dn5* resistance genes are cultivated in South Africa (Marasas *et al.*, 1997; Prinsloo, 2000).

The resistance mechanisms can be categorized into three functional groups. They are antibiosis (reduce herbivore survival and reproduction on a host plant), antixenosis (deter herbivore) and tolerance (the ability of a plant to withstand herbivore damage) (Goggin, 2007; Marasas *et al.*, 1997; Smith *et al.*, 1992). An example of antibiosis is PI137739 (*Dn1*), PI262660 (*Dn2*) is tolerance or antixenosis and PI294994 (*Dn5*) is antibiosis, antixenosis and tolerant (du Toit, 1987, 1989; Marais and du Toit, 1993; Rafi *et al.*, 1996). After RWA infestation, the chloroplasts and cell membranes of susceptible plants become disrupted or disintegrated (Belefant-Miller *et al.*, 1994; Burd and Burton, 1992; Fouché *et al.*, 1984). The leaves of antibiosis resistant wheat cultivars are however able to maintain their chlorophyll content at a relative stable level for much longer periods compared to

susceptible cultivars (Haile *et al.*, 1999; Macedo *et al.*, 2009; Ni and Quisenberry, 2006).

1.3. Conclusion

A proper understanding of the interactions between the RWA and its host plant (plant-insect) and plant-microbe interactions is important for developing effective strategies for controlling plant pathogen invasion and pest attack. In particular, increasing the knowledge on the biochemical mechanisms of plant defence responses, such as eliciting events, signal molecules, transduction pathways and involvement of defence enzymes and other products, would provide useful information to improve resistance to pathogens and pests and to stay ahead of evolving new biotypes.

SA has been identified as a critical signal molecule which is involved in defence against pathogens (Vlot *et al.*, 2009). Previous studies showed that SA was also involved in the resistance response of wheat against the RWASA1 (Mohase and van der Westhuizen, 2002). There are, however, no related reports on the RWASA2.

The objectives of this study were to:

1. Investigate and compare the effects of infestation of different resistant wheat cultivars with RWASA1 and RWASA2 on SA content,
2. Elevate the current understanding of SA biosynthesis and metabolism with regard to aphid resistance in wheat,
3. Determine the interaction of SA with enzyme(s) of the biochemical pathway(s) and signal transduction that are induced in the RWA resistance response of wheat.

LITERATURE REVIEW

2.1. Plant defence

Plants can suffer from a wide range of abiotic stresses, including drought, flooding, heat, cold, salinity, extreme light intensity and mechanical damage, as well as biotic stresses such as attacks by a wide array of pathogens and insects (Buchanan *et al.*, 2000). Unlike mammals, who have an immune system to protect themselves, plants possess an innate cell immunity and systemic signals emanating from infection sites (Chisholm *et al.*, 2006; Jones and Dangl, 2006).

The interaction between plant and pathogen can be classified as either compatible (susceptible) or incompatible (resistance). When a pathogen overcomes the plant's defence response, the interaction is compatible (Johal *et al.*, 1995). Although plants are constantly exposed to pathogens, diseases rarely develop from these contacts and most plant species are resistant to the attack of potential pathogens and pests. The host or species incompatibility is also described as nonhost resistance (Johal *et al.*, 1995; Mysore and Ryu, 2004). Nonhost resistance is described as resistance occurring between all genotypes of a plant species to all genotypes of a pathogen species (Jones and Dangl, 2006; Mysore and Ryu, 2004; Niks and Marcel, 2009). Incompatible interaction includes passive and active defence responses. Passive defence mechanisms embrace preformed or constitutive physical barriers (such as cell wall barriers and cuticles) as well as chemical factors (such as phenolics and alkaloids) against invading pathogens (Jones and Dangl, 2006; Mysore and Ryu, 2004). When the pathogen has overcome the plants'

passive defences, the active defence mechanism will be switch on. Plant resistance using active resistance involves the activation of a diverse set of defence responses, such as cell wall cross-linking, cell wall appositions, hypersensitive reaction (HR), phytoalexin accumulation, synthesis of pathogenesis-related (PR) proteins and systemic acquired resistance (SAR) (Buchanan *et al*., 2000; Johal *et al*., 1995). The active response includes the recognition of invading pathogens or insects, early events, signal transduction, defence gene activation and induction of local and systemic defence responses (Buchanan *et al*., 2000).

Plant defence towards herbivores can be divided into direct and indirect defences. Similar to pathogen resistance mechanisms, the direct defences include physical barriers for herbivores, such as trichomes and thorns, and chemical factors which have toxic, repellant or anti-digestive effects on herbivores, such as cyanogenic glucosides, phenolics, alkaloids and proteinase inhibitors (Bennett and Wallsgrove, 1994). The emitting of volatile compounds or production of extrafloral nectar that attract the predators of insect herbivores for self protection is an example of indirect defence mechanism (Heil, 2008; Kessler and Baldwin, 2001).

2.1.1. Perception

The initiation of induced (active) defence responses depends upon the successful recognition of the pathogen, or other intruders. The molecules that can be perceived by plant receptors and induce defence responses are called elicitors (Heil, 2009). Elicitors may originate from the plant or the attacker, such as a pathogen or an insect e.g. the chemical elicitors derived from insect oral secretions and oviposition fluids or the molecules originating from specific ways of wounding (Baker *et al*., 1997; Wu and Baldwin,

2009). Such elicitors could include poly- or oligosaccharides, cell wall fragments, proteins or peptides, glycoproteins, as well as fatty acid derivatives (Baker and Orlandi, 1999; Nürnberger *et al.*, 2004; Walters *et al.*, 2005; Zhao *et al.*, 2005).

Elicitor receptors in plants were characterized in several plants. These receptors are mostly located on the plasma membrane, but some of them can also be localized in endosomal compartments or in the cytoplasm (Nürnberger *et al.*, 2004). For instance, a 13-amino acid peptide elicitor has been well studied in a parsley suspension culture, and its receptor on the cell membrane has also been identified (Nennstiel *et al.*, 1998). Bacterial flagellin can be recognized by some plant species, for example, a receptor-like kinase with a high binding affinity for a 22-amino acid peptide (flg 22) was identified in *Arabidopsis* (Bauer *et al.*, 2001; Gómez-Gómez *et al.*, 1999).

Elicitors can be divided into race-specific and non-specific. In the plant immune system, race-specific elicitors induce the resistance (R) gene-mediated resistance and non-specific elicitors induce a basal resistance (general defence, basal defence or basal immunity) (Bent and Mackey, 2007; Smith and Boyko, 2007; Taylor, 1998). The pathogen (or microbe) - associated molecular patterns (PAMPs or MAMPs) are non-specific elicitors and result in PAMP-triggered immunity (PTI, also called basal resistance) (Chisholm *et al.*, 2006; Vlot *et al.*, 2009). Basal resistance confers low-level resistance to virulent pathogens expressed in susceptible plants and also involve the general defence responses that occur in susceptible plants as well as resistant hosts (Collins *et al.*, 2003; Goggin, 2007; Vlot *et al.*, 2009). In order to infect a host successfully, a pathogen either evades or interferes with recognition of its PAMPs or suppresses or alters plant defences immediately by secreting effector protein into the plant cell cytosol after plant recognition which results in effector-triggered susceptibility (ETS) (Chisholm *et al.*, 2006; Ingle *et al.*,

2006; Jones and Dangl, 2006).

During the evolution process, plants developed a specific recognition of the effector protein resulting in effector-triggered immunity (ETI, called *R* gene-mediated resistance), which overcomes ETS (Jones and Dangl, 2006; Vlot *et al.*, 2009). *R* gene-mediated resistance includes rapid activation of plant defences, many of which are common to the slower basal defence response found in susceptible genotypes (Goggin, 2007). The successful induction of *R* gene-mediated resistance depends on interaction between the plant resistance gene product and the pathogen avirulence (*Avr*) gene product according to gene-for-gene model provided by Flor (1971). This model predicts that an incompatible interaction between a plant and pathogen will occur only when a plant possesses a dominant *R* gene and the pathogen expresses the complementary dominant *Avr* gene (Buchanan *et al.*, 2000; Jones and Dangl, 2006). The architecture of R proteins have common features such as containing a nucleotide binding site (NBS) and variable-length leucine-rich repeat (LRR) domains, which functions to mediate protein-protein interactions (Bent and Mackey, 2007). To date, R proteins are classified in five groups: intracellular protein kinases; receptorlike protein kinases with an extracellular LRR domain; intracellular LRR proteins with a NBS and a leucine zipper (LZ) motif; intracellular NB-LRR proteins with a region with the Toll and interleukin-1 receptor (TIR) proteins or a predicted coiled-coil (CC) domain; and LRR proteins that encode membrane bound extracellular proteins (Bent and Mackey, 2007; Gachomo *et al.*, 2003; Odjakova and Hadjiivanova, 2001).

2.1.2. Early events and signal transduction cascades

Following perception, the early events or the immediate responses are initiated, which is a multiple component network with various sequential reactions to establish an efficient defense and further amplify the signal to other down stream reactions (Boller, 1995; Ebel and Mithöfer, 1998; Wu and Baldwin, 2009; Zhao *et al.*, 2005). Early events involve calcium and other ion fluxes, cell membrane depolarization, cytoplasmic acidification, activation of guanosine triphosphate (GTP)-binding proteins (G-proteins), regulation of various enzymes such as NADPH oxidases, phospholipases, phosphatases and mitogen-activated protein kinases (MAPK) as well as the oxidative burst which includes reactive oxygen species (ROS) generation, nitric oxide (NO) generation, early defence gene activation and the HR (Ebel and Mithöfer, 1998; Maffei *et al.*, 2004, 2006; Orozco-Cárdenas *et al.*, 2001; Orozco-Cárdenas and Ryan, 2002; Zhao *et al.*, 2005). These particular rapid changes are likely to have immediate effects on various metabolic pathways which further results in large scale biochemical and physiological changes locally and systemically. This may include activation of enzymes to undertake specific modifications to primary and secondary metabolism, synthesis of compounds or precursors to act as signal molecules of defence responses and expression of defence-related genes (Somssich and Hahlbrock, 1998). The signal transduction pathway may vary with perception of different elicitors (Zhao *et al.*, 2005).

The HR results in rapid and localized cell death at the site of infection and induction of intense metabolic alterations in the cells surrounding necrotic lesions which cause local responses (Baker *et al.*, 1997; Hammond-Kosack and Jones, 1996). The HR is also thought to play a causal role in resistance to biotrophic pathogens by depriving access to further nutrients (Buchanan *et al.*, 2000; Johal *et al.*, 1995). The local responses include

alterations in secondary metabolic pathways, cessation of the cell cycle, synthesis of a broad range PR proteins, accumulation of compounds with antibiotic activity or act as signal molecules (such as salicylates, ethylene, jasmonates, ROS and lipid-derived metabolites), and fortification of cell walls (Buchanan *et al.*, 2000; Fritig *et al.*, 1998).

The types of PR proteins include fungal cell wall-degrading enzymes, antimicrobial polypeptides and components of signal transduction cascades (Table 2.1) (Buchanan *et al.*, 2000; Fritig *et al.*, 1998; Gachomo *et al.*, 2003). The PR proteins are induced both locally around the infection sites and systemically (Bowles, 1990; Lamb *et al.*, 1992). After HR development, the pathogen uninoculated area of the plant often displays increased levels of *PR* gene expression and the development of SAR, a long-lasting, broad-based resistance to infection by a wide variety of pathogens (Durrant and Dong, 2004; van Loon, 1997; Vlot *et al.*, 2008). For SAR to develop systemically, a signal generated in the inoculated site is transmitted via the phloem to the uninfected portions of the plant (Vlot *et al.*, 2009).

Differences in plant responses to herbivore attack and pathogen invasion exist. The HR mainly occurs in pathogen-induced defence responses while herbivores normally induce wounding responses, which are caused by mechanical tissue damage (Johal *et al.*, 1995; Smith and Boyko, 2007; Wasternack *et al.*, 2006). However, there are some similarities of defence responses and signal transduction between invading pathogens and insect attack. Both may cause cell wall modifications, generation of SA, jasmonic acid (JA), ethylene, ROS and induce enzymes that participate in the signalling pathways and synthesis of signal molecules (Maffei *et al.*, 2007; Smith and Boyko, 2007; Wu and Baldwin, 2009).

Table 2.1 A list of pathogenesis-related protein (PRs) families in plants and their putative functions (Gorjanović, 2009; van Loon *et al.*, 2006).

Family	**Type member**	**Properties**
PR-1	Tobacco PR-1a	Antifungal
PR-2	Tobacco PR-2	β-1,3-glucanase
PR-3	Tobacco P, Q	Chitinase I-II, IV-VII
PR-4	Tobacco ‘R’	Chitinase I, II
PR-5	Tobacco S	Thaumatin-like protein
PR-6	Tomato Inhibitor I	Protease inhibitor
PR-7	Tomato P_{69}	Endoproteinase
PR-8	Cucumber chitinase	Chitinase type III
PR-9	Tobacco “lignin-forming peroxidase”	Peroxidase
PR-10	Parsley “PR1”	‘Ribonuclease-like’
PR-11	Tobacco “class V” chitinase	Chitinase, type I
PR-12	Radish Rs-AFP3	Defensin
PR-13	Arabidopsis THI2.1	Thionin
PR-14	Barley LTP4	Lipid-transfer protein
PR-15	Barley OxOa (germin)	Oxalate oxidase
PR-16	Barley OxOLP	‘Oxalate oxidase-like’
PR-17	Tobacco PRp27	Unknown

2.2. Signal molecules in defence responses

After the perception and early events, the amplification of the defence response occurs through the generation of additional signal molecules, such as ROS, NO, lipid peroxides (oxylipins), benzoic acid, SA, JA and ethylene (ET). The function of signal molecules is the activation of defence-related gene expression, modification of defence proteins and enzymes, concomitant alterations to cellular redox status or cellular damage leading to activation of the cell protection mechanism and induction of genes that encode various cell protectants, stimulation of downstream defence responses and establishment of SAR (Buchanan *et al.*, 2000). The role of ROS, NO, oxylipins, JA, ET and SA during the defence responses will be discussed below in more detail.

2.2.1. Reactive oxygen species

During the HR, one of the early events is the rapid accumulation of ROS [includes superoxide anions (O_2^-), hydroxyl radicals (OH^-) and hydrogen peroxide (H_2O_2)], also known as the oxidative burst, which is a common feature of the plant's response to pathogen invasion and herbivore attack (Alvarez *et al.*, 1998; Leitner *et al.*, 2005; Maffei *et al.*, 2006; Wojtaszek, 1997). In different plant species, biphasic H_2O_2 generation during the oxidative burst is observed. Phase I includes an immediate and transient H_2O_2 generation (non-specific response) and Phase II is a delayed and prolonged H_2O_2 generation that is stimulated by incompatible plant-pathogen or elicitor-treated interactions (Baker *et al.*, 1997; Bolwell and Wojtaszek, 1997; Grant *et al.*, 2000; Zhao *et al.*, 2001). Moreover, evidence points to the involvement of H_2O_2 in early events as the signal for the induction of defence responses during plant-pathogen interactions (Alvarez

et al., 1998; Levine *et al.*, 1994; Orozco-Cárdenas *et al.*, 2001). In plants, there are several sources for the generation of ROS, including NADPH oxidase, cell wall peroxidase, superoxide dismutase (SOD), xanthine oxidase, oxalate oxidase and amine oxidase (Bolwell and Wojtaszek, 1997; Desikan *et al.*, 1996; Lamb and Dixon, 1997). Evidently, NADPH oxidases are responsible for the production of pathogen-elicited, wounding- and herbivory-induced ROS (Desikan *et al.*, 1996; Orozco-Cárdenas *et al.*, 2001; Simon-Plas *et al.*, 2002).

The H_2O_2 might act as a prerequisite for further defence signal transduction events, such as induced SA biosynthesis (Bradley *et al.*, 1992). It also contributes to structural reinforcement of plant cell walls during lignification and cross-linking of cell wall structural proteins, phytoalexin production, induce programmed cell death (PCD) during the HR and is involved in the activation of defence genes (Alvarez *et al.*, 1998; Dempsey and Klessing, 1994; Grant and Loake, 2000; Shirasu and Schulze-Lefert, 2000; Wojtaszek, 1997). At low concentrations, ROS acts as a defence signal and activates detoxification mechanisms, whereas at high concentrations ROS can have antimicrobial activity and also cause cell damage (Lamb and Dixon, 1997). To control the level of ROS and to protect cells under stress conditions, plants are equipped with ROS scavenging enzymes, such as SOD, catalase (CAT), ascorbate peroxidase (APX) and glutathione peroxidase (GPX). The SOD converts O_2^- into H_2O_2, after which CAT, APX and GPX detoxify the H_2O_2 to H_2O (Apel and Hirt, 2004).

2.2.2. Nitric oxide

NO has been established as a key signal molecule during the resistance responses in *Pseudomonas syringae* pv. *glycinea* inoculated soybean suspension cultures and tobacco mosaic virus (TMV) inoculated tobacco leaves (Delledonne *et al.*, 1998; Durner *et al.*, 1998). NO is also involved in the regulation of physiological processes such as stomatal closure and repression of flowering (Delledonne *et al.*, 1998; He *et al.*, 2004; Neill *et al.*, 2002). It synergistically acts with ROS to induce the HR and the expression of various defence related genes, including the PR genes, such as PAL1, PR-1 and GST during plant-pathogen interactions (Delledonne *et al.*, 1998, 2002; Durner *et al.*, 1998). It was reported that NO activates MAPK in tobacco and *Arabidopsis* (Clarke *et al.*, 2000; Kumar and Klessig, 2000). In addition, NO also participate in regulating the expression of many genes involved in the synthesis of JA and response to JA (Orozco-Cárdenas and Ryan, 2002). NO may also directly interact with SA since SA treatment enhances NO production in soybean (Klepper, 1991). Moreover, treatment of NO results in a significant accumulation of SA in tobacco leaves (Durner *et al.*, 1998).

2.2.3. Oxylipins

Oxylipins is a collective name of diverse oxidized fatty acids derived from the lipoxygenase (LOX) pathway (Wasternack *et al.*, 2006). The LOX pathway can be divided into 9-LOX and 13-LOX pathways. The 9-lipoxygenase and 13-lipoxygenase catalyzed the oxygenation of linolenic acid at carbon atom 9 or 13 respectively leading to two groups of compounds (Blée, 2002; Feussner and Wasternack, 2002; Shah, 2005). In addition, each branch is further divided into several sub-branches with different enzymes participating. For example, the JA biosynthetic pathway is first catalyzed from 13-LOX,

followed by the allene oxide synthase (AOS) branch. The products of 13-LOX include fatty acid hydroperoxides, hydroxyl-, oxo-, and keto-fatty acids, divinyl ethers, volatile aldehydes, and the plant hormone JA (Grechkin, 1998). Oxylipins such as hydroperoxy, hydroxyl, and keto fatty acids accumulate in plants in response to pathogens attack and treatment with inducers of plant defence responses (Shah, 2005). In plants, oxylipins can act as signals to induce defence mechanisms in response to wounding, pathogen and pest attacks (Ryan and Pearce, 1998; Shah, 2005). Many oxylipins display cell toxicity and have antimicrobial effects (Prost, 2005; Rustérucci *et al.*, 1999). They also provide building units of physical barriers against pathogen invasion and regulate plant cell death (La Camara *et al.*, 2004; Shah, 2005). Recently, a study of the 9-lipoxygenase gene in *Arabidopsis thaliana* revealed the role of 9-LOX branch that participates in plant defence and developmental responses through the activation of specific signalling pathways (Vellosillo *et al.*, 2007).

2.2.4. Jasmonic acid

Jasmonates, a collective name of JA and its methyl ester, are key regulators in the development and physiology of plants and also associated with a wide range of plant defence responses (Dong, 1998; Szczegielniak *et al.*, 2005). In plant defence responses, JA acts as a wound hormone, especially against chewing insects and necrotrophic pathogens, which are able to induce resistance pathways and defence gene expression (Balbi and Devoto, 2008; Farmer *et al.*, 2003). Proteinase inhibitors, defence-related volatile compounds and secondary metabolites, such as nicotine, active phenolics and phytoalexins, have all been associated with jasmonate induction (Balbi and Devot, 2008; Farmer *et al.*, 2003). An endogenous increase of JA was demonstrated in cell suspension

cultures after elicitor treatment (Gundlach *et al.*, 1992; Mueller *et al.*, 1993). In *Arabidopsis*, application of JA enhances resistance against thrips feeding via JA-regulated defence responses (Abe *et al.*, 2008).

2.2.5. Ethylene

The phytohormone ET is a signal molecule for plant development, such as ripening and senescence, and in response to biotic and abiotic stimuli (Guo and Ecker, 2004; Wang *et al.*, 2002). Enhanced production of ET is an early response of plants after perception of pathogen attack and is related to the induction of defence reactions (Boller, 1991). It acts in concert with JA as a systemic signal of wound-induced gene activation (O'Donnell *et al.*, 1996). Although ET has no effect on defence-related callose deposition, it has been reported to be involved in several defence responses including xylem occlusions, cell wall-strengthening by the production of hydroxyproline-rich glycoproteins, phytoalexins and induction of PR proteins (Adie *et al.*, 2007; Ton and Mauch-Mani, 2004).

2.2.6. Salicylic acid

SA is a molecule naturally found in plants. Since the 4th century B.C., plants containing large quantities of salicylates, have been used medicinally for pain relief and anti-inflammatory reasons (Raskin, 1992a). In 1938, SA was named by Raffaele Piria after Johann Buchner (1928) isolated salicyl alcohol glucoside (Salicin) from the willow (*Salix helix*) bark (Raskin *et al.*, 1990). The derivative of SA, acetylsalicylic acid, which is well known as aspirin, is the world’s first synthetic drug and was produced in 1887 (Weissman, 1991). Afterwards aspirin and its derivatives became famous in the world.

Chemically, SA belongs to the group plant phenolics, which have an aromatic ring bearing a hydroxyl group and other functional derivatives (Figure 2.1). Free SA is a crystalline powder with a melting point of 157-159 ℃. It is po orly soluble in water (0.2 g 100 ml^{-1} H_2O at 20 ℃), but highly soluble in other polar organi c solvents, for example, methanol (Raskin, 1992b). A saturated aqueous solution of SA has a pH value of 2.4; pKa of 2.98 and log K_{OW} equal to 2.26 (Hsu and Kleier, 1990; Kleier, 1988).

SA is ubiquitously distributed throughout the whole plant kingdom, including 36 agronomically important species confirming the universal distribution of SA in plants (Raskin *et al.*, 1990). During the last couple of decades, the role of SA's diverse regulation in the metabolism of plants, has received more attention in botanical studies. In 1992, Raskin (1992b) classified SA as a group of plant hormones. SA acts by influencing plant growth, thermogenesis, flower induction, uptake of ions, stomatal movement and responses to abiotic stresses (Hayat *et al.*, 2007). The first researcher that mentioned the role of SA in plant defence responses was White (1979), who found the enhanced resistance to TMV in infected tobacco leaves after injection of aspirin. This discovery opened a new avenue for plant defence mechanisms. Up until now, it remains an important on-going research topic amongst signalling in plant defence responses.

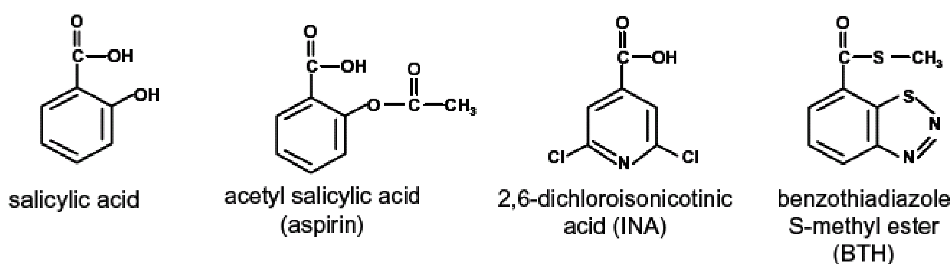

Figure 2.1 Salicylic acid and its synthetic functional analogs (adapted from Chaturvedi and Shah, 2007).

2.2.6.1 Biosynthesis of SA

SA in plants can be generated via two distinct enzymatic pathways. Both require the primary metabolite chorismate, an intermediate in shikimate pathway, operative in the synthesis of phenolic compounds. The following section gives a brief discussion of the recent information regarding these two SA biosynthesis pathways and its involvement in defence responses.

Phenylpropanoid-derived SA synthesis pathway (phenylalanine pathway)

Production of SA, starting from phenylalanine, occurs in the cytoplasm of a variety of plants such as tobacco and cucumber (Kawano and Furuichi, 2007). Phenylalanine ammonia lyase (PAL) is a key regulator of the phenylpropanoid pathway and is induced under a variety of biotic and abiotic stress conditions (Figure 2.2). In higher plants, SA biosynthesis via the phenylalanine pathway has been studied by isotope feeding experiments since the early 1960s (Coquoz *et al.*, 1998; Lee *et al.*, 1995). SA can be formed through two routes: from cinnamate via *o*-coumarate or benzoate. It depends on whether the hydroxylation of the aromatic ring takes place before or after the chain-shortening reactions. After PAL has converted phenylalanine into cinnamic acid, it can be further hydroxylated to form *o*-coumaric acid, followed by oxidation of the side chain (Coquoz *et al.*, 1998; Hayat *et al.*, 2007). The conversion of cinnamic acid to *o*-coumaric acid is catalyzed by cinnamate 4- hydroxylase (C4H) (Dixon *et al.*, 2002). However, the enzyme that activates the conversion of *o*-coumaric acid to SA has not yet been identified (Hayat *et al.*, 2007).

Alternatively, the side chain of cinnamic acid can be decarboxylated to generate benzoic acid undergoing hydroxylation at the *ortho* position to synthesise SA (Ellis and Amrhein, 1971). Furthermore, a benzoic acid-2-hydroxylase (BA2H), which converts benzoic acid to SA, is a key enzyme induced by TMV inoculation of tobacco leaves (León *et al.*, 1993; 1995b). In healthy young tomato seedlings, SA appeared to be formed mainly from cinnamate via benzoate. However, after infection with *Agrobacterium tumefaciens*, the pathway from cinnamate via *o*-coumarate was favoured (Chadha and Brown, 1974). Moreover, the study on tobacco suggested that SA is synthesized from cinnamate via benzoate both in healthy and TMV-infected tobacco leaves (Yalpani *et al.*, 1993). Similar results were obtained in rice, potato and cucumber (Coquoz *et al.*, 1998; Meuwly *et al.*, 1995; Silverman *et al.*, 1995).

In wheat, increased PAL activity has been reported after leaf-rust fungus infections (Southerton and Deverall, 1990). In a previous study, suppression of *PAL* gene expression in transgenic tobacco resulted in a lower level of SA accumulation after TMV inoculation than in non-transgenic plants (Pallas *et al.*, 1996). Application of a PAL inhibitior, 2-aminoindan-2-phosphonic acid, inhibits SA accumulation in pathogen-infected *Arabidopsis* and elicitor-treated potato (Coquoz *et al.*, 1998; Mauch-Mani and Slusarenko, 1996). These studies suggest that PAL plays an important role during the defence response in SA synthesis.

Isochorismate-derived SA synthesis pathway (Isochorismate pathway)

More recently, evidence showed that chorismate can also be converted into SA via isochorismate in a two-step process involving isochorismate synthase (ICS) and isochorismate pyruvate lyase (IPL) (Figure 2.2). In *Arabidopsis*, tobacco and potato, SA

could be produced even when the phenylalanine pathway is inhibited (Coquoz *et al.*, 1998; Mauch-Mani and Slusarenko, 1996; Yalpani *et al.*, 1993). Interestingly, some bacteria can synthesize SA from chorismate through two reactions catalyzed by ICS and IPL (Serino *et al.*, 1995). In the study of a *Catharanthus roseus* cell culture, the first plant ICS was isolated and this ICS was shown to be highly homologous to the bacterial ICS isozymes (van Tegelen *et al.*, 1999). Further approaches confirmed that this pathway exists in different species, such as *Arabidopsis*, tobacco and tomato (Catinot *et al.*, 2008; Uppalapati *et al.*, 2007; Wildermuth *et al.*, 2001). The studies show that the bulk of SA induced by pathogens or ozone stress derives from isochorismate pathway in *Arabidopsis* (Ogawa *et al.*, 2007; Wildermuth *et al.*, 2001). In contrast, studies show that the phenylalanine pathway is the main route for SA biosynthesis, and not the isochorismate pathway, as shown in TMV-infested tobacco and ozone fumigated tobacco (Ogawa *et al.*, 2005, 2006). Although SA synthesis from these two pathways has been identified, it seems that the involvement of different SA biosynthesis pathways depend on plant species and the type of the biotic and abiotic stress.

2.2.6.2 Metabolism of SA

In plants, SA is known to be converted into a number of molecules by glycosylation, esterification, methylation and amino acid conjugation (Figure 2.2). These molecules might be involved in various functions such as physiological roles and defence responses.

Salicylic acid glucosides

Most of the SA produced, is converted into SA glucosides, such as SA 2-*O*-β-glucoside

(SAG) and SA glucose ester (SGE) (Dean *et al.*, 2003; Edwards, 1994; Enyedi and Raskin, 1993; Lee and Raskin, 1998; Yalpani *et al.*, 1992). In potato leaves and cell cultures, the free SA and SAG contents are reportedly elevated following interaction with a pathogenic fungus and elicitors (chitosan and elicitors derived from a certain pathogen) (Keller *et al.*, 1996; Panina *et al.*, 2005). Similar evidence was obtained in tobacco, oat and soybean cell suspension cultures (Dean *et al.*, 2003; Lee and Raskin, 1998; Yalpani *et al.*, 1992).

In both virus- and bacteria- inoculated tobacco, SAG and SGE accumulated. However, the experimental results revealed that SGE is less stable compared to SAG and they suggested that SGE might be an intermediate in the formation of SAG (Lee and Raskin, 1998). Other studies also indicated that SA is mostly converted to SAG and less frequently to SGE (Lee *et al.*, 1995; Popova *et al.*, 1997). The possible functions of the glucosylation of SA in the defence mechanism might be detoxification of SA, i.e. protection of plants from the phytotoxic effects and regulation of free SA levels. In addition, SAG is actively transported from the cytosol into the vacuole, where it may function as an inactive storage form that can be converted back to SA, which may maintain SAR over extended periods of time (Dean and Mills, 2004; Dean *et al.*, 2005; Enyedi and Raskin, 1993; Lee and Raskin, 1998).

SA UDP-glucosyl transferase (SAGT) activity leading to the formation of SA glucosides has been detected in oat, tobacco, soybean, potato and *Arabidopsis* (Dean *et al.*, 2003; Enyedi and Raskin, 1993; Lee and Raskin, 1998; Song, 2006; Yalpani *et al.*, 1992). Biochemical studies indicated that increased SAGT activity is closely associated with enhancement of endogenous free SA levels during pathogen infection, elicitor treatment and exogenous SA application (Enyedi and Raskin, 1993; Panina *et al.*, 2005; Yalpani *et*

al., 1992). It is suggested that the high level of free SA can trigger SAGT. In addition, genetic evidence suggests that SAGT may participate in the early disease response by modulating SA metabolism during pathogenesis. In tobacco, the gene encoding *SAGT* was induced during bacterial and viral pathogen infections and it was also paralleled by an increased endogenous free SA level in inoculated tissue (Lee and Raskin, 1999). The *SAGT* gene, *AtSGT1* (*Arabidopsis thaliana* SA glucosyltransferase1), was characterized in *Arabidopsis* and was induced by bacterial infection (Song, 2006).

Methyl salicylate

The methylated form of SA (methyl salicylate, MeSA) is also an alternative storage form of SA (Shulaev *et al*., 1997). SA methyl- transferase (SAMT) is the enzyme that catalyzes the synthesis of MeSA from SA (Effmert *et al*., 2005). The gene, which encodes a methyltransferase that catalyzes MeSA synthesis, was also identified in *Arabidopsis* (Chen *et al*., 2003). MeSA could be hydrolyzed by esterases to release SA. Recently, the SA-binding protein 2 (SABP2) which has strong esterase activity with MeSA as substrate, was reported (Forouhar *et al*., 2005). SABP2-silenced tobacco plants failed to develop SAR after inoculation with TMV (Kumar and Klessig, 2003). As SAR development is dependent on SA, activation of SABP2 may regulate SA-dependent signalling in defence responses (Forouhar *et al*., 2005; Vlot *et al*., 2008). The study shows that MeSA triggers disease resistance and mediates the expression of defence related genes in neighbouring plants and in healthy tissue of infected plants (Shulaev *et al*., 1997). Similar evidence of MeSA accumulation is provided from transgenic *Arabidopsis* plants overexpressing OsBSMT1 (*Oryza sativa* SA/benzoic acid carboxyl methyltransferase 1), which encodes SAMT in rice. However, pathogen infection of transgenic plants resulted in increased susceptibility and reduced accumulation of SA, SAG and PR1 compared to the wild-type

plants. The OsBSMT1 overexpressors triggered *PR1* induction in neighbouring wild-type plants (Koo *et al.*, 2007). It is suggested that MeSA is ineffective in inducing a defence response, but can function as a mobile or volatile signalling molecule inducing defence responses of remote tissue (Chen *et al.*, 2003; Loake and Grant, 2007; Shulaev *et al.*, 1997; Song *et al.*, 2008).

SA amino acid conjugation

Twenty years ago, SA amino acid conjugates, such as N-salicyloyl aspartic acid, was identified in wild grapes, some of grape cross-bred hybrids and French beans (Bourne *et al.*, 1991; Steffan *et al.*, 1988). The role of SA amino acid conjugates recently emerged as bioactive inducers of defence responses. SA amino acid synthase might be identified in the study of the *Arabidopsis pbs3* mutants (Nobuta *et al.*, 2007; Staswick *et al.*, 2005). PBS3 is a member of the acyl-adenylate/ thioester-forming enzyme family (also known as GH3 proteins), which adenylates the plant hormones auxin, indoleacetic acid (IAA), JA and SA into amino acid conjugates (Jagadeeswaran *et al.*, 2007; Staswick *et al.*, 2005). The amino acid conjugates of plant hormones play an important role in phytohormone regulation by activating or inactivating their functions (Wildermuth, 2006). A study on an *Arabidopsis pbs3*-1 mutant showed a reduction of SA accumulation and subsequent pathogen resistance. Nobuta *et al.* (2007) also indicated that induction of PBS3 by pathogen infestation is highly correlated with the expression of *ICS1* gene**.** Exogenous SA or its functional analogs application rescued the compromised resistance phenotypes of these mutants. This indicated that PBS3 might act upstream of SA in defence signalling (Jagadeeswaran *et al.*, 2007; Nobuta *et al.*, 2007). In addition, PBS3 plays a role in both basal and *R* gene-mediated defence responses (Nobuta *et al.*, 2007). On the other hand,

Okrent *et al*. (2009) found that SA is not the favoured substrate of PBS3, but instead 4-substituted benzoates. Furthermore, SA specifically and reversibly inhibits PBS3 activity. Therefore, they proposed that PBS3's product, 4-hydroxybenzoate-glutamic acid, might induce or prime SA biosynthesis, with SA feedback inhibiting PBS3's activity and thereby modulating its own synthesis (Okrent *et al*., 2009).

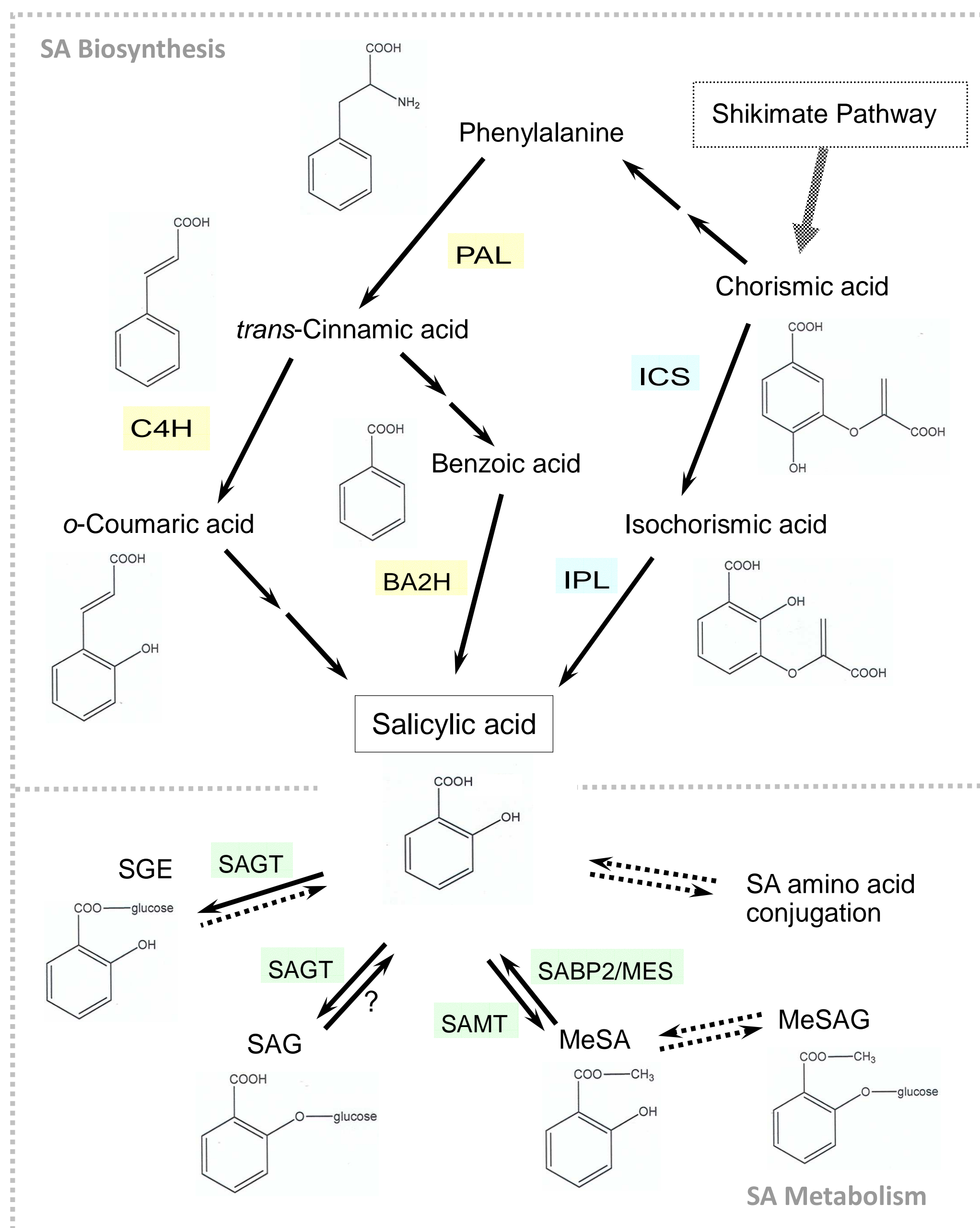

Figure 2.2 Simplified schematic representation of pathways for SA biosynthesis and metabolism (Modified from Métraux, 2002 and Vlot *et al*., 2009). *Abbreviations: PAL, phenylalanine ammonia lyase; C4H, cinnamate-4-hydroxylase; BA2H, benzoic acid-2-hydroxylase; ICS, isochorismate synthase; IPL, isochorismate pyruvate lyase; SA, salicylic acid; SGE, SA glucose ester; SAG, SA 2-O-β-glucoside; MeSA, methyl salicylate; MeSAG, methyl salicylate O-β-glucoside; SAGT, SA glucosyltransferase; SAMT, SA methyltransferase; SABP2, SA-binding protein 2; MES, methyl esterase.*

2.2.6.3 SA signal transduction in defence responses

Downstream of SA signal transduction include SA-protein and protein-protein interactions as well as genetic interactions (Volt *et al.*, 2009). SA-binding proteins (SABPs) are receptors for SA. To date, three types of SABP have been identified. The first SABP isolated from tobacco was identified as a CAT (Chen *et al.*, 1993a, 1993b). SA inhibits CAT activity by binding its active site (heme-iron) which results in the inhibition of H_2O_2 degradation (Durner and Klessig, 1995). The increase in H_2O_2 was proposed to activate defence gene expression or act as an antimicrobial barrier at the site of pathogen invasion (Chen *et al.*, 1993b). In addition, SA also binds to APX, another H_2O_2-scavenging enzyme (Durner and Klessig, 1995).

Another SABP, SABP2, with the highest affinity for SA was found in tobacco which is associated with TMV-induced SAR development (Du and Klessig, 1997). SABP2 has an esterase and a SA-inducible lipase activity. When SA binds to the active site of SABP2, the feedback inhibits SABP2's esterase activity and enables MeSA accumulation in the infected site and subsequent transportation to the uninfected site (Forouhar *et al.*, 2005). On the other hand, MeSA is also a substrate with a high binding activity for SABP2 which can convert MeSA to SA (Forouhar *et al.*, 2005; Seskar *et al.*, 1998).

SABP3 was identified as a chloroplast carbonic anhydrase which has antioxidative activity as well (Slaymaker *et al.*, 2002). This study also showed the role of carbonic anhydrase in plant defence by silencing carbonic anhydrase gene in *Nicotiana benthamiana,* it suppressed the HR in a race-specific plant-pathogen interaction (Slaymaker *et al.*, 2002). Carbonic anhydrase was found to contain S-nitrosylate activity which can suppress its SA binding and enzymatic activities indicating that S-nitrosylation could be part of a negative

feedback loop for modulating the defence response (Wang *et al*., 2009).

The Non-expressor of PR1 (NPR1) plays an important role in SA signal transduction and it acts as a co-regulator of gene expression (Dong, 2004; Pieterse and van Loon, 2004). It acts downstream from SA and activates the expression of PR-1. A study of *npr1* mutants showed enhanced disease symptoms after pathogen infection. In addition, SA treatment could not activate *PR* genes of *npr1* mutant plants and was also unable to mount effective SAR responses (Pieterse *et al*., 1998). Under normal conditions, NPR1 is present in the cytoplasm when the level of SA is low (Mou *et al*., 2003). When the SA level increases, monomers of disulfide-connected NPR1 oligomers are formed (Mou *et al*., 2003). The monomers are subsequently translocated from the cytosol into the nucleus, where they interact with TGA transcription factors (Dong, 2004). In plant cells, interaction between NPR1 and TGA1 and TGA4 was detected upon SA treatment of leaves (Durrant and Dong, 2004). It is suggested that TGA1 and TGA4 form part of the signalling cascade for SA-induced PR expression (Despres *et al*., 2003; Durrant and Dong, 2004).

MATERIALS AND METHODS

3.1. Plant material

The wheat (*Triticum aestivum*) cultivar, Tugela, is susceptible to both biotypes of the Russian wheat aphid (RWASA1 and RWASA2) (*Diuraphis noxia* Kurdjumov). Two resistant wheat cultivars were used i.e. Tugela DN, containing the *Dn1* resistance gene which provides resistance to biotype RWASA1; and PAN 3144, with the resistance gene *Dn5* providing resistance to biotype RWASA1 and RWASA2 (du Toit, 1989; du Toit, 1992; Tolmay *et al.*, 2007). Both RWA biotypes were supplied by the ARC-SGI, Bethlehem, South Africa. Seeds were pre-germinated and planted in a peat and red soil (1:1) mixture. Wheat plants were grown under controlled conditions in the greenhouse at day and night temperatures ~24 ^{o}C and ~14 ^{o}C, respectively. Plants were infested at the early third leaf stage (about two to three weeks after planting) by gently brushing approximately 20 RWAs onto the leaves. All plants were placed in cages, covered with sterilised nets, to prevent the aphids from escaping. A set of plants was left uninfested as control.

The leaves were collected at specific time intervals after aphid infestation [0, 4, 8, 12, 24, 48, 72, 96 and 120 hours post infestation (h.p.i)]. For the LOX activity assay the following time intervals were used: 0, 3, 6, 9, 12, 24, 48, 72 and 96 h.p.i. A half hour period was given for the aphids to settle on the plants, after which the infestation time lapse started. At each sampling time, aphids were removed from randomly selected second and third leaves of the wheat plants. The leaves were collected and immediately frozen in liquid nitrogen. Leaf samples were subsequently stored at -20 ^{o}C and -80 ^{o}C for further assay

purposes for all experiments.

3.2. Chemicals

All chemicals used were of analytical grade or liquid chromatographic grade where applicable.

3.3. Assay of salicylic acid (SA) content

3.3.1. Extraction of SA

The procedure used for SA extraction was slightly modified from the method described by (Verberne *et al.*, 2002). Leaf material (0.5 g) was ground in liquid nitrogen using a mortar and pestle and homogenised in 1 ml pre-cooled 90% (v/v) methanol. The extraction mixtures were vortexed for 1 min and subsequently sonicated for 5 min before centrifugation for 5 min (8,000 g, 4 ^{o}C) in a micro-centrifuge (Hettich D-7200). The supernatant was collected in 2 ml eppendorf tubes. The pellet was re-suspended in 0.5 ml pre-cooled 100% methanol, and the sonication and centrifugation steps repeated. The supernatants were combined. After adding 10 µl of 0.2 M sodium hydroxide (NaOH) to the pooled supernatant, it was mixed well and evaporated in the rotavapor (BÜCHI) under vacuum at 45 ^{o}C. The dried sample was resuspended in 250 µl of 5% (w/v) trichloroacetic acid (TCA) and extracted twice with 800 µl ethyl acetate: cyclohexane (1:1, v/v). To the combined extracts (top phase), 60 µl of 0.2 M sodium acetate buffer (pH 5.5) was added. Samples were mixed and gently evaporated to dryness at 45 ^{o}C, under vacuum. The residue was resuspended in 600 µl of HPLC mobile phase [40% (v/v) methanol with 1% (v/v) phosphoric acid] and centrifuged for 5 min. After filtering with a syringe equipped with

a microfilter (0.45 µm GHP Acrodisc, Separations), the sample was ready for high performance liquid chromatography (HPLC) analysis to determine free SA content.

To measure conjugated SA levels, the leftover TCA fraction was subjected to acid hydrolysis by adding 300 µl of 8 M hydrogen chloride (HCl). The sample was mixed well and incubated in a water bath at 80 ^{o}C for 1 hour. After that the acid fraction was partitioned again with the ethyl acetate:cyclohexane mixture and the procedure was continued as described above. The sample which now contains free SA released from the conjugated SA during hydrolysis was used for the determination of the conjugated SA content. It should be noted, to prevent SA loss during evaporation procedure, 0.2 M NaOH and 0.2 M sodium acetate buffer (pH 5.5) were added.

3.3.2. High performance liquid chromatography (HPLC) analysis of SA

The HPLC system was equipped with a C_{18} reverse phase column (Luna 5 µm C_{18} 150 x 4.60 mm, Phenomenex, Separations). The mobile phase consisted of 40% (v/v) methanol in water and 1% (v/v) phosphoric acid. The flow rate was 0.7 ml min^{-1} and the column temperature was 40 ^{o}C. SA was detected at 240 nm. The content of SA was calculated from SA standard curve which was prepared by analysing different concentrations of SA (in a range of 0.005 to 0.1 mM) in 40% (v/v) methanol in the same HPLC system. SA standard solution was also added into free and conjugated SA extracts separately to distinguish the SA peak. SA content was expressed as µg SA g^{-1} fresh leaves.

3.4. Assay of phenylalanine ammonia-lyase (PAL) activity

PAL enzyme activity was measured as reported by Arz and Grambow (1995) with minor modifications. Plant material (0.5 g) was ground with a pre-cooled mortar and pestle [containing 60 mg regenerated Dowex, 60 mg polyvinylpyrrolidone (PVP) and 60 mg acid washed sea-sand] and homogenized in 5 ml sodium borate buffer, pH 8.8 [containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM dichlorodiphenyltrichloroethane (DTT)]. The extract was centrifuged at 15,000 g for 20 min at 4 ^{o}C. The supernatant was collected for the enzyme activity assay.

For the enzyme activity assay, the reaction mixture consisted of 400 µl enzyme extract and 500 µl sodium borate buffer (pH 8.8, containing 1 mM DTT and 1 mM EDTA). After incubation at 40 ^{o}C for 2 min, 100 µl 60 mM L-phenylalanine was added to start the reaction. The blank consisted of 900 µl sodium borate buffer and 100 µl 60 mM L-phenylalanine. Change in absorbancy was measured at 290 nm after 20 min with a double beam spectrophotometer (Cary 100 Bio UV-Visible spectrophotometer, Varian). PAL activity was calculated using a calibration curve of cinnamic acid (CA), which was in a concentration range from 0.1 to 10 µg ml^{-1}. Enzyme activity was expressed as nmol CA mg^{-1} protein min^{-1}.

For Dowex [1x2 (200-400) anion exchange resin] regeneration, 5 g Dowex was boiled and stirred in 500 ml 2x distilled water for 30 min. After cooling down, the solution was filtered by suction through a sintered glass funnel. The pellet was then transferred into 500 ml 0.5 M HCl and stirred for 30 min at room temperature. After the treatment, it was filtered and rinsed with 2x distilled water (500 ml) before transferring to the next solution. Afterwards, the pellet was transferred into 500 ml 0.5 M NaOH and 500 ml 0.5 M sodium chloride

(NaCl) consecutively, wash and rinse steps were repeated. In the final step, the pellet was stirred twice in 0.1 M sodium borate buffer (pH 8.8) and the regenerated Dowex was stored in 0.1 M sodium borate buffer (pH 8.8) at 4 ^{o}C. Before it was used for enzyme extraction, the regenerated Dowex suspension was filtered and 100 ml 0.1 M sodium borate buffer (pH 8.8), containing 1 mM DTT and 1 mM EDTA, was added to resuspend 1 g regenerated Dowex by stirring for 30 min at room temperature.

3.5. Assay of lipoxygenase (LOX) activity

Extraction was performed using a modified method of Bohland *et al.* (1997). Frozen leaves (1 g) were ground using a pestle and mortar at 4 ^{o}C in 6 ml of 100 mM potassium phosphate buffer (pH 7.5) [containing 1 mM EDTA, 100 mg acid washed sea-sand and 1% (m/v) insoluble PVP]. After centrifugation (25,000 g for 15 min at 4 ^{o}C), the supernatant was used for LOX assay.

The reaction mixture of LOX activity assay consisted of 1 ml 0.1 M sodium citrate phosphate buffer (pH 6.2), 50 µl of enzyme extract and 150 µl of 2.5 mM linoleic acid (in 0.15% Tween20). The change in absorbance was measured at 234 nm for 15 min at 30 ^{o}C using a spectrophotometer. The specific LOX activity was expressed as nmol HPOD (hydroperoxy octadecanoic acid) mg^{-1} protein min^{-1}.

The preparation of the linoleic acid substrate was done according to (Ocampo *et al.*, 1986). Linoleic acid (400 µl), 768 µl Tween20 and 40 ml methanol were added into a round - bottomed flask and evaporated to dryness. The residue was resuspended in 500 ml 0.05 M sodium phosphate buffer (pH 9). The substrate was divided into smaller volumes and stored in air tight bottles at -20 ^{o}C. During the transfer of the substrate,

nitrogen gas was bubbled into the round-bottomed flask and small bottles to prevent air contact. The substrate was kept on ice during experiments, and discarded afterwards (do not store and reuse).

3.6. Assay of catalase (CAT) activity

The procedure for CAT extraction was modified from the method described by Gong *et al.* (2000). Frozen leaf material (0.5 g) was ground in a pre-cooled pestle and mortar on ice in 5 ml extraction buffer [0.1 M Tris-HCl buffer (pH 8.5) containing 2 mM EDTA, 10% (w/v) PVP and 10% (v/v) glycerol]. The homogenate was centrifuged (15,000 g at 4 ^{o}C) for 15 min. A suitable dilution of the supernatant was made for the enzyme activity assay and the residues were stored at -20 ^{o}C.

CAT activity was determined by means of the disappearance of H_2O_2 in the enzyme reaction mixture. The reaction mixture consisted of 10 µl diluted enzyme extract, 765 µl assay buffer (50 mM Tris-HCl buffer, pH 6.8), 100 µl 25 mM H_2O_2 and 125 µl titanium reagent (20% titanium tetrachloride in concentrated HCl, v/v). The reaction proceeded for 10 min at room temperature and was terminated by adding 125 µl of titanium reagent. For the time zero sample, 125 µl of titanium reagent was added before adding the enzyme extract to the assay mixture. The absorbance was measured at 415 nm in a spectrophotometer. The difference in absorbance at 10 min and time zero was calculated. The consumption rate of H_2O_2 was calculated using a calibration curve of H_2O_2 in a concentration range from 0.25 to 1.25 mM. CAT activity is expressed as enzyme units mg^{-1} protein (1 unit is defined as 1 mM H_2O_2 consumed per min).

3.7. Determination of protein content

Protein concentration was determined using the dye-binding assay technique according to Bradford (1976) and measuring the absorbance on a Bio-Rad Microplate Reader (model 3550) at 595 nm. The assay mixture consisted of 150 µl distilled water, 40 µl BioRAD reagent and 10 µl enzyme extract. For the protein standard 10 µl of a 0.5 µg $µl^{-1}$ γ-globulin solution was used.

3.8. Reverse transcriptase quantitative polymerase chain (RT-qPCR) reaction

3.8.1. Extraction of total RNA

Frozen leaves were ground to a fine powder in liquid nitrogen and transferred to a 1.5 ml eppendorf tube (up to a 100 µl mark). To the ground tissue, 500 µl Trizol® Reagent (Invitrogen™) was added and vortexed for 10 sec before incubation at room temperature for 10 min. After the incubation, 100 µl chloroform was added and the tubes were inverted 15 times. After incubation at room temperature for 5 min, samples were centrifuged, at 12,000 g for 15 min at 4 ^{o}C. The cleared supernatant (250 µl) was transferred to a new eppendorf tube containing 250 µl isopropanol for precipitating the RNA. After incubation at room temperature for 10 min, the tubes were centrifuged to pellet the RNA (12,000 g for 10 min, 4 ^{o}C). The supernatant was removed by water jet pump suction and 500 µl 70% ethanol (v/v) was added to wash out the salts from the remaining pellet. After centrifugation at 7,500 g for 10 min at 4 ^{o}C, the supernatant was completely removed and the sample was left for 5 minutes to dry. The dried RNA was finally dissolved in 200 µl diethyl pyrocarbonate (DEPC) treated water. After incubation on ice or in the fridge for 1 hour or overnight, the tubes were centrifuged (12,000 g for 5 min, 4 ^{o}C) to pellet

undissolved RNA. The supernatant (RNA) was transferred to a new tube. For DNase treatment, 5 U DNase I (Fermentas) in 10 mM Tris-HCl (pH 7.5) and 2.5 mM magnesium chloride ($MgCl_2$) were added to the RNA. After incubation at 37 ^oC for 30 min, 5 mM EDTA was added to stop the reaction and samples were incubated at 65 ^oC for 10 min. The chloroform extraction procedure was repeated and RNA was precipitated as previously described. All solutions used for RNA extraction were prepared using DEPC treated water (0.1% DEPC, v/v) to ensure that it was RNase-free. The treated water was left overnight and autoclaved to destroy the DEPC.

3.8.2. RNA concentration

Each RNA sample was diluted 50 times with DEPC water for concentration determination. The absorbance was measured spectrophotometrically at 260 and 280 nm.

To determine the quality of the RNA, it was separated on a 1.0% (w/v) denaturing agarose gel containing 0.41 M formaldehyde. For the running buffer, a 1x MOPS [3-(N-morpholino) - propanesulfonic acid] buffer was used, which was diluted from the stock 10x MOPS [200 mM MOPS, 50 mM sodium acetate, pH8, 0.5 mM EDTA and NaOH] solution. The RNA (100 ng) was dissolved in 18 µl RNA buffer [500 mM MOPS, 50% (v/v) formamide, 6.5% (v/v) formaldehyde and 50 µg ml^{-1} ethidium bromide (EtBr)] and denatured at 65 ^oC for 15 min, then cooled on ice. Before loading the RNA samples on the gel, 2 µl 10x RNA loading buffer [50 % (v/v) glycerol, 1 mM EDTA pH 8.0, 0.25% (w/v) bromophenol blue] was added and well mixed. The separation was performed at 10 $V.cm^{-1}$ for 60 min and the gel photographed using the Bio-Rad gel documentation system.

3.8.3. RT-qPCR analysis

RT-qPCR analysis was performed using the CFX96 (Bio-Rad) machine and the iScript™ One-Step RT-PCR kit with SYBR® Green (Bio-Rad). For one RT-qPCR analysis in a 96-well PCR plate (Bio-Rad), a 5-fold dilution series (from 100 ng total RNA to 0.0064 ng) was used for all standard curve analysis while 10 ng of total RNA was used for all the other reactions. All the reactions were done in triplicate. Each RT-qPCR reaction consisted of 5 µl 2x SYBR Green RT-PCR reaction mix (Bio-Rad), 1 µl of each primer pair (10 pmol μl^{-1} in stock) and 0.2 µl iScript reverse transcriptase, 1.3 µl nuclease-free water and 2.5 µl total RNA (4 ng μl^{-1} in stock). The RT-qPCR protocol was as follows: an initial 50 °C for 10 min RT step, a denaturing step at 95 °C for 5 min, 40 cycles of 95 °C for 10 sec and 30 sec at the specific annealing temperature and lastly a melting curve from 65 °C increasing every 5 sec by 0.5 °C to 95 °C. The primer pairs used in this study are listed in Table 3.1. All primer pairs were optimized for RT-qPCR analysis.

Calculation of the relative quantification of the target gene was according to the mathematical model of Pfaffl (2001):

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta CP_{\text{target}}(\text{control-sample})}}{(E_{\text{reference}})^{\Delta CP_{\text{reference}}(\text{control-sample})}}$$

E_{target} represents the reaction efficiency of target gene transcript and $E_{reference}$ represents the reaction efficiency of reference gene transcript. The equation for the reaction efficiency (E) was $E = 10^{(-1/slope)}$ (Rasmussen, 2001). The value of slope is from the standard curve of each reaction. The crossing point (CP, also called quantification cycle,

Cq) was defined as the point at which the fluorescence rises obviously above the background fluorescence. The fluorescence threshold needed for the calculations was the default value by the CFX96's analytical software. The relative expression ratio of a target gene (*ICS* and *SAGT*) is calculated based on *E* and the CP deviation (ΔCP) of an unknown sample versus a control sample ($CP_{control}$ - CP_{sample}). In this experiment, the sample of 0 h.p.i was used as control. The relative expression levels of target gene were obtained in comparison to a reference gene (*GAPDH*).

3.9. Processing of results

For all assays, at least two separate experiments (planting sets) were conducted. Within each experiment, determinations or assays were done in triplicate. The plotted values represented the average of the triplicate values. Standard deviations were calculated for each data point (n=3). The data were analyzed using Sigma Plot 2001 version 7.0 and Microsoft Office Excel 2003 software.

Table 3.1 A list of the target (*ICS* and *SAGT)* and reference (*GAPDH*) genes and their primer sequences for RT-qPCR analysis.

Gene	Primer sequences	Tm
Reference gene:		
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	5'-CAACGCTAGCTGCACCACTAACT-3' 3'-GACTCCTCCTTGATAGCAGCCTT-5'	60 °C
Target gene:		
Isochorismate synthase (ICS)	5'-GGCACCGGCATAAACTAAAG-3' 3'-ACAGTTTGAGCAGCAGGAGT-5'	60.4 °C
Salicylic acid UDP-glucosyl transferase (SAGT)	5'-ATGGGAAACAAGACGAATGC-3' 3'-ACATGCGAGAAAGTACCGTC-5'	60 °C

RESULTS

4.1. Effect of RWASA1 and RWASA2 infestation on the salicylic acid (SA) content

Figure 4.1 represents HPLC chromatographs showing the separation of SA from a standard solution containing authentic SA (Fig 4.1 a), wheat plant extracts for free SA (Fig 4.1 b) and conjugated SA (Fig 4.1 c) respectively. A satisfactory resolution was obtained. Retention time of the eluting SA is shown in each case. Identification of SA in the plant extracts was done by co-injection of authentic SA.

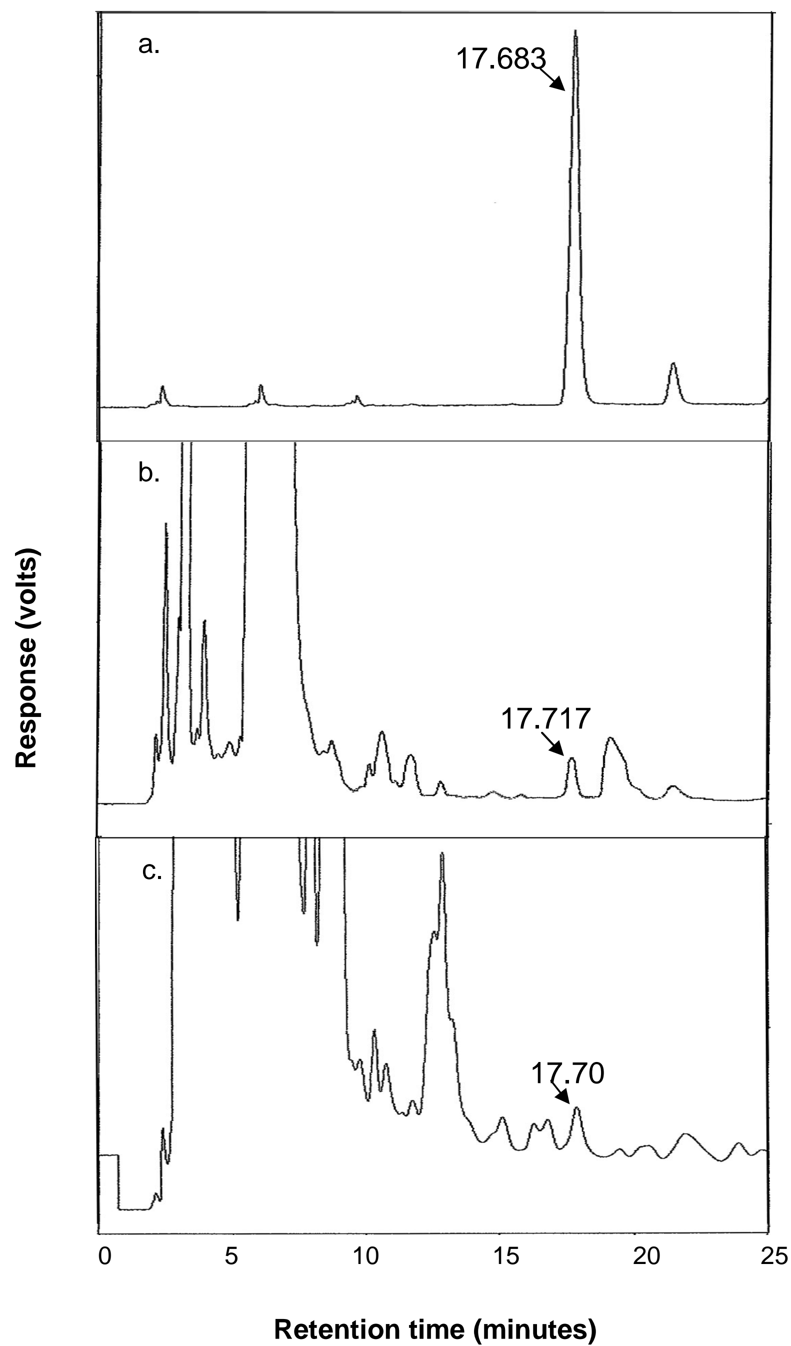

Figure 4.1 HPLC chromatograph of SA. (a) SA standard, (b) wheat plant extract for free SA and (c) wheat plant extract for conjugated SA. The retention times of the SA peaks are indicated.

In order to compare the effect of RWASA1 and RWASA2 infestation on different cultivars directly, results of free, conjugated and total SA contents were presented separately (Figs 4.2, 4.3 and 4.4). In the susceptible cultivar (Tugela), there were relative small changes in the free SA contents of uninfested and infested plants (Fig 4.2 a). In RWASA1 infested Tugela DN, free SA started to accumulated after 24 hours of infestation and peaked (6-fold increase compared to uninfested control) at 96 h.p.i (Fig 4.2 b). In RWASA2 infested Tugela DN, a later accumulation of free SA was observed at 96 h.p.i, with approximately 3.8-fold higher (120 h.p.i) than uninfested plants (Fig 4.2 b). RWASA1 and RWASA2 infestation of the resistant cultivar, PAN 3144, resulted in a continuous increase after 24 h of infestation in free SA content at a similar level. RWASA1 and RWASA2 infestation resulted in an 11-fold and a 9-fold higher increase (120 h.p.i) than in uninfested plants respectively (Fig 4.2 c). In uninfested plants of both resistant cultivars, Tugela DN and PAN 3144, there were no significant changes in free SA contents (Fig 4.2 b and c).

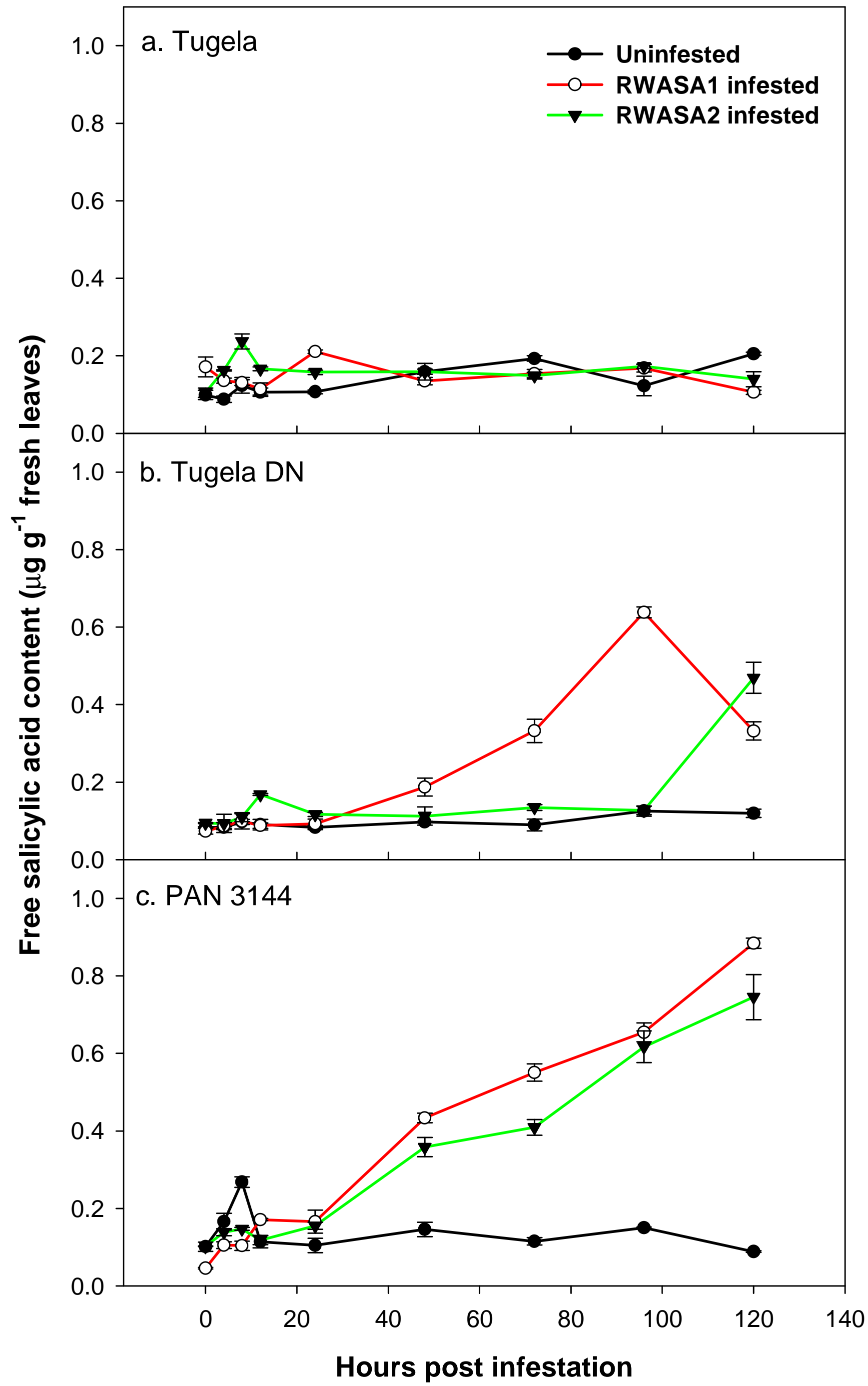

Figure 4.2 Effect of RWASA1 and RWASA2 infestation on the free SA content of susceptible and resistant wheat cultivars. (a) Tugela, (b) Tugela DN and (c) PAN 3144. Values are means ± SD (n=3).

There were no significant differences in the conjugated SA contents of the infested and uninfested susceptible cultivar (Fig 4.3 a). In RWASA1 infested resistant wheat cultivar, Tugela DN, conjugated SA started increasing at 48 h.p.i (Fig 4.3 b). In contrast, RWASA2 infestation of Tugela DN resulted in a later (72 h.p.i) induction of conjugated SA production (Fig 4.3 b). At 120 h.p.i, the conjugated SA level of RWASA1 infested Tugela DN was much higher than that of RWASA2 infested Tugela DN, representing a 4.5-fold and an 1.9-fold increase, respectively (Fig 4.3 b). In resistant PAN 3144, an early increase of conjugated SA content was observed after RWASA1 infestation (12 h.p.i) and after RWASA2 infestation (24 h.p.i). At 120 h.p.i, a 3.1-fold and a 2-fold increase for RWASA1 and RWASA2 infested Tugela DN were obtained (Fig 4.3 c). In both resistant cultivars, there were no significant changes in conjugated SA content in uninfested plants (Fig 4.3 b and c).

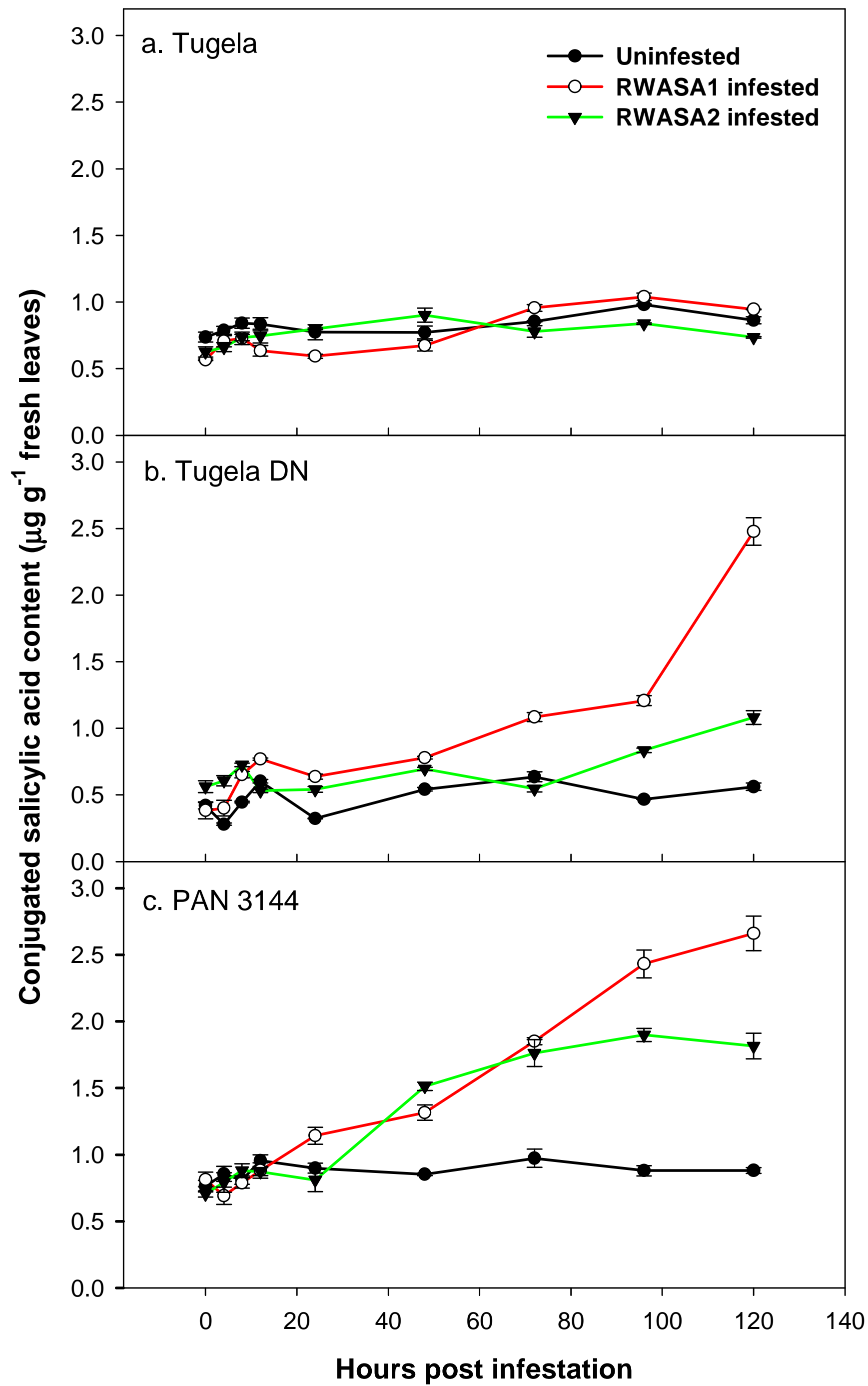

Figure 4.3 Effect of RWASA1 and RWASA2 infestation on the conjugated SA content of susceptible and resistant wheat cultivars. (a) Tugela, (b) Tugela DN and (c) PAN 3144. Values are means ± SD (n=3).

Total SA is the total of free and conjugated SA. In uninfested and infested susceptible Tugela, the total SA content remained relatively unchanged throughout the investigation period (Fig 4.4 a). In RWASA1 infested resistant Tugela DN, the total SA content started to increase after 48 h.p.i and it eventually resulted in a 3.2-fold higher content (120 h.p.i) when compared to the uninfested plants (Fig 4.4 b). A later increase in total SA content was observed in the RWASA2 infested Tugela DN and at 120 h.p.i, the total SA content was 1.6-fold higher than in the uninfested plants (Fig 4.4 b). RWASA1 infestation of resistant PAN 3144 resulted in a slightly earlier (12 h.p.i) increase in total SA than RWASA2 infestation (24 h.p.i). At 120 h.p.i, the total SA of RWASA1 and RWASA2 infested plants were respectively 3.4-fold and 2.5-fold higher than uninfested plants (Fig 4.4 c). Throughout the experiment, low levels of total SA were always associated with uninfested plants in the first instance and secondly with infested susceptible plants (Fig 4.4).

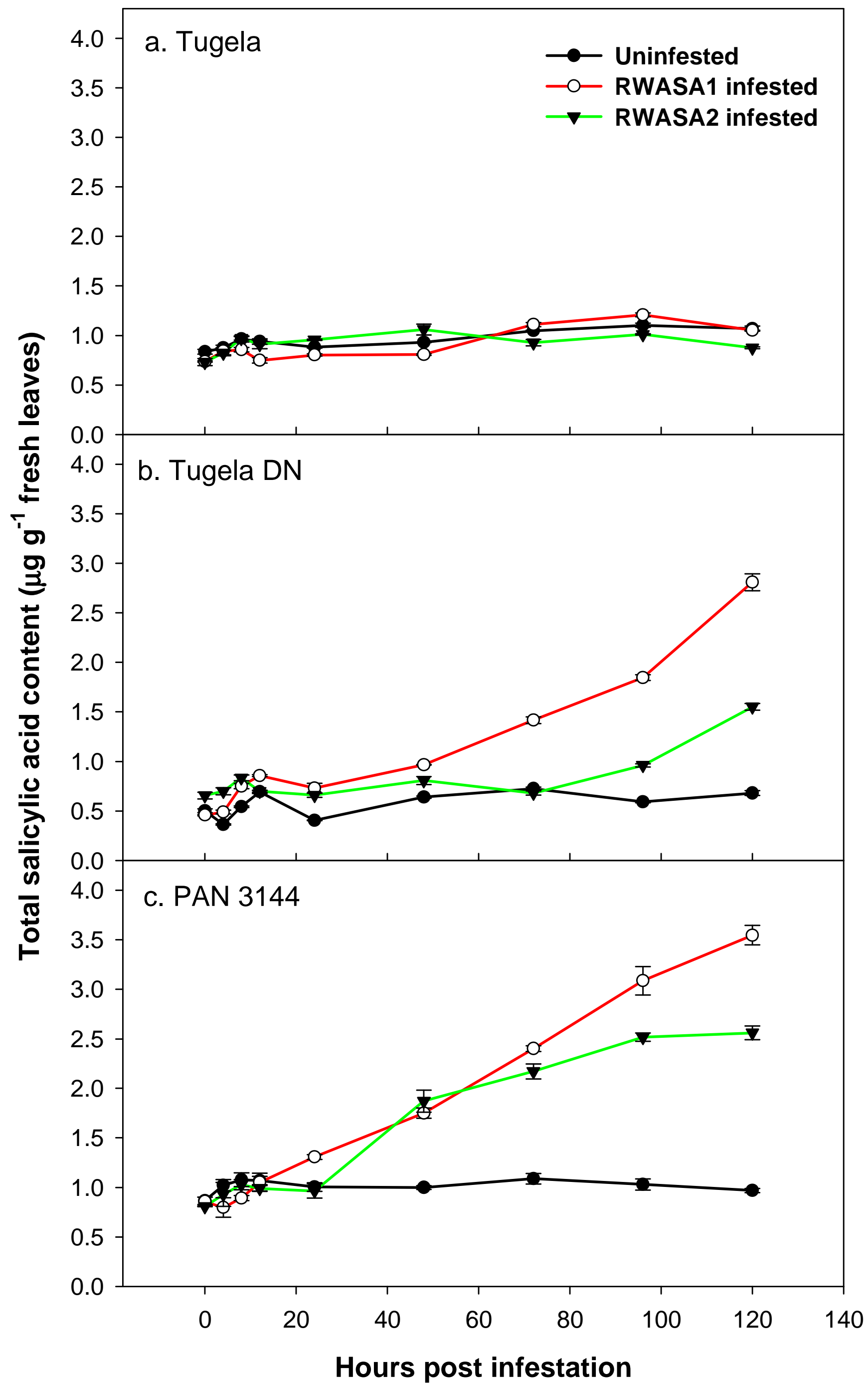

Figure 4.4 Effect of RWASA1 and RWASA2 infestation on the total SA content of susceptible and resistant wheat cultivars. (a) Tugela, (b) Tugela DN and (c) PAN 3144. Values are means ± SD (n=3).

Figures 4.5 to 4.7 depict the free, conjugated and total SA contents for cultivars Tugela, Tugela DN and PAN 3144 during infestation with RWASA1 and RWASA2. It is anticipated that the relation with regard to changes in the different cultivars and the different forms of SA is better illustrated in this way.

Relative small changes in the free, conjugated and hence total SA contents occurred in the uninfested susceptible cultivar, Tugela, during the course of the experiment (Fig 4.5 a). A small increase in the conjugated and hence total SA contents was observed after 48 hours of RWASA1 infestation of Tugela (Fig 4.5 b). RWASA2 infestation of the same cultivar resulted in an even lower increase 48 h.p.i (Fig 4.5 c). It should be noted that in the susceptible cultivar under conditions of infestation and non-infestation very small transient increases appeared to occur at 12 h.p.i (Fig 4.5). The SA (conjugated and free) content of uninfested Tugela DN was relatively unaffected during the course of the experiment (Fig 4.6 a). Both free and conjugated SA contents increased to much higher levels in the resistant cultivar, Tugela DN, upon RWASA1 infestation (Fig 4.6 b) than in the susceptible cultivar, Tugela (Fig 4.5 b). This was accompanied by a continual increase in the total SA content, but the free SA content peaked at 96 h.p.i (Fig 4.6 b). RWASA2 infestation of Tugela DN resulted in a much lower SA content increase than RWASA1 infestation (Fig 4.6 c). The small transient SA content increases at 12 h.p.i, especially conjugated and total SA, were also observed for Tugela DN under conditions of infestation and non-infestation (Fig 4.6). In uninfested resistant cultivar, PAN 3144, both free, conjugated and hence total SA contents remained relatively constant during the course of the experiment (Fig 4.7 a). The SA (conjugated and free) content of the infested PAN 3144 was induced to higher levels than infested resistant Tugela DN in both instances of RWASA1 and RWASA2 infestation and induction also occurred earlier (Fig 4.7 b and c). It should be noted that induction was at a higher level after RWASA1 infestation than after

RWASA2 infestation (Fig 4.7 b and c). Again small transient increases in SA content occurred at 12 h.p.i under conditions of infestation and non-infestation (Fig 4.7).

The results of independent replicated experiments on SA contents are shown in Appendix 1 (Figs 7.1 to 7.6). Results of replicate experiments showed similar tendencies than those found above.

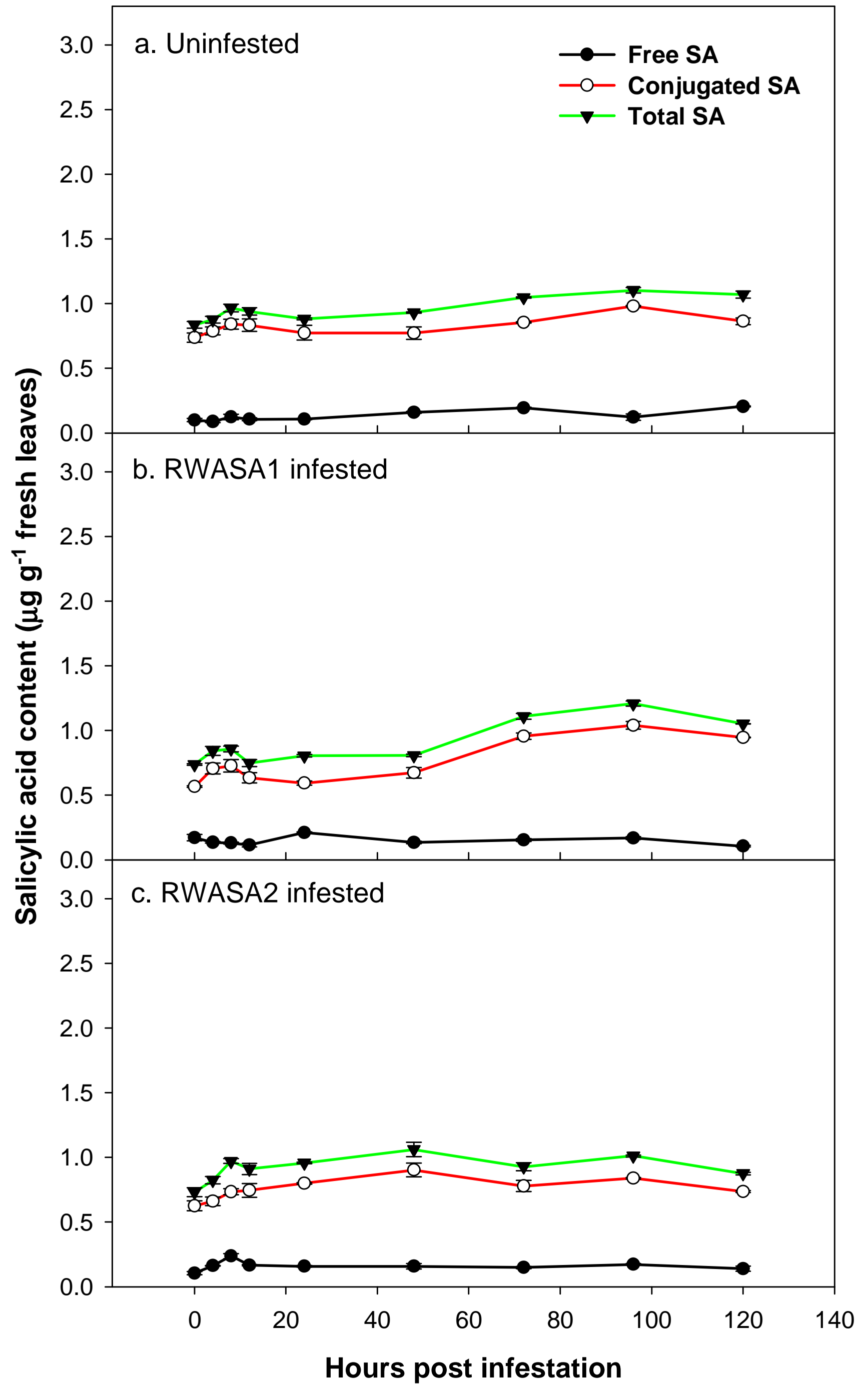

Figure 4.5 Effect of RWASA1 and RWASA2 infestation on the SA content of the susceptible (Tugela) wheat cultivar. Uninfested (a) and RWASA1 infested (b) and RWASA2 infested (c). Values are means ± SD (n=3).

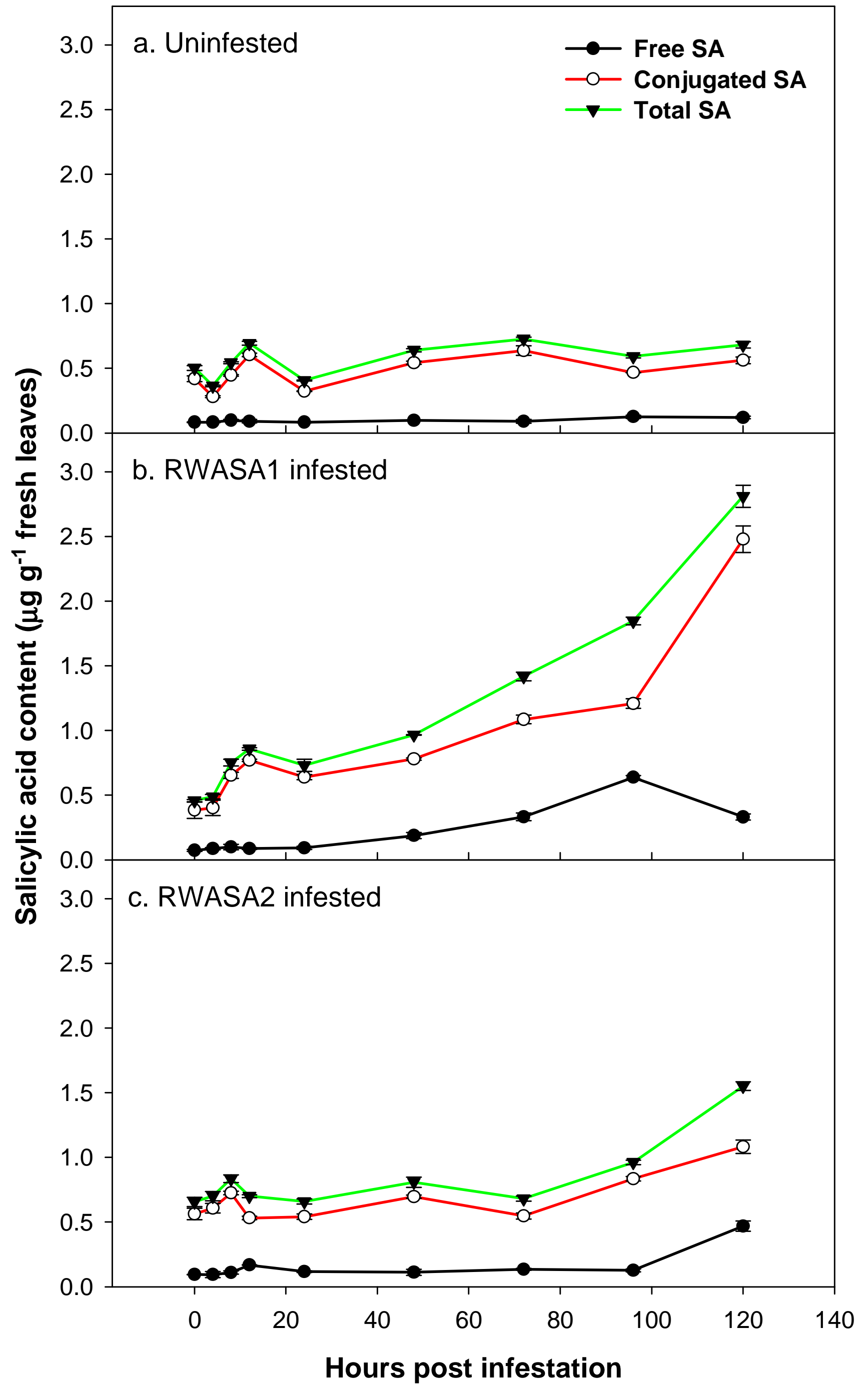

Figure 4.6 Effect of RWASA1 and RWASA2 infestation on the SA content of the resistant (Tugela DN) wheat cultivar. Uninfested (a) and RWASA1 infested (b) and RWASA2 infested (c). Values are means ± SD (n=3).

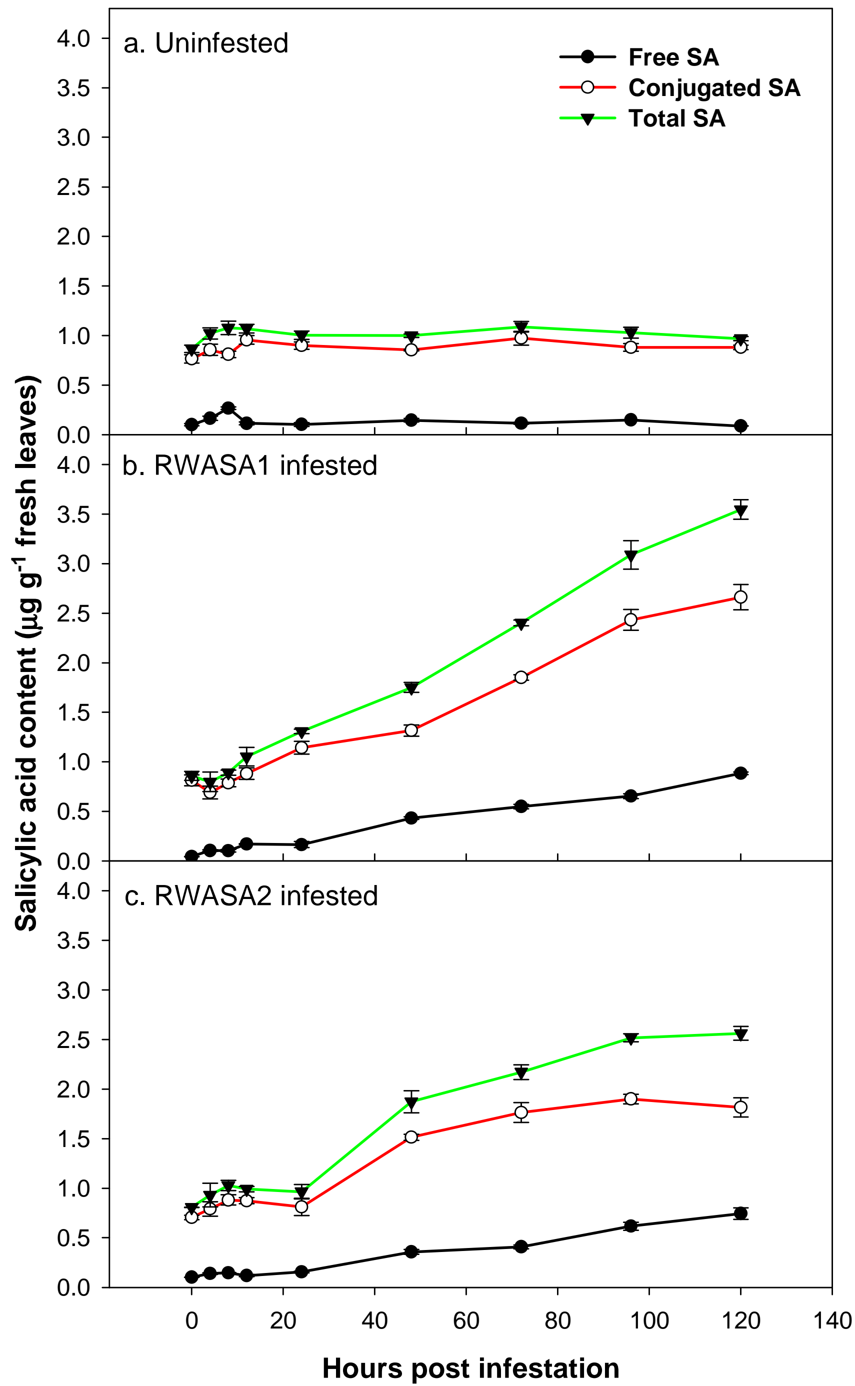

Figure 4.7 Effect of RWASA1 and RWASA2 infestation on the SA content of the resistant (PAN 3144) wheat cultivar. Uninfested (a) and RWASA1 infested (b) and RWASA2 infested (c). Values are means ± SD (n=3).

4.2. Effect of RWASA1 and RWASA2 infestation on phenylalanine ammonia lyase (PAL) activity

Infestation by both biotypes of RWA led to a selective induction of PAL activity in the resistant cultivars while the PAL activity remained relative constant in infested susceptible and uninfested susceptible and resistant cultivars during the investigation period (Fig 4.8). In RWASA1 infested resistant cultivar, Tugela DN, a major transient increase in PAL activity started 24 h.p.i and reached peak activity at 72 h.p.i (1.9-fold) (Fig 4.8 b). In contrast, the major induction occurred in RWASA2 infested Tugela DN after 72 hours of infestation to reach an 1.7-fold increase at 120 h.p.i relative to the initial level (Fig 4.8 b). It should be noted that smaller earlier increases can be observed for RWASA1 and RWASA2 infested Tugela DN at 12 h.p.i and 48 h.p.i, respectively (Fig 4.8 b). An early induction of PAL activity occurred in both the RWASA1 and the RWASA2 infested resistant cultivar, PAN 3144 (Fig 4.8 c). Induction occurred as early as 12 h.p.i and both peaked 24 h.p.i (2.4-fold increase for RWASA1 infested and 5.3-fold for RWASA2 infested plants). A later second increase followed after 72 hours of infestation (Fig 4.8 c). At 120 h.p.i, RWASA1 infestation resulted in a higher (2.4-fold) induction level than RWASA2 infestation (2-fold) (Fig 4.8 c).

For the results of an independent replicate experiment, refer to Appendix 1 (Fig 7.7). Similar tendencies were observed in a replicate experiment.

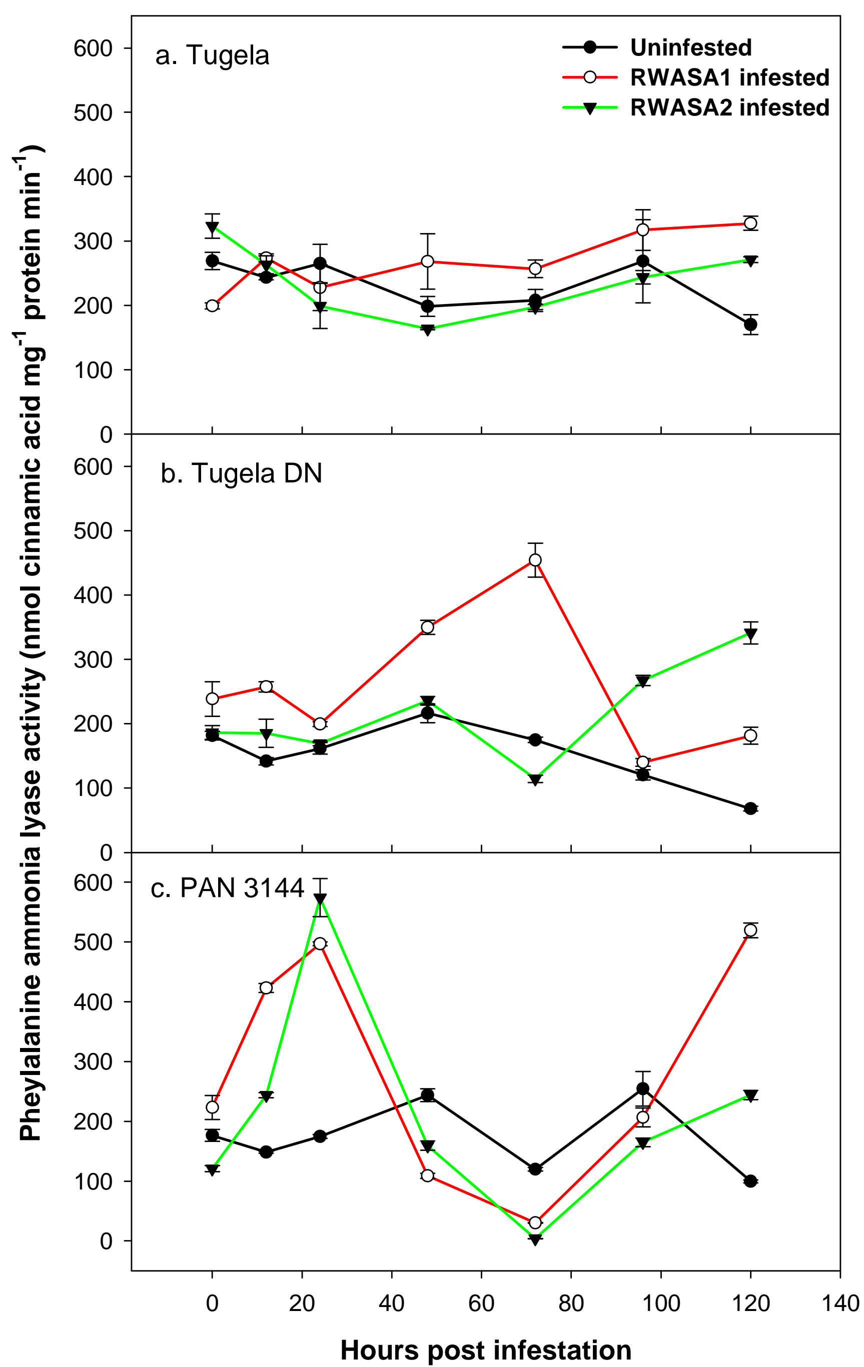

Figure 4.8 Effect of RWASA1 and RWASA2 infestation on the PAL activity of susceptible (Tugela) (a), and resistant wheat cultivars, Tugela DN (b) and PAN 3144 (c). Values are means ± SD (n=3).

4.3. Effect of RWASA1 and RWASA2 infestation on isochorismate synthase (*ICS)* gene expression

In order to determine ICS gene expression, RT-qPCR analyses were done (see section 3.8.3). For more information that shows the efficiency of RT-qPCR reactions, including RNA quality, gradient primer analysis, standard curve and melting peaks of target genes for each experiment, refer to Appendix 2.

In this investigation, three time intervals were selected for *ICS* gene expression analyses; i.e. 0, 48 and 96 h.p.i. The expression levels of the *ICS* gene were calculated as described in section 3.8.3. The expression levels at 48 and 96 h.p.i were then expressed relative to the levels at 0 h.p.i.

According to ICS mRNA expression profiles, induced *ICS* gene expression of RWASA1 (1.24-fold) and RWASA2 (1.4-fold) infested Tugela was observed at 48 h.p.i, however, a decrease in expression levels were found in uninfested (0.6-fold) and RWASA2 (0.8-fold) infested plants at 96 h.p.i (Fig 4.9 a). In uninfested resistant Tugela DN, inductions occurred at 48 h.p.i (1.4-fold) and 96 h.p.i (1.1-fold), but after RWASA1 and RWASA2 infestation the expression levels tended to decrease at 48 and 96 h.p.i (Fig 4.9 b). In resistant PAN 3144, a clear induction occurred in RWASA2 infested plants at 96 h.p.i (1.35-fold) while *ICS* gene expression decreased in uninfested plants at 48 h.p.i (0.72-fold) (Fig 4.9 c). In the replicate experiment, *ICS* expression tended to decrease at 48 and 96 h.p.i after RWASA1 and RWASA2 infestation in susceptible and resistant cultivars (Appendix 1, Fig 7.8). There was a relative high induction in uninfested Tugela at 96 h.p.i (1.85-fold), which might have arisen from an experimental error (Fig 7.8 a). Even though

some inductions seemed to occur, taking the standard deviations to in account, the differences of induced expression become relatively insignificant (Fig 4.9). Therefore, results of both experiments were too inconclusive to proof that induction of *ICS* gene expression has occurred.

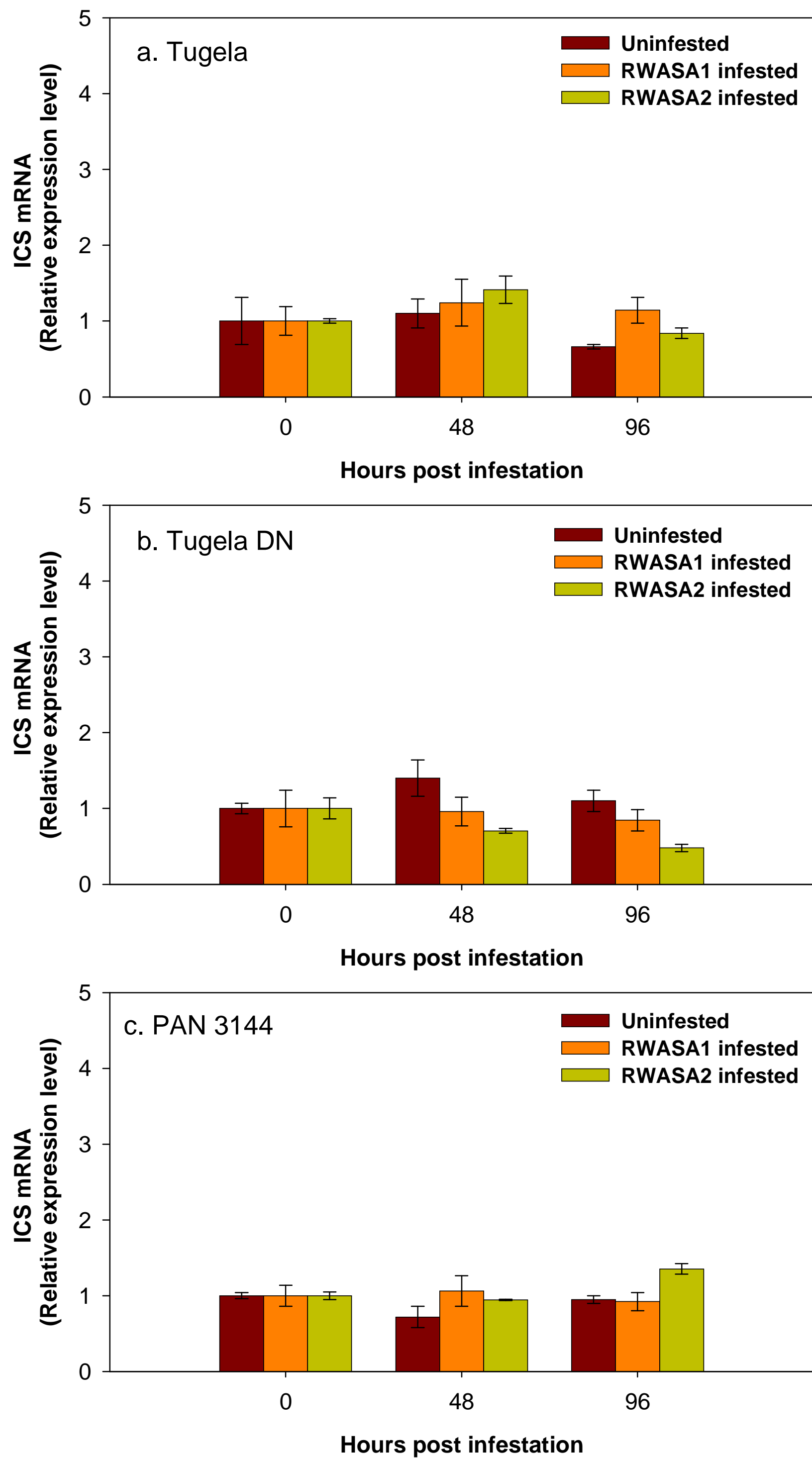

Figure 4.9 Real-time expression analysis of the *ICS* gene following RWASA1 and RWASA2 infestation. Tugela (a), Tugela DN (b) and PAN 3144 (c). Values are means ± 3 SD (n=3).

4.4. Effect of RWASA1 and RWASA2 infestation on salicylic acid UDP-glucosyltransferase (*SAGT)* gene expression

Instead of examining SAGT enzyme activity, RT-qPCR was used for the detection of *SAGT* gene expression in response to RWASA1 and RWASA2 infestation. For more information about RT-qPCR experimental parameters and calculation, refer to Appendix 2.

Results on *SAGT* mRNA expression analysis indicated increased expression at 48 and 96 h.p.i (1.54-fold and 5.28-fold, respectively) in the RWASA1 infested susceptible cultivar, Tugela (Fig 4.10 a). At 96 h.p.i, lower increased expressions were observed in uninfested and RWASA2 infested susceptible Tugela at similar levels (Fig 4.10 a). RWASA1 infestation of the resistant cultivar, Tugela DN, resulted in higher expression levels, also at 48 (2.82-fold) and 96 (3.80-fold) h.p.i. RWASA2 also led to increased expression levels, but at a lower level than RWASA1 infestation at 48 h.p.i (1.76-fold) and 96 (1.46-fold) h.p.i (Fig 4.10 b). In the resistant cultivar, PAN 3144, both RWASA1 and RWASA2 infestation resulted in higher expression levels compared to uninfested plants. In addition, the expression levels of RWASA1 infested plants were higher than RWASA2 infested plants at 48 h.p.i and 96 h.p.i; amounting to a 2.09-fold and 5.68-fold increase, respectively after RWASA1 infestation and a 1.15-fold and 3.95-fold increase, respectively after RWASA2 infestation (Fig 4.10 c). This gene was expressed in susceptible and resistant cultivars after RWASA1 infestation, however, after RWASA2 infestation, a significant increased expression was only observed in the resistant cultivar, PAN 3144, compared to uninfested plants (Fig 4.10). The results of replicate experiment showed similar tendencies, however, a relative low induction in PAN 3144 in response to RWASA1 and RWASA2 was observed (Appendix 1, Fig 7.9).

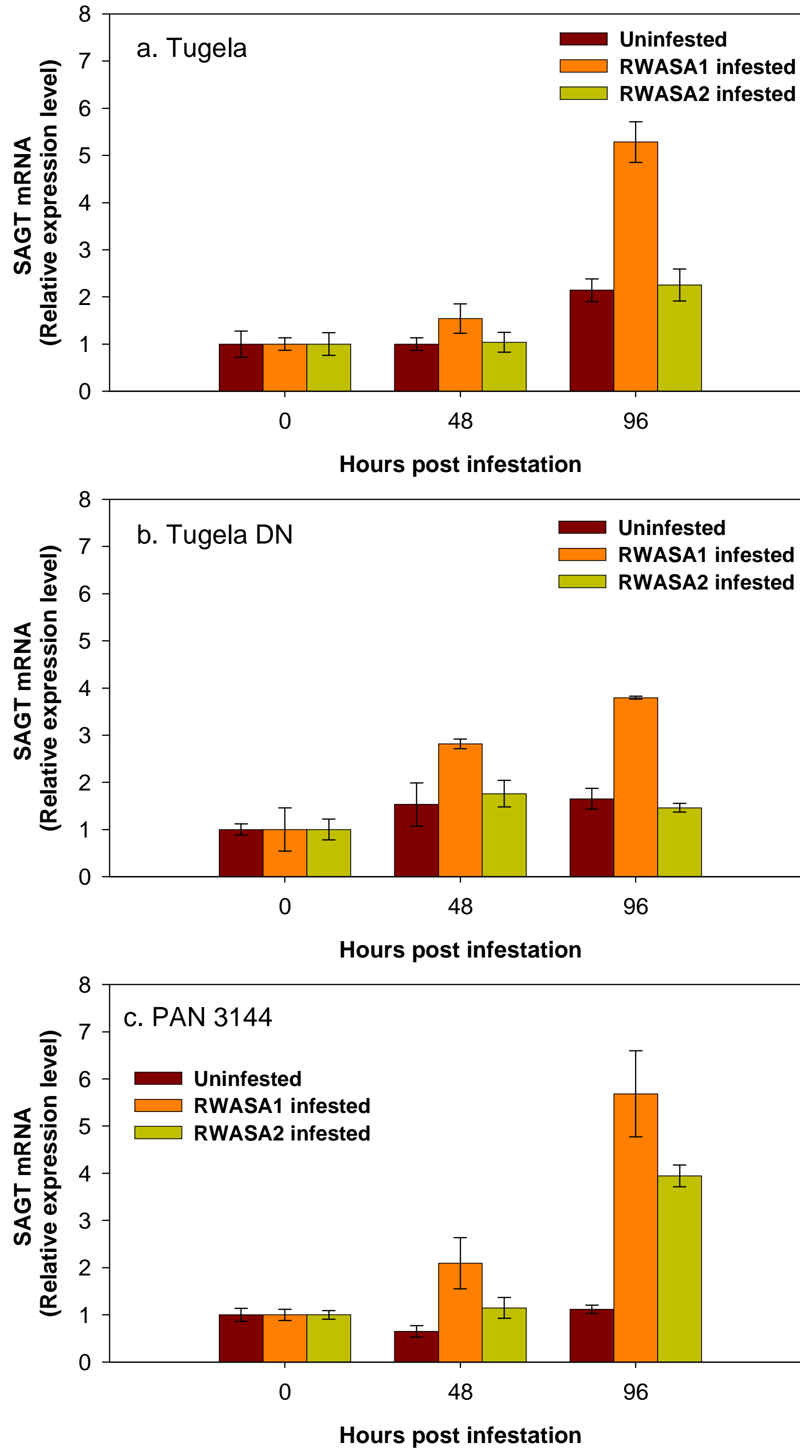

Figure 4.10 Real-time expression analysis of the *SAGT* gene following RWASA1 and RWASA2 infestation. Tugela (a), Tugela DN (b) and PAN 3144 (c). Values are means ± 3 SD (n=3).

4.5. Effect of RWASA1 and RWASA2 infestation on catalase (CAT) activity

Infestation with RWASA1 and RWASA2, respectively induced a general decrease in CAT activity in resistant cultivars (Fig 4.11). In the resistant cultivar, Tugela DN, decreases in CAT activity were observed after 24 h of RWASA1 infestation and after 48 h of RWASA2 infestation. The lowest point of CAT activity represented a 31 % decrease in RWASA1 infested plants and a 29 % decrease in RWASA2 infested plants (Fig 4.11 b). In the resistant cultivar, PAN 3144, CAT activity tended to decrease after 24 h in both RWASA1 and RWASA2 infested plants (Fig 4.11 c). However, in RWASA1 infested plants, a transient increase occurred between 48 h.p.i and 72 h.p.i in RWASA1 infested plants (Fig 4.11 c). The lowest activities after RWASA1 and RWASA2 infestation represent a 25 % and a 31 % reduction respectively (Fig 4.11 c). There was very little change in CAT activity of the infested and uninfested susceptible cultivar (Fig 4.11 a), as well as, of the uninfested resistant cultivars (Fig 4.11 b and c). For the results of an independent replicate experiment, refer to Appendix 1 (Fig 7.10). Similar tendencies were obtained in the replicate experiment.

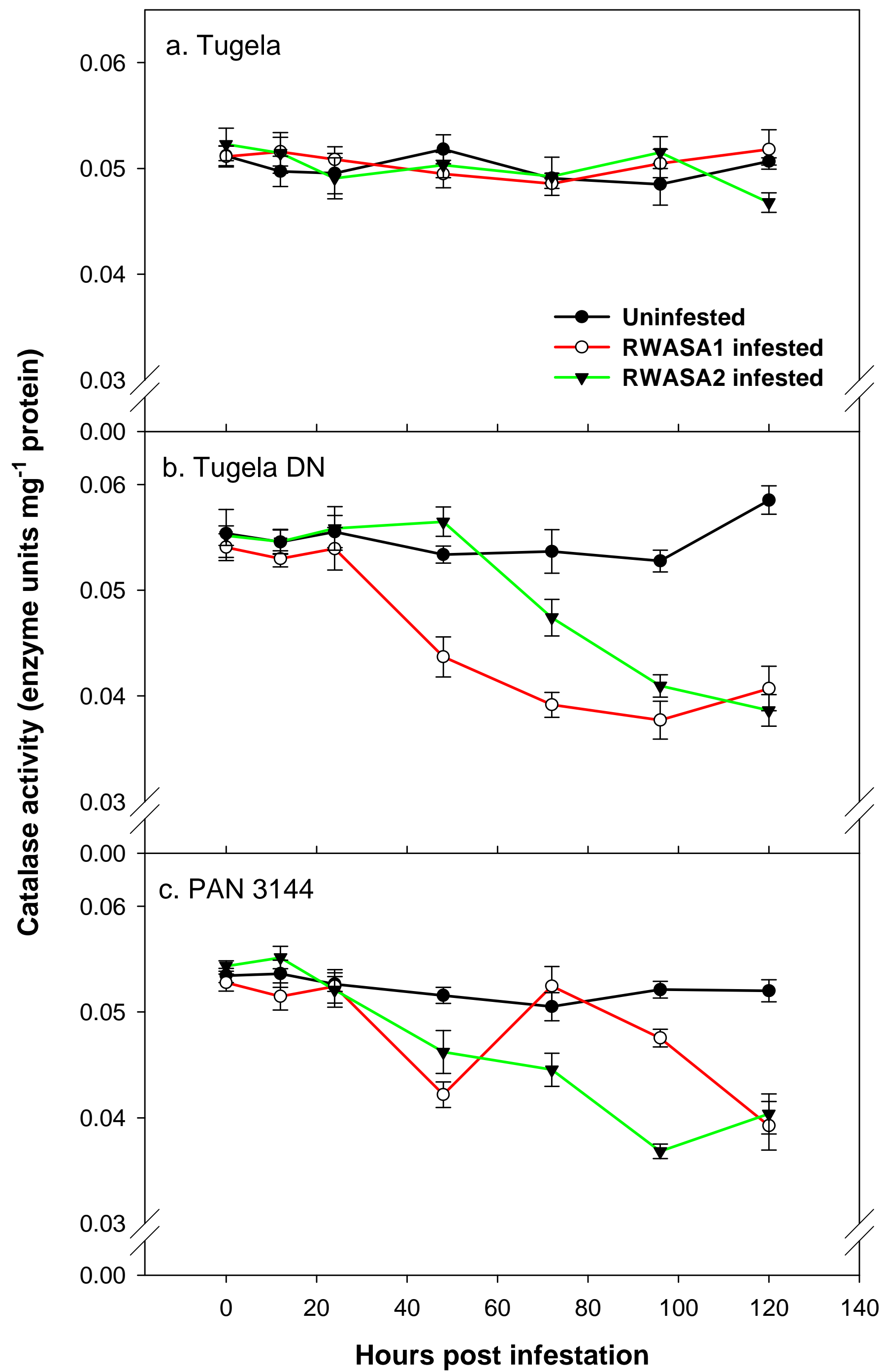

Figure 4.11 Effect of RWASA1 and RWASA2 infestation on the CAT activity of susceptible (Tugela) (a), and resistant wheat cultivars, Tugela DN (b) and PAN 3144 (c). Values are means ± SD (n=3).

4.6. Effect of RWASA1 and RWASA2 infestation on lipoxygenase (LOX) activity

LOX activities were selectively induced in both resistant cultivars (Tugela DN and PAN 3144) after RWASA1 and RWASA2 infestation (Fig 4.12 b and c). There was, however, a difference between these induced responses. In Tugela DN, the highest level of induction occurred after RWASA1 infestation, while RWASA2 infestation resulted in a lower level of induction. The early transient increase in LOX activity (3 h.p.i) was followed by a second prolonged increase in RWASA1 infested Tugela DN, reaching about 2.7-fold higher level compared to the initial activity (Fig 4.12 b). A similar dual induction pattern was also observed in RWASA2 infested Tugela DN, but at a lower level (Fig 4.12 b). Again, RWASA1 infested PAN 3144 showed an early transient peak activity at 12 h.p.i and a second prolonged increase. The earlier peak reached an activity nearly 2.2-fold that of the initial activity, while the second prolonged increase was 2.8-fold higher than the initial activity (Fig 4.12 c). RWASA2 infestation of PAN 3144 resulted in two peaks of LOX induction, i.e. at 24 h.p.i (1.9-fold) and 72 h.p.i (3-fold), respectively (Fig 4.12 c).

It should be noted that RWASA2 infestation of PAN 3144 resulted in higher induction of LOX activity than of Tugela DN (Fig 4.12 b and c). The LOX activity remained relatively constant at a low level in uninfested resistant and susceptible cultivars, as well as in the infested susceptible cultivar (Fig 4.12). For the results of an independent replicate experiment, refer to Appendix 1 (Fig 7.11). Similar tendencies were obtained in the replicate experiment.

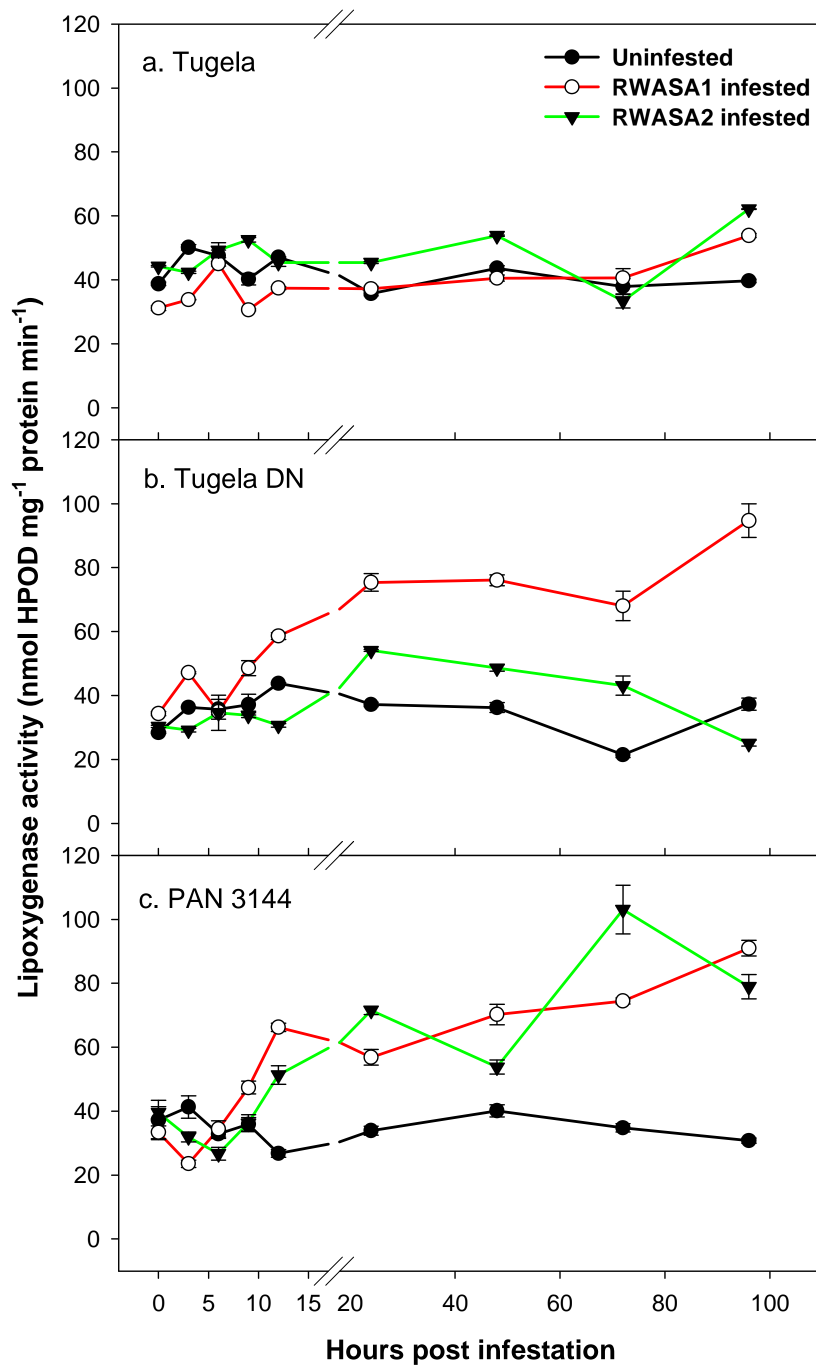

Figure 4.12 Effect of RWASA1 and RWASA2 infestation on the LOX activity of susceptible (Tugela) (a), and resistant wheat cultivars, Tugela DN (b) and PAN 3144 (c). Values are means ± SD (n=3)

DISCUSSION

The Russian wheat aphid (RWA) is a destructive pest of wheat crops that can drastically impair wheat production in South Africa and other countries (Marasas *et al.*, 1997). Since chemical control is expensive and harmful to the environment, wheat resistance is the favoured control tactics for the RWA. It is cheaper, safe and environmentally friendly. However, the co-evolution battle between plants and pathogens or pests is never ending. It is anticipated that resistance-breaking biotypes can evolve and hamper current disease and pest control measures. A better understanding of the plant defence mechanisms is imperative to stay ahead of this problem and also to establish effective strategies for controlling diseases and pests.

There are currently 27 resistant wheat cultivars in use for commercial cultivation in South Africa (Tolmay *et al.*, 2007). Resistance in cultivars, such as Tugela DN and Betta DN containing the *Dn1*-resistance gene is only effective against the old RWA biotype (RWASA1) (Tolmay *et al.*, 2007). The resistance breaking biotype RWA (RWASA2), recently discovered in South Africa, causes severe damage to commercial resistant wheat cultivars containing the *Dn1*-resistance gene (Tolmay *et al.*, 2007; Tolmay, 2006). The newly released commercial wheat cultivar, PAN 3144, however, has high resistance towards RWASA1 and RWASA2 (Tolmay *et al.*, 2007). PAN 3144 was screened and believed to contain the resistant gene, *Dn5* (Dr. A Jankielsohn, personal communication[1]).

[1]Dr. Astrid Jankielsohn, Crop Protection: Entomologist, ARC-Small Grain Institute, South Africa.

A newly developed RWA biotype (RWA2) was also found in Colorado, United State, which is virulent to commercially available resistant wheat cultivars containing the *Dn4* resistance gene (Haley *et al.*, 2004). In addition, it was found that the resistant cultivar, 94M370 containing the *Dn7* resistance gene, was resistant to the RWA2. However, *Dn1* and *Dn5* containing resistant cultivars are highly susceptibile towards RWA2 (Haley *et al.*, 2004). A study of nuclear and mitochondrial DNA genetic divergence among different United State biotypes and two South African biotypes, showed that the South African biotypes significantly differed from the Unites State biotypes (Lapitan *et al.*, 2007). In addition, a recent study on the endosymbiont variation in the United State and South Africa RWA biotypes also showed differences (Swanevelder *et al.*, 2010). This evidence revealed that different *Dn* genes have diverse resistance abilities towards different RWA biotypes.

It was also suggested that the resistance response of wheat plants towards RWA follows the gene-for-gene hypothesis (Botha *et al.*, 2006; 2005). It has been predicted that aphid saliva contains some elicitors that are recognized by the corresponding plant *R* gene-encoded receptor, which then leads to *R* gene-mediated resistance in plants (Goggin, 2007; Miles, 1999). The *R*-gene candidates for RWA resistance are from the nucleotide binding site leucine-rich repeat (NBS-LRR) gene family (Botha *et al.*, 2006; Lacock *et al.*, 2003). A study on the cloning of tomato *Mi-1.2*, a NBS-LRR gene, showed that it confers resistance against certain aphid biotypes (*Macrosiphum euphorbiae*, *Myzus persicae* and potato aphids). It showed that *R* gene imparted resistance to aphids likewise pathogens. (Goggin *et al.*, 2001; Rossi *et al.*, 1998).

When the RWA probes the leaf cells for a suitable feeding site, some cells might be penetrated before feeding occurs in the phloem cells. On the way, salivary compounds

are secreted into the intercellular space (Miles, 1999; Tjallingii and Esch, 1993). The recognition might occur by directly identifying salivary proteins, which bind to the receptor proteins, or indirectly through the recognition of the cell wall fragments that are broken down by pectinase from RWA saliva (de Vos *et al.*, 2007; Miles, 1999; Tjallingii, 2006). It is also suggested that mechanisms controlling virulence in RWA is saliva-based, in fact, several genes have been identified that significantly differentiate various RWA biotypes (CSIRO, 2007). A study involving intercellular injection of different RWA (RWASA1 and RWASA2) crude salivary materials into different resistant wheat cultivars showed selective induced defence responses (Mohase and Taiwe, 2010).

In *R* gene-mediated resistance, the recognition process triggers a signalling cascade that rapidly and effectively activates plant defences, many of which are superimposed to the slower basal defence response found in susceptible genotypes (Dangl and Jones, 2001). Many defence responses in plant-pathogen interactions were also found to be involved in RWA-wheat interactions. Instead of wounding responses, a typical characteristic of herbivore attacks, the RWA resistance response causes the HR which is a characteristic response in plant resistance to pathogens (Belefant-Miller *et al.*, 1994; van der Westhuizen *et al.*, 1998a, 1998b). The HR is associated with the early production of ROS which are toxic to insects or act as signal molecules to trigger downstream defence responses (Kehr, 2006; Klessig *et al.*, 2000; Levine *et al.*, 1994). León *et al.* (1995a) have demonstrated that treatment of tobacco leaves with H_2O_2 induced accumulation of free benzoic acid and SA and suggested that H_2O_2 activates SA biosynthesis. A study done by Moloi and van der Westhuizen (2006) indicated that H_2O_2, produced as a result of NADPH oxidase activation, is involved in the resistance response of wheat against the RWA. It acts as a signal for the activation of downstream defence enzymes, such as intercellular β-1,3-glucanase and peroxidase (Moloi and van der Westhuizen, 2006). The involvement

of xanthine oxidase in the production of ROS during the RWA resistance response was recently reported (Berner and Van der Westhuizen, 2010a).

In addition to ROS, several other defence-related products have been reported to rapidly accumulate in RWA resistant wheat cultivars, such as NO, phenolics, SA, oxylipins, proline, and PR proteins (intercellular β-1,3-glucanase, peroxidases and chitinases) (Berner, 2006; Berner and van der Westhuizen, 2010b; Mohase and van der Westhuizen, 2002; Moloi and van der Westhuizen, 2006, 2008; Moloi, 2010; van der Westhuizen and Pretorius, 1995; van der Westhuizen *et al.*, 1998a, 1998b). These studies addressed the biochemical resistance responses of the interaction between *Dn1* resistant wheat and RWASA1. The biochemistry of the interaction between RWASA2 and different resistant wheat cultivars remains unclear. The aim of this study was therefore to further explore the similarities or differences on biochemical level between defence responses in *Dn1* and *Dn5* resistant wheat cultivars to RWASA1 and RWASA2 infestation with emphasis on SA. This information could be useful for producing efficient resistant wheat cultivars.

Evidence suggests that the resistance response against the RWA involves both the SA- and JA / ET- dependent signalling pathways by mimicking aspects of both pathogen and herbivorous insect attacks, including aphids (Smith and Boyko, 2007; Thompson and Goggin, 2006). According to Swart (1999), JA is induced to higher levels in both resistant (*Dn1*) and susceptible wheat cultivars during RWASA1 infestation i.e. it is not a feature of the resistance response *per se*. More recently evidence was found that RWA infestation of a *Dnx* resistant wheat cultivar increased the gene expression for JA synthesis and for the JA-mediated defence reaction (Boyko *et al.*, 2006). Accumulation of total SA was observed in the *Dn1* resistant wheat cultivar after RWASA1 infestation (Mohase and van der Westhuizen, 2002). The role of SA as an endogenous signal molecule involved in the

RWA resistance response, has also been emphasized by the exogenous application of SA (Mohase and van der Westhuizen, 2002). In this study the earlier induction of total SA to much higher levels in Tugela DN after RWASA1 than after RWASA2 infestation is indicative of *Dn1* resistance towards RWASA1 and susceptibility towards RWASA2 (Fig 4.4 b). A later and a weaker defence response is sometimes characteristic of susceptibility (Fritig *et al.*, 1998). The results therefore clearly illustrated that the resistance response regarding SA accumulation conferred by the *Dn1* gene towards RWASA1 differs substantially from that towards RWASA2. The later and much weaker accumulation of SA during RWASA2 infestation corresponds to the susceptibility of Tugela DN (Fig 4.4 b). The patterns of total SA accumulation after RWASA1 and RWASA2 infestation of a *Dn5* containing wheat cultivar (PAN 3144) are quite similar (Fig 4.4 c). RWASA1 infestation resulted in a somewhat earlier SA development and somewhat higher SA accumulation during later stages (Fig 4.4 c). RWASA2 infestation in PAN 3144 caused a much higher accumulation than was the case with the *Dn1* containing wheat (Fig 4.4 b and c).

The results on *Dn5* resistance are reminiscent of an effective defence response and hence resistance towards both RWA biotypes. These results confirm that SA is not only involved in *Dn1* resistance to RWASA1, but also in *Dn5* resistance to RWASA1 and RWASA2. According to Tolmay *et al.* (2007) the symptoms (damage rating) caused by RWASA1 and RWASA2 infestation of different resistant cultivars, indicated that Tugela DN is resistant to RWASA1, but susceptible to RWASA2 whereas PAN 3144 is resistant to RWASA1 and RWASA2 infestation. This study, furthermore, provides evidence on a biochemical level that PAN 3144 possesses a SA mediated effective resistance response towards RWASA1 and RWASA2. The higher accumulation during later stages of RWASA1 infestation might even indicate better resistance towards RWASA1.

Total SA is the total of free and conjugated SA. In tobacco, oat and soybean cell suspensions, newly synthesized free SA is known to be converted into conjugated forms, such as SA glucosides (Dean *et al.*, 2003; Edwards, 1994; Enyedi and Raskin, 1993; Lee and Raskin, 1998; Yalpani *et al.*, 1992). To investigate the role of free and conjugated SA in defence mechanisms of the RWA-wheat interaction, free and conjugated SA were assayed separately.

Free SA was induced in both RWASA1 and RWASA2 infested Tugela DN and PAN 3144 (Fig 4.2). However, SA accumulation in RWASA2 infested Tugela DN occurred later (96 h.p.i) and at a lower level compared to RWASA1 infested Tugela DN (24 h.p.i) (Fig 4.2 b). In PAN 3144, both RWASA1 and RWASA2 infestation led to a continuous accumulation of free SA after 24 h.p.i (Fig 4.2 c). Free SA is an active form for defence signalling transduction (Garcion and Métraux, 2006). In tobacco, Yalpani *et al.* (1991) showed that 54 ng of SA (g^{-1} fresh leaves) is sufficient for the induction of detectable amounts of PR-1 protein, with progressively stronger induction at higher SA levels. In addition, the basal level of SA varies amongst plant species (Raskin *et al.*, 1990). It is considered that the levels of SA required to trigger the defence are also different. In this study the significant inductions in free SA levels during the resistance responses, portray the role of free SA in defence signalling.

Free SA plays a role as a defence signalling molecule and, in contrast, SA glucosides are inactive storage forms, which can be hydrolyzed back to the active free SA. The formation of SA glucosides has a detoxifying role i.e. to remove free SA to establish non-toxic levels, especially when not needed (Dean and Mills, 2004; Dean *et al.*, 2005; Hennig *et al.*, 1993; Lee and Raskin, 1998).

In the case of TMV and *Pseudomonas syringae* pv. *phaseolicola*- inoculated tobacco leaves, it seems that free SA is newly synthesized and accompanied by an increase in

conjugated SA (Enyedi and Raskin, 1993; Lee and Raskin, 1998). A common phenomenon to plants infected with an avirulent pathogen is the induction of the formation of conjugated SA concomitantly with the induction of the formation of free SA or a slightly delayed induction (Delaney *et al.*, 1994; Malamy *et al.*, 1990). In contrast, a study on phytophthora infected and elicitor (chitosan) treated potato tubers indicated an increase in free SA, which is accompanied by a decrease in conjugated SA, suggesting that free SA is hydrolysed from the conjugated SA (Panina *et al.*, 2005). This suggests the possibility of a SA burst in case of emergency and thereafter prolonged resistance maintained by progressive release of SA from the conjugated forms (Panina *et al.*, 2005).

The question is whether the accumulation of free SA is mainly due to *de novo* synthesis or hydrolyses from conjugated SA in the RWA-wheat interactions. Conjugated SA started to accumulate in Tugela DN 48 h after RWASA1 infestation which was later than free SA accumulation (Figs 4.2 b, 4.3 b and 4.6 b). In RWASA2 infested Tugela DN an increase in conjugated SA started 72 h.p.i (Fig 4.3 b), which is earlier than free SA accumulation (Fig 4.2 b, 4.3 b and 4.6 c). However, the results of the second experiment indicated similar initiation times for free and conjugated SA accumulation (Figs 7.1 b, 7.2 b and 7.5 c). In RWASA1 infested PAN 3144 induction of conjugated SA started slightly earlier (12 h.p.i) than the induction of free SA (24 h.p.i) (Fig 4.3 c, 4.4 c and 4.7 b), however in the second experiment, free and conjugated SA accumulation started simulaneously (Figs 7.1 c, 7.2 c and 7.6 b). The conjugated SA started to accumulate after 24 h.p.i in RWASA2 infested PAN 3144 concomitant with free SA accumulation (Figs 4.2 c, 4.3 c and 4.7 c).

The correlation between free and conjugated SA contents of each wheat cultivar is apparent in Figures 4.5, 4.6 and 4.7. According to these results the free and conjugated SA contents of all infested resistant cultivars mostly increased concurrently, but the rates may change during the course of the experiment (Figs 4.6, 4.7, 7.5 and 7.6). It thus

seems that the increase in free SA can predominately be ascribed to *de novo* synthesis. The release of some free SA from conjugated SA however not can be excluded totally.

To further investigate the formation of conjugated SA, the conjugation rate of glucose and SA was investigated using RT-qPCR to monitor SAGT mRNA formation. Freeman *et al.* (2007) demonstrated that RT-qPCR results correspond favourably with enzyme activities.

SAGT converts free SA to SA glucosides (Dean *et al.*, 2005). According to several studies the induction of SAGT activity can be accomplished by an increase in endogenous free SA and also SA application (Dean *et al.*, 2003; Edwards, 1994; Enyedi and Raskin, 1993; Yalpani *et al.*, 1992). The role of SAGT in plant defence has also been confirmed. It was found that TMV and *P. syringae* pv. *phaseolicola* inoculation of tobacco leaves induces SAGT activity which was confirmed by increased expression of *SAGT* mRNA (Lee and Raskin, 1998). The expression of the *AtSGT1* gene was rapidly induced by MeSA application and infection with a bacterial pathogen (Song, 2006). This indicated that SAGT is involved in an early disease response in *Arabidopsis* (Song, 2006). Rather unexpectedly, it was found in this study that *SAGT* expression increased in uninfested plants as time proceeded, hinting that *SAGT* expression is not defence-related. On the other hand, induction of *SAGT* expression in RWA infested wheat plants was also observed in this study (Fig 4.10) which suggests a possible role in the wheat's resistance response. A relative higher induction of *SAGT* expression was observed in RWASA1 infested Tugela (susceptible), Tugela DN and PAN 3144 than in RWASA2 infested plants (Fig 4.10). The level of *SAGT* expression in RWASA2 infested Tugela and Tugela DN was not always significantly different from that in uninfested plants (Fig 4.10 a and b). In PAN 3144, RWASA2 infestation resulted in higher *SAGT* expression levels than in uninfested plants, but still lower than in RWASA1 infested plants (Fig 4.10 c). Positive correlations were found between *SAGT* expression levels and SA (free and conjugated) levels in PAN

3144 infested with RWASA1 and RWASA2 (Figs 4.2 c, 4.3 c and 4.10 c), as well as RWASA1 infested Tugela DN (Figs 4.2 b, 4.3 b and 4.10 b).

As mentioned previously, conjugated SA accumulation started earlier than accumulation of free SA in RWASA2 infested Tugela DN. The results showed an induction of *SAGT* expression at 48 h.p.i, which was much earlier than the induction of free and conjugated SA in Tugela DN after RWASA2 infestation (Figs 4.2 b, 4.3 b and 4.10 b). Therefore, it is speculated that early increased *SAGT* expression provided the potential to immediately convert newly synthesised free SA into conjugated SA which results in the undetectability of free SA accumulation prior to the detection of accumulated conjugated SA.

The induction of *SAGT* expression in uninfested plants may be associated with regulation of growth development in wheat plants. Glycosylation is a modification of plant secondary metabolites, which is involved in the regulation of hormone homeostasis, the detoxification of xenobiotics and the biosynthesis and storage of secondary compounds (Gachon *et al.*, 2005). Fraissinet-Tachet *et al.* (1998) showed that treatment with a HR-inducing-elicitor did not significantly stimulate accumulation of *TOGT* (tobacco glucosyltransferase) transcripts in NahG tobacco i.e. no significant difference from that of the wild-type. They speculated that even though the *TOGT* genes were induced by exogenous SA, their induction during the HR is SA-independent (Fraissinet-Tachet *et al.*, 1998). The tomato *Twi1* gene (homology to *TOGT*) was induced during the HR or by exogenous SA application, and has also been shown to be induced by wounding and remained wound-inducible in tomato transgenic lines expressing *nahG* (O'Donnell *et al.*, 1998). In a recent study, a transient increase in SA and SA glucose conjugates was also observed after wounding of *Arabidopsis*, suggesting a possible role for SA in wound healing (Ogawa *et al.*, 2010).

Jimoh *et al.* (2011) indicated that RWASA2 caused earlier and more severe leaf roll symptoms on the resistant wheat than RWASA1 infestation. However, RWASA1 caused greater levels of chlorosis in RWA-resistant plants, which may due to a difference in feeding behaviour or salivary biochemistry of this biotype. According to the information above, we speculate the feeding behaviour or saliva compounds of RWASA1 might trigger *SAGT* expression in infested susceptible and resistant wheat cultivars, whereas the specific induced *SAGT* expression only occurred in the incompatible interaction between RWASA2 and the *Dn5* resistant wheat cultivar.

To conclude, the results in this study provide evidence that increasing conjugated SA levels correlated with the induction of *SAGT* expression and hence SAGT activity. The higher induction of *SAGT* expression during the incompatible RWA-wheat interaction may indicate that SAGT is involved in the RWA-wheat resistance mechanism.

All the results on the changes in free, conjugated and total SA contents during the RWA-wheat interaction, indicated that the *Dn5* resistant wheat cultivar (PAN 3144) has higher accumulation levels of free, conjugated and total SA contents than *Dn1* resistant wheat cultivar (Tugela DN) (Figs 4.2 to 4.7). It is suggested that the level of the response to RWA infestation varies in different resistant wheat cultivars containing different resistance genes. The genetic background into which a specific *Dn* gene is bred also plays a role in the effectiveness of the resistance response (van der Westhuizen *et al.*, 1998a, 1998b). The resistance mechanism of the *Dn1* containing resistant wheat cultivars is antibiosis and that of *Dn5* containing wheat cultivars is antixenosis and antibiosis (du Toit, 1989; du Toit, 1992). Botha *et al.* (2008) reported that the *Dn1* gene is responsible for antibiosis which includes increased levels of SA and the oxidative burst. Antixenosis is associated with the expression of volatile organic compounds, in addition, more crosstalk

between SA- and JA / ethylene- mediated pathways occurs [confirmed by Affymetrix gene technology and cDNA-amplified fragment length polymorphism (cDNA-AFLP) transcript profiling studies] (Botha *et al.*, 2008).

Our study also reveals that SA participates in *Dn1* and *Dn5* resistance responses. Furthermore, a relative high SA content was observed in *Dn5* resistance response to RWA infestation. MeSA, a volatile form of SA and an important signal molecule for SAR, is synthesized from SA during plant-pathogen resistance responses (Shulaev *et al.*, 1997). It is unknown whether MeSA is involved in the RWA-wheat resistance response, but if so, it could be involved in the antixenosis resistance mechanism. In a field experiment, MeSA, when applied as a semiochemical for RWA control, had repellent effects on the RWA. It is deduced that this might be a direct effector or indirect effect whereby the host plant defences are induced, resulting in repellent effects (Prinsloo *et al.*, 2007).

To date, two SA biosynthesis pathways were found in plants, i.e. the phenylalanine and isochorismate pathways (Vlot *et al.*, 2009). PAL is a key enzyme in the phenylpropanoid pathway for the production of phenolics and is correlated with plant defence responses to herbivores and pathogens (Gerasimova *et al.*, 2005; Southerton and Deverall, 1990). Plant phenolic compounds produced during host-pathogen interactions function by means of several mechanisms in plant defence, such as antimicrobial, defence signal molecules and involvement in cell wall lignification (Dixon, 2001). The induction of phenolics in resistant wheat cultivars against RWA infestation indicates a role for phenolic compounds in RWA resistance (Berner and van der Westhuizen, 2010b; van der Westhuizen and Pretorius, 1995). Wheat cultivars with high levels of phenolic compounds are less attractive to the cereal aphid (*Sitobion avenae*) compared to cultivars with low phenolic contents (Leszczynski *et al.*, 1989). Leszczynski *et al.* (1989) also suggested that that

quantity of phenolic compounds is of essence for antibiotic resistance. SA may be an essential part of antibiotic resistance against RWASA1 and RWASA2 in *Dn1* and *Dn5* resistant wheat cultivars.

In a recent study, Berner and van der Westhuizen (2010b) have demonstrated that an increased PAL activity is involved in the resistance response of Tugela DN against RWASA1. In addition, the induction patterns of several phenolic compounds corresponded to an increased PAL activity. The current study on PAL activity includes *Dn5* resistance and RWASA2 infestation in addition to *Dn1* resistance and RWASA1 infestation. The induction of PAL activity started 24 h.p.i and peaked at 72 h.p.i in Tugela DN after RWASA1 infestation (Fig 4.8 b). Compared to the results of free SA, PAL activity and free SA contents increased simultaneously and the decrease in PAL activity is accompanied by a later decrease of free SA content (Fig 4.2 b and 4.8 b). PAL activity was also induced in RWASA2 infested Tugela DN, but earlier (72 h.p.i) than free SA (96 h.p.i) (Fig 4.2 b and 4.8 b). Two peaks of PAL activity were observed in PAN 3144 infested with RWASA1 and RWASA2 (Fig 4.8 c). The induction of PAL activity was earlier (before 12 h.p.i) than the induction of free SA (24 h.p.i) in both RWASA1 and RWASA2 infested PAN 3144 (Fig 4.2 c and 4.8 c). In addition, a continuous accumulation of free SA content was observed (Fig 4.2 c). This is consistent with the results of He *et al.* (2011) who found two peak PAL activities after aphid infestation of resistant chrysanthemum cultivars. The highly resistant cultivar, Keiun, had a primary peak that was earlier and stronger than in the moderately resistant cultivar, Han6. Furthermore, PAL activity was also induced in aphid infested susceptible chrysanthemum cultivars with a single peak pattern. Considering that speed and strength of a defence response is of essence in determining the resistance potential (Fritig *et al.*, 1998), one may be tempted to speculate that the earlier and higher response of PAN 3144 may contribute to a better resistance than Tugela DN.

It has been reported that SA might play a role in regulating PAL expression. SA treatment of grape berries induces PAL mRNA expression and as a result, enhances PAL protein amounts and activity and leads to the accumulation of phenolics (Chen *et al.*, 2006; Wen *et al.*, 2005). However, the relationship between the continuous increase of free SA and two phase PAL induction in RWASA1 and RWASA2 infested PAN 3144 remains unclear.

Another SA synthesis pathway, isochorismate pathway, has been implicated in a study of Wildermuth *et al.* (2001). They indicated that ICS is required to synthesize SA during SAR activation in pathogen infested *Arobidopsis*. In ozone (O_3) exposed *Arabidopsis*, researchers proposed that SA is mainly synthesized from the isochorismate pathway, by increasing ICS activity rather than via the phenylalanine pathway (Ogawa *et al.*, 2007). In tobacco, after *ICS* expression was silenced, no SA accumulation occurred in response to ultraviolet (UV) light exposure or to bacterial [*Pseudomonas syringae* pv. *tomato* (Pto) DC3000] infection (Catinot *et al.*, 2008). This provides evidence for a possible role of *ICS* in SA synthesis in tobacco (Catinot *et al.*, 2008). In contrast, the expression level of *ICS* decreased after both TMV inoculation and mock inoculation of tobacco (Ogawa *et al.*, 2006). Moreover, in O_3-exposed tobacco, no increase of ICS activity and *ICS* gene expression was observed and instead a remarkable elevation of PAL activity and *PAL* gene expression was found (Ogawa *et al.*, 2005).

To clarify the possible contribution of the isochorismate pathway, we investigated the possible participation of ICS in SA biosynthesis in RWA infested susceptible and resistant wheat cultivars. *ICS* gene expression was monitored by using RT-qPCR. Expression profiles of *ICS* revealed small changes at different time intervals in wheat cultivars infested with RWASA1 and RWASA2 (Fig 4.9). The second experiment showed a relative steady decreased expression of *ICS* during the infestation period (Fig 7.8). This is

consistent with the results of Liu *et al.* (2011) which indicated that there are no significant differences in *ICS* expression in RWA1 (incompatible) and RWA2 (compatible) infested *Dn 4* resistant wheat cultivars (Liu *et al.*, 2011). Our results and these previous studies suggest that the phenylalanine pathway and not the isochorismate pathway, is the main route of SA biosynthesis in RWA-wheat interactions.

SABP is involved in SA signal transduction in plant defence (Garcion and Métraux, 2006). The first SABP, isolated from tobacco, was identified as a CAT. Its H_2O_2-degrading activity is specifically inhibited by SA or other SA analogs (Chen *et al.*, 1993a; 1993b). It suggests a possible role for ROS in SA signalling, i.e. that SA may facilitate H_2O_2 accumulation during the oxidative burst induced by infection with avirulent pathogens (Durner and Klessig, 1995). RWASA1 and RWASA2 infestation had an inhibiting effect on CAT activity in both resistant wheat cultivars (Fig 4.11 b and c). Results on RWASA1 infested Tugela DN showed an earlier and higher inhibition than what was found after RWASA2 infestation (Fig 4.11 b). The inhibition of CAT activity coincided with the induction of free SA accumulation (Fig 4.2 b and 4.11 b). The inhibition pattern in PAN 3144 infested with RWASA2 revealed a continuous inhibition of CAT activity which is similar to the pattern in the RWASA1-*Dn1* interaction (Fig 4.11 b and c). In comparison, the inhibition pattern for RWASA1 infested PAN 3144 was different from that of RWASA2 infestation, which showed a restored CAT activity at 72 h (Fig 4.11 c). It may be that the response of RWASA1 infestation is different in *Dn1* and *Dn5* resistant wheat cultivars. However, the causal effect of this phenomenon is unknown. According to a previous study of Mohase and van der Westhuizen (2002), CAT activity is inhibited in both the resistant (Tugela DN) and susceptible (Tugela) wheat cultivars after RWASA1 infestation, but inhibition occurred earlier and stronger in resistant plants.

For successful invasion, a possible strategy may be to detoxify ROS during plant defence responses. Antioxidant enzymes were detected in aphid's (RWA and bird cherry-oat aphid) saliva (Ni and Quisenberry, 2003; Ni *et al*., 2000). Ni *et al.* (2000) found differences in the activity of SOD and CAT between saliva from RWA and the bird cherry-oat aphid. SOD and ascorbate peroxidase activities were detected in the saliva of both aphids, whereas CAT activity was detected only in RWA saliva. In addition, an early induction of antioxidant enzymes (SOD, APX and glutathione reductase) were also found in RWA-wheat interactions (Moloi and van der Westhuizen, 2008).

LOX acts on cell membrane lipids and produces plant defence signal molecules such as oxylipins, including JA. Induced LOX activities have been reported for the resistance response in *Dn1* (Tugela DN and Betta DN), *Dn 2* (Tugela-*Dn2*) and *Dn5* (Tugela-*Dn5*) resistant wheat cultivars against RWASA1 (Berner, 2006; Swart, 1999). In this study it was found that LOX activities increased in both RWASA1 and RWASA2 infested resistant cultivars, Tugela DN and PAN 3144 (*Dn5*) (Fig 4.12). The LOX activity in infested susceptible Tugela was slightly induced, but to a much lower level than in infested resistant wheat cultivars (Fig 4.12). Similar tendencies of LOX activity in infested susceptible plants were observed in the study of Swart (1999). The LOX activity in RWASA2 infested Tugela DN was induced to a much lower level than in RWASA1 infested plants (Fig 4.12 b) confirming the compatible interaction between RWASA2 and the *Dn1* resistant wheat cultivar. This evidence is consistent with the results of Liu *et al.* (2011) i.e. induction of *LOX* expression occurred in compatible and incompatible RWA-wheat interactions, but the induction level was significantly higher in incompatible interactions than in compatible interactions. Therefore, LOX assumedly may participate in the basal resistance as well as *R* gene-mediated resistance in the wheat plant against RWA.

Swart (1999) indicated that the highest induced level observed for LOX was in RWA infested Tugela DN followed by RWA infested Tugela-*Dn2* and Tugela-*Dn5*. In addition, various induction levels of LOX activity in different resistant wheat cultivars also correlated to resistance. Although the previous study showed that Tugela-*Dn5* was less resistant towards RWA infestation, our results show PAN 3144, which also contains *Dn5* resistant gene, has a high induction level of LOX activity after RWA infestation (Fig 4.12 b and c). These differences are probably due to the complicated genetic background in different wheat cultivars.

SA is capable of interacting with iron ions and inhibiting heme-containing enzymes, such as peroxidase and CAT, by its ability to donate electrons. This converts SA molecule to a free radical, which affects lipid peroxidation (Anderson *et al.*, 1998; Durner and Klessig, 1995; 1996). Theoretically, SA may interact with LOX, which is a non-heme iron containing fatty acid dioxygenase (Feussner and Wasternack, 2002). An *in vitro* study of LOXs from plants and animals, indicated that application of SA could inhibit LOX-catalyzed lipid peroxidation (Lapenna *et al.*, 2009). In rice, the application of SA inhibited LOX activity and hence partially alleviated the cell membrane damage from heavy metal treatment (Mishra and Choudhuri, 1999). The interaction between endogenous SA and LOX is unclear; however, the induction of LOX activity is earlier than induction of free SA (Fig 4.2 and 4.12).

Zhao *et al.* (2009) reported that SA and JA signal transduction pathways are both involved in wheat after *Sitobion avenae* infestation. This was evident through the detection of PAL, β-1,3-glucanase, LOX and poyphenoloxidase activities. Although LOX is associated with the JA signalling pathway, it is also a key enzyme for initiating many branches of the LOX pathway (Wasternack *et al.*, 2006). These branches are associated with the synthesis of

different groups of oxylipins e.g. JA whose synthesis is catalysed downstream by the AOS (Wasternack *et al*., 2006). JA is involved in the wounding response and hence its role in defence responses attracted more attention. It is considered that the induction of LOX during defence responses may not only contribute to JA synthesis, but also produce diverse oxylipins which participate in different defence signalling events. As mentioned previously, Swart (1999) indicated that selective increase in LOX activity did not correlate with a selective increase in JA content, therefore, speculated JA was not the primary product of RWA induced LOX activity and that it was not the signal molecule in the RWA-wheat resistance response. In addition, other research demonstrated that several lipid-like compounds (oxylipins), which were indentified as hydroxyl and keto fatty acids, were newly induced after RWA infestation of the resistant wheat (Berner, 2006). A more detailed investigation is required to understand the roles of signal molecules, such as SA and oxylipins, in RWA-wheat resistance and the cross-talking between different signal transduction pathways.

CONCLUSIONS

The discovery of a new RWA biotype (RWASA2) in South Africa which has overcome most of the available resistance in the field is the cause of great concern. Resistance conferred by the *Dn1* gene, which was abundantly used in wheat breeding programmes, has been overcome by the RWASA2, but *Dn5* resistance is still intact. Obviously, RWASA2 presents a changed elicitor or a total new elicitor which is not any more detected or just ineffectively detected by the receptor (*R* gene product) of *Dn1* containing wheat, but still effectively detected in *Dn5* containing wheat. Another study is ongoing on the identification of such elicitors, but this study concentrates on the main signalling events with emphasis on SA. In the case of poor detection of the elicitor the one scenario would be that signalling is just at a too low level or too late to induce an effective hypersensitive reaction (HR), the other scenario is no detection and no signal development.

Previous studies have indicated that SA is mainly responsible for the induction of the HR during the RWASA1-*Dn1* wheat interaction (Mohase and van der Westhuizen, 2002). One study also hints towards the possible participation of oxylipins in signalling pathways (Berner, 2006). This study was launched mainly to broaden our insight on the induction of SA under these circumstances. Not only SA levels *per se*, but also enzymes that could affect SA levels were investigated in a comparative study using *Dn1*, *Dn5* and no resistance containing wheat cultivars and biotype 1 and 2 RWAs. To obtain some insight on the possible formation of lipid signals LOX activities were investigated as well.

It was anticipated that this research might provide further insights in the understanding of the race-specific (*R* gene-mediated) resistance responses of RWASA1 and RWASA2 infested in *Dn1* and *Dn5* resistant wheat cultivars. Various aspects of the interaction between incompatible and compatible RWA-wheat responses include the following:

1. An earlier and higher induction of total SA occurred during the incompatible RWA-wheat interactions (RWASA1-*Dn1*, RWASA1-*Dn5* and RWASA2-*Dn5*) compared to that in the compatible RWASA2-*Dn1* interaction. This suggests that the timing and level of accumulation of SA could be correlated to the resistance level e.g. the ineffectiveness of *Dn1* resistance towards RWASA2 corresponded to a late and very low SA accumulation. An early and high accumulation of SA in *Dn5* containing wheat (PAN 3144) in response to RWASA1 and RWASA2 infestation is reminiscent of effective resistance against both biotypes, a result of the recognition of elicitors from both biotypes, *Dn5* resistance towards RWASA1 resulted in a higher accumulation of SA than *Dn1* resistance. It is clear that resistance conferred by the *Dn1* and *Dn5* genes is associated with SA accumulation and hence the systemic induction of the HR.

2. The remarkable increase of PAL activity in incompatible RWA-wheat interaction indicates to the involvement of the phenylpropanoid pathway, rather than the isochorismate pathway, in the synthesis of SA. Induction of *ICS* expression was not obvious in this study. Noteworthy, the differences in the PAL activity induction patterns of *Dn1* and *Dn5* resistant wheat cultivars demonstrate the variation in biochemical responses associated with different *Dn* genes.

3. The formation of SA conjugates plays a role in controlling free SA levels. To establish to what extend conjugate formation accounted for changes in free SA

levels, *SAGT* expression was investigated. *SAGT* expression (activity) could have an effect on the level of SA and hence resistance response. RWASA1 infestation, specifically, induced *SAGT* expression in all wheat cultivars. In contrast, RWASA2 infestation only induced significant *SAGT* expression in the *Dn5* resistant wheat cultivar. In resistant plants these levels of induction is consistent with levels of conjugated SA, but not inversely correlated with free SA levels. This points to the possibility that free SA accumulation is due to *de novo* synthesis. The abundant availability of free SA might also be favourable for SA conjugation. However, induction of *SAGT* expression in susceptible plants after RWASA1 infestation is unclear.

4. The inhibition of CAT activity occurred concomitantly with increases in free SA contents in the incompatible RWA-wheat interactions. This finding is consistent with hypothesis that inhibition of CAT, a SABP, by free SA conveys SA signalling by enhancing the H_2O_2 concentration which triggers downstream defence responses. It is questionable, a transient restored CAT activity occurred in RWASA1 infested *Dn5* resistant wheat cultivar (72 h.p.i), which was not found in RWASA2 infested plants.

5. A high induction of LOX activity occurred in the incompatible RWA-wheat interactions whereas the compatible interaction resulted in a lower induced LOX activity. LOX activity induction occurred earlier than the accumulation of free SA. This may point to upstream from SA, the involvement of the LOX pathway in early defence responses, during the RWA-wheat interaction. However, the possible interaction between LOX and SA in the RWA resistance response needs to be further explored.

In conclusion, the *Dn1* and *Dn5* resistant wheat cultivars respond in a specific manner to infestation with different RWA biotypes which is in agreement with gene-for-gene model. In addition, the involvement of PAL, SAGT, CAT and LOX were all found in *Dn1* and *Dn5* resistance responses against RWASA1 and RWASA2 with differences in enzyme activity levels and pattern of changes related to RWA resistance levels. Information of this investigation could be helpful for the design of resistant cultivars in the future. Research in natural defence signal molecules and biochemical responses could contribute to the enhancement and development of bio-chemical control. Especially, SA is an essential signal molecule in plant defence responses; recent advances in biopesticide development include the application of MeSA. As a whole, this study was performed based on previous information on *Dn1* resistance towards RWASA1. The new information on the resistance response towards RWASA2 may serve as a foundation for future studies in this field.

ABSTRACT

The effect of the Russian wheat aphid (RWA) (*Diuraphis noxia*, Kurdjumov), South African biotype 1 (RWASA1) and biotype 2 (RWASA2) infestation, on signalling events, with emphasis on salicylic acid (SA), in different resistant wheat (*Triticum aestivum* L.) cultivars, containing *Dn1* (Tugela DN) and *Dn5* (PAN 3144) resistant genes, was investigated. This study was mainly conducted in an attempt to compare changes in SA content and factors affecting it during the resistance response of different wheat cultivars towards RWASA1 and RWASA2 infestation. SA contents were determined by high performance liquid chromatography (HPLC). To obtain insight into SA metabolism during the resistance responses the involvement of phenylalanine ammonia lyase (PAL), isochorismate synthase (ICS), SA UDP-glucosyl transferase (SAGT) and SA binding protein catalase (CAT) were investigated. In addition, lipoxygenase (LOX) activities, related to the synthesis of the signalling molecules, jasmonates and oxylipins, were determined during the resistance responses. PAL, CAT and LOX activities were assayed spectrophotometrically while ICS and SAGT mRNA expression was measured on a molecular level, using reverse transcriptase quantitative polymerase chain reaction (RT-qPCR).

The resistance responses during the incompatible interactions, RWASA1-*Dn1,* RWASA1-*Dn5* and RWASA2-*Dn5* were characterized by an earlier and higher induction of total SA (total of free and conjugated forms). In contrast, in the compatible RWASA2-*Dn1* interaction, a much later and lower induction of SA accumulation was found. In addition, the level of SA induction during the *Dn5* resistance response was higher than during the *Dn1* resistance response. Furthermore, RWASA1 infestation of *Dn5* resistant wheat

caused a higher SA induction than that of RWASA2 infestation. These results reflect a correlation between the level of SA accumulation and the resistance level.

Increases in PAL activity and not significant expression of *ICS* indicate that the phenylpropanoid pathway for SA synthesis was preferred over the isochorismate pathway during the resistance responses towards both RWA biotypes. Increased free SA contents were accompanied by increased *SAGT* expression and conjugated SA contents, indicating that free SA was mainly synthesized *de novo* and further metabolised in the conjugation reactions catalyzed by SAGT.

The finding that increased free SA contents were accompanied by inhibition of CAT activities during RWA-wheat interactions is consistent with SA signalling transduction via increased H_2O_2 levels. However, the inhibition patterns of CAT activity were also different in RWASA1 and RWASA2 infested resistant cultivars.

Significant induction of LOX activities occurred before the accumulation of SA in the incompatible RWA-wheat interactions. This may point to an early defence function of LOX, upstream from SA, which may include the production of signal molecules such as oxylipins. The levels of LOX induction were consistent with resistance against the two RWA biotypes.

The involvement of SA, PAL, SAGT, CAT and LOX in both *Dn1* and *Dn5* resistance responses towards infestation by both RWA biotypes is indicative of the similarity between different RWA-wheat interactions. However, differences in the timing, level and pattern of changes occurred. The fact that *Dn1* resistance was overcome by RWASA2 and that *Dn5* resistance is effective against both RWA biotypes was clearly illustrated by results of this study.

Keywords: Wheat, Russian wheat aphid, resistance, salicylic acid, phenylalanine ammonia lyase, isochorismate synthase, salicylic acid UDP-glucosyl transferase, catalase, lipoxygenase

OPSOMMING

Die invloed van Russiese koringluis (RKL) (*Diuraphis noxia*, Kurdjumov), Suid-Afrikaanse biotipe 1 (RKLSA1) en biotipe 2 (RKLSA2), infestering op seingebeure, met die klem op salisielsuur (SA), in verskillende weerstandbiedende koring-(*Triticum aestivum* L.) kultivars, wat *Dn1* (Tugela DN) en *Dn5* (PAN 3144) weerstandsgene bevat, is ondersoek. In hierdie studie is hoofsaaklik gepoog om die veranderinge in SA-inhoud en faktore wat dit tydens die weerstandsrepons van verskillende koringkultivars teen RKLSA1- en RKLSA2-infestering beïnvloed, te bestudeer. SA-inhoude was met behulp van hoëverrigtingvloeistofchromatografie (HPLC) bepaal. Om insig in SA-metabolisme tydens die weerstandsresponse te verkry, is die betrokkenheid van fenielalanienammoniakliase (PAL), isochorismaatsintase (ICS), SA-UDP-glukosieltransferase (SAGT) en SA-bindingsproteïenkatalase (CAT) ondersoek. Hierbenewens, is lipoksigenase (LOX)-aktiwiteite, wat met die sintese van seinmolekules, jasmonate en oksilipiene, verband hou tydens die weerstandsresponse bepaal. PAL-, CAT- en LOX-aktiwiteite was spektrofotometries bepaal terwyl ICS en SAGT mRNA-uitdrukking op molekulêre vlak, deur van die omgekeerdetranskriptase kwantitatiewe polimerasekettingreaksie (RT-qPCR) gebruik te maak, gemeet is.

Die weerstandsresponse tydens die onververenigbare interaksies, RKLSA1-*Dn1,* RKLSA1-*Dn5* en RKLSA2-*Dn5* word deur ‘n vroeër en hoër induksie van totale SA (totaal van vrye en gekonjugeerde vorme) gekenmerk. In teenstelling, tydens die verenigbare RKLSA2-*Dn1* interaksie, was ‘n veel later en laer induksie van SA-akkumulasie gevind. Hierbenewens was die vlak van SA-induksie tydens die *Dn5*-weerstandsrespons hoër as tydens die *Dn1*-weerstandsrespons. Boonop het RKLSA1-infestering van *Dn5*-weerstandbiedende koring ‘n hoër SA-induksie as RKLSA2-infestering veroorsaak.

Hierdie resultate was aanduidend van ‘n verband tussen die vlak van SA-akkumulering en die weerstandsvlak.

Volgens die PAL aktiwiteits- en *ICS*-uitdrukkkingsresultate was dit ooglopend dat die fenielpropanoïedroete vir SA-sintese voorrang bo die isochorismaatroete tydens die weerstandsresponse teen beide RKL-biotipes geniet. Hoë vrye SA-inhoude het met hoë *SAGT*-uitdrukkingsvlakke en gekonjugeerde SA-inhoude gepaard gegaan wat ‘n aanduiding was dat SA hoofsaaklik *de novo* gesintitiseer was en verder in konjugasiereaksies, gekataliseer deur SAGT, gemetaboliseer is.

Die bevinding dat verhoogde vrye SA-inhoude met die remming van CAT-aktiwiteite tydens RKL-koringinteraksies gepaard gegaan het, is in ooreenstemming met SA-seinoordraging via verhoogde H_2O_2-vlakke. Nietemin, die CAT-aktiwiteitremmingspatrone was ook verskillend in RKLSA1 en RKLSA2 geïnfesteerde weerstandbiedende kultivars.

Beduidende induksies van LOX-atiwiteite het voor die akkumulasie van SA in die onverenigbare RKL-koringinteraksies voorgekom. Dit kan dui op ‘n vroeë verdedigingsfunksie van LOX, stroomop van SA, wat die produksie van seinmolekules soos oksilipiene kan insluit. Die LOX-induksievlakke was in ooreenstemming met weerstand teen die twee RKL-biotipes.

Die betrokkenheid van SA, PAL, SAGT, CAT en LOX in beide *Dn1*- en *Dn5*-weerstandsresponse teen infestering met beide RKL-biotipes is aanduidend van die ooreenkomste tussen die verskillende RKL-koringinteraksies. Nieteenstaande het verskille in die tyd van voorkoms, vlak en patroon van veranderings voorgekom. Die feit dat *Dn1*-weerstand deur RKLSA2 oorkom is en dat *Dn5*-weerstand effektief is teen beide RKL-biotipes is duidelik deur resultate in hierdie studie geïllustreer.

Sleutelwoorde: Koring, Russiese koringluis, weerstand, salisielsuur, fenielalanien-ammoniakliase, isochorismaatsintase, salisielsuur-UDP-glukosieltransferase, katalase, lipoksigenase

APPENDIX 1

- **Results of independent replicate experiments on SA contents, PAL activity, *ICS* expression, *SAGT* expression, CAT activity and LOX activity**

Results of independent replicate experiments are presented here.

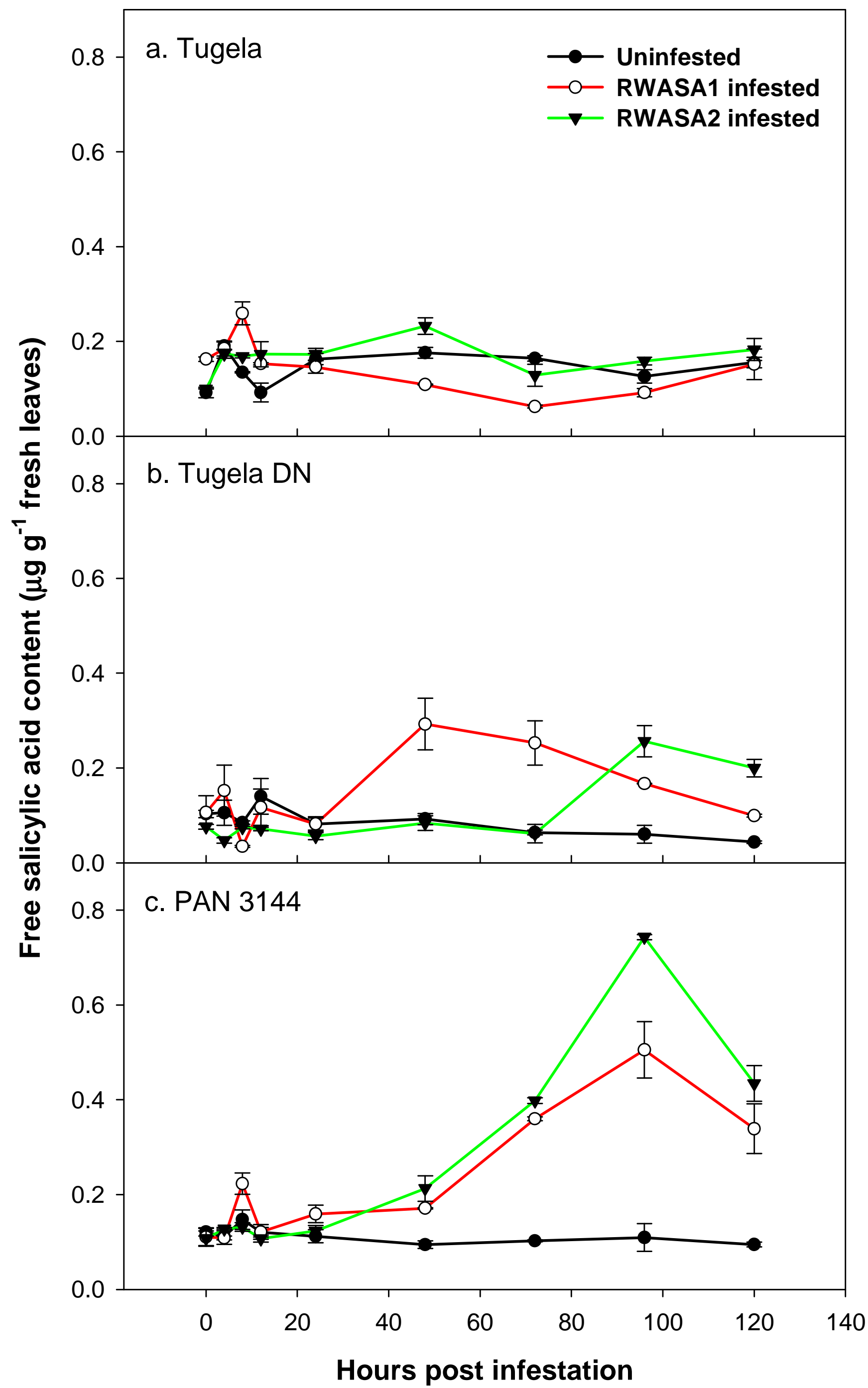

Figure 7.1 Effect of RWASA1 and RWASA2 infestation on the free SA content of susceptible and resistant wheat cultivars. (a) Tugela, (b) Tugela DN and (c) PAN 3144. Values are means ± SD (n=3).

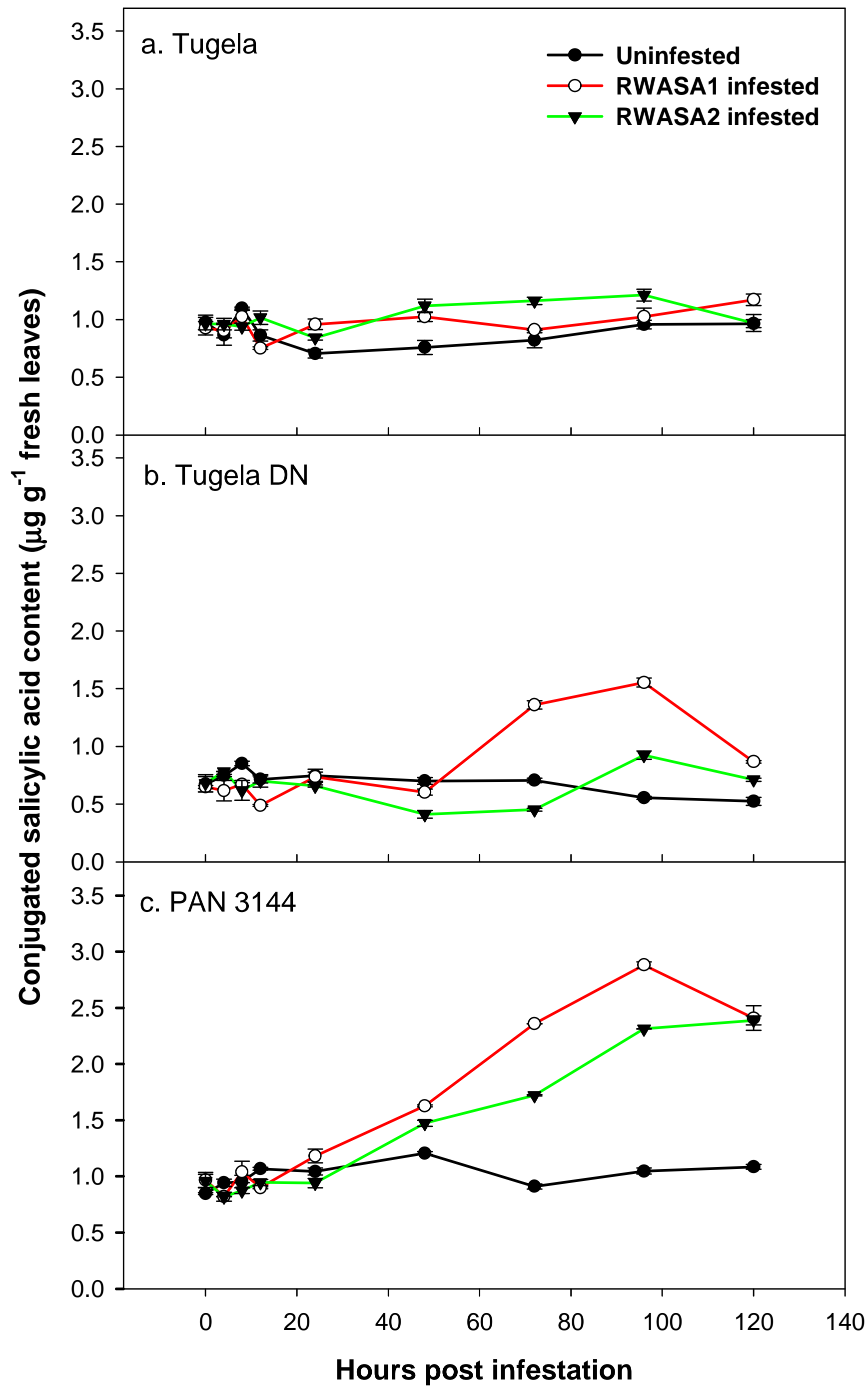

Figure 7.2 Effect of RWASA1 and RWASA2 infestation on the conjugated SA content of susceptible and resistant wheat cultivars. (a) Tugela, (b) Tugela DN and (c) PAN 3144. Values are means ± SD (n=3).

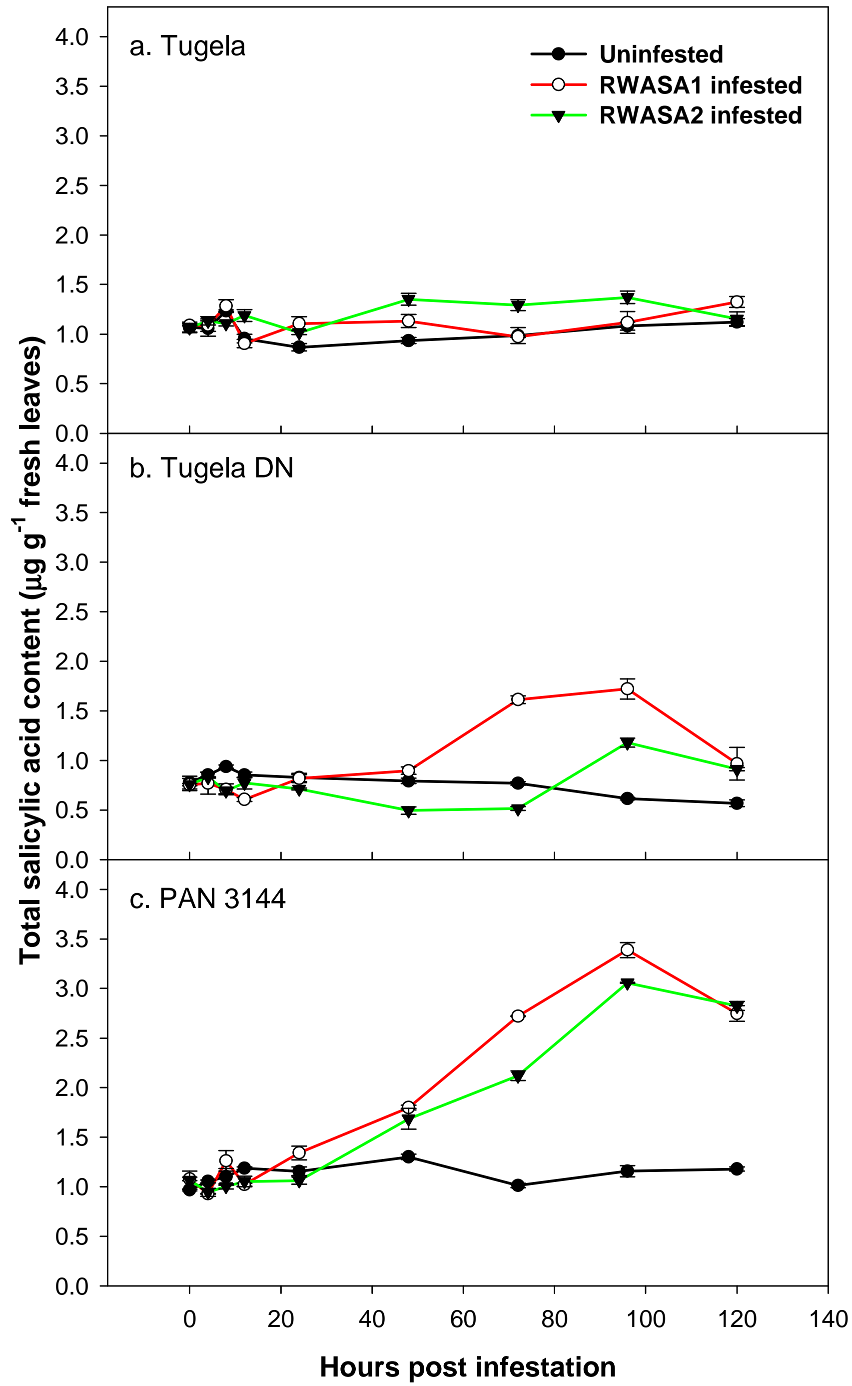

Figure 7.3 Effect of RWASA1 and RWASA2 infestation on the total SA content of susceptible and resistant wheat cultivars. (a) Tugela, (b) Tugela DN and (c) PAN 3144. Values are means ± SD (n=3).

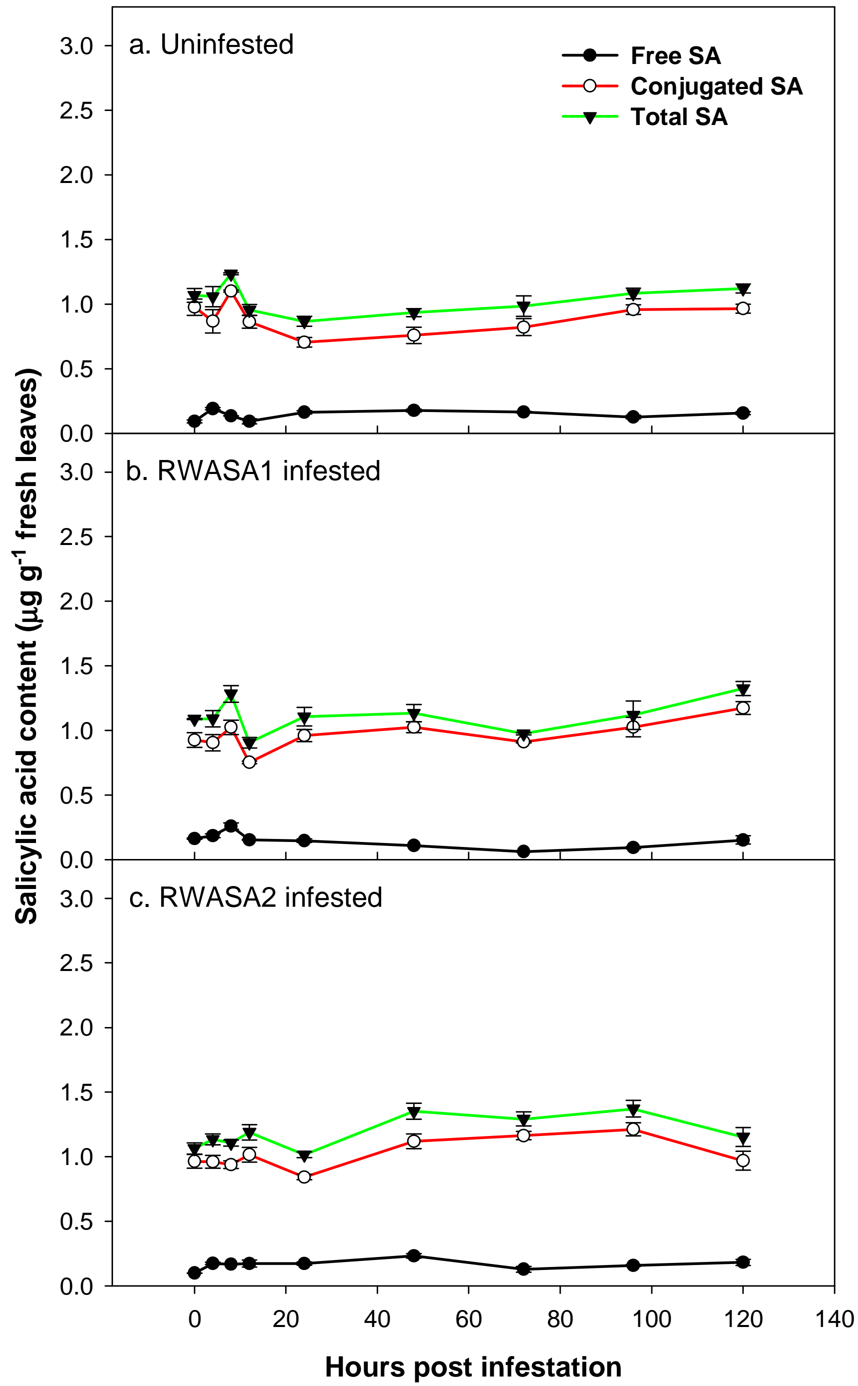

Figure 7.4 Effect of RWASA1 and RWASA2 infestation on the SA content of the susceptible (Tugela) wheat cultivar. Uninfested (a) and RWASA1 infested (b) and RWASA2 infested (c). Values are means ± SD (n=3).

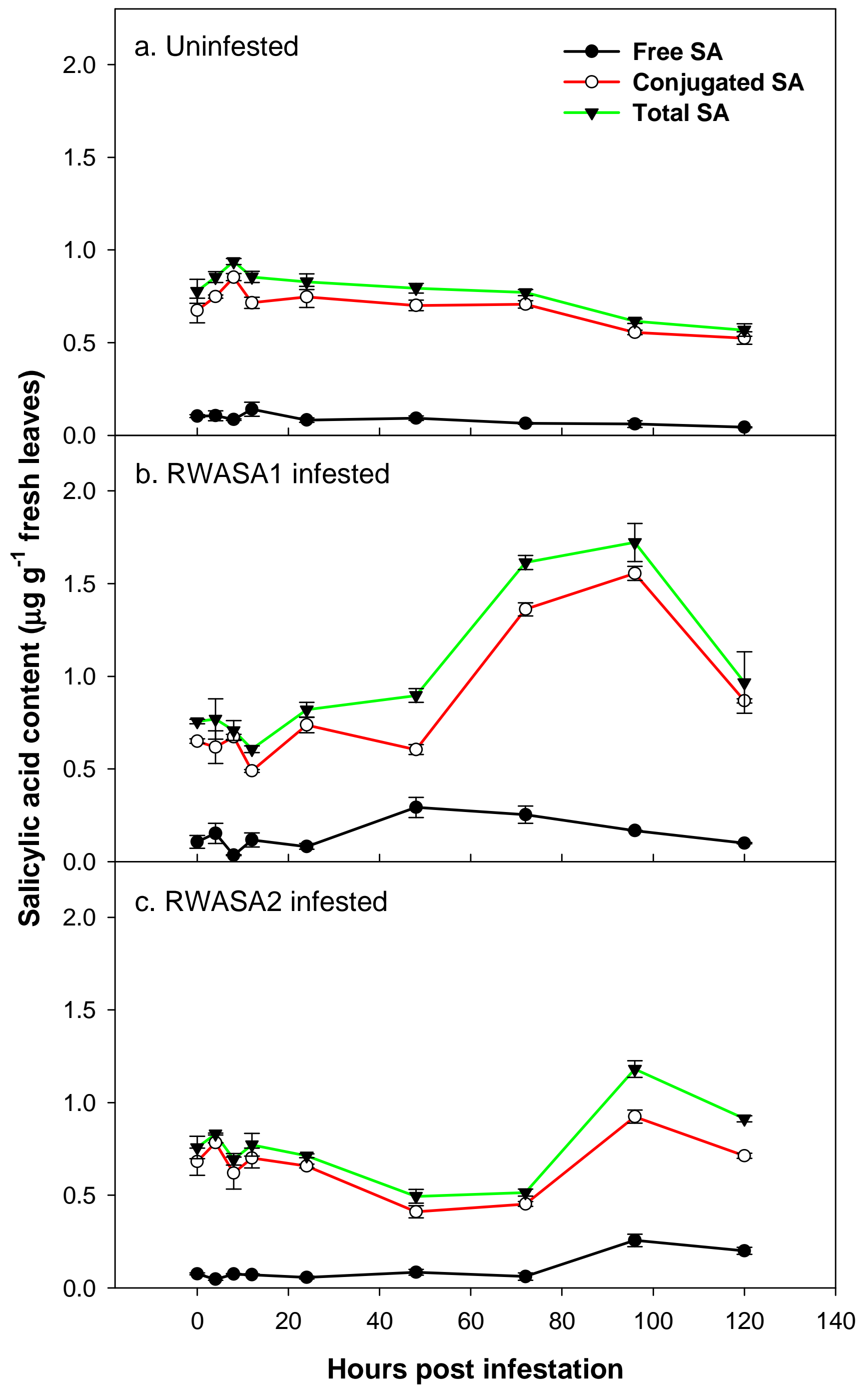

Figure 7.5 Effect of RWASA1 and RWASA2 infestation on the SA content of the resistant (Tugela DN) wheat cultivar. Uninfested (a) and RWASA1 infested (b) and RWASA2 infested (c). Values are means ± SD (n=3).

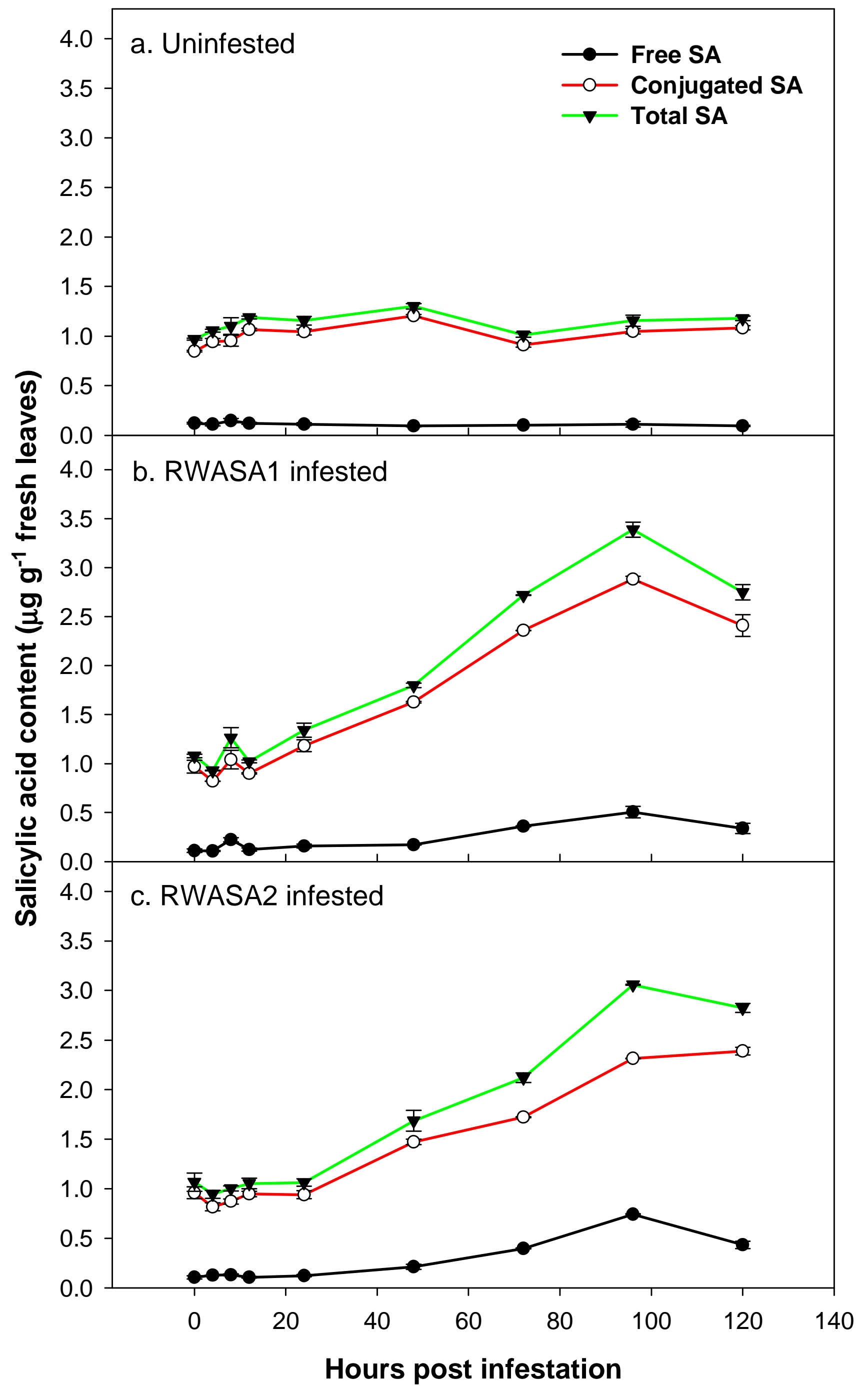

Figure 7.6 Effect of RWASA1 and RWASA2 infestation on SA content of the resistant (PAN 3144) wheat cultivar. Uninfested (a) and RWASA1 infested (b) and RWASA2 infested (c). Values are means ± SD (n=3).

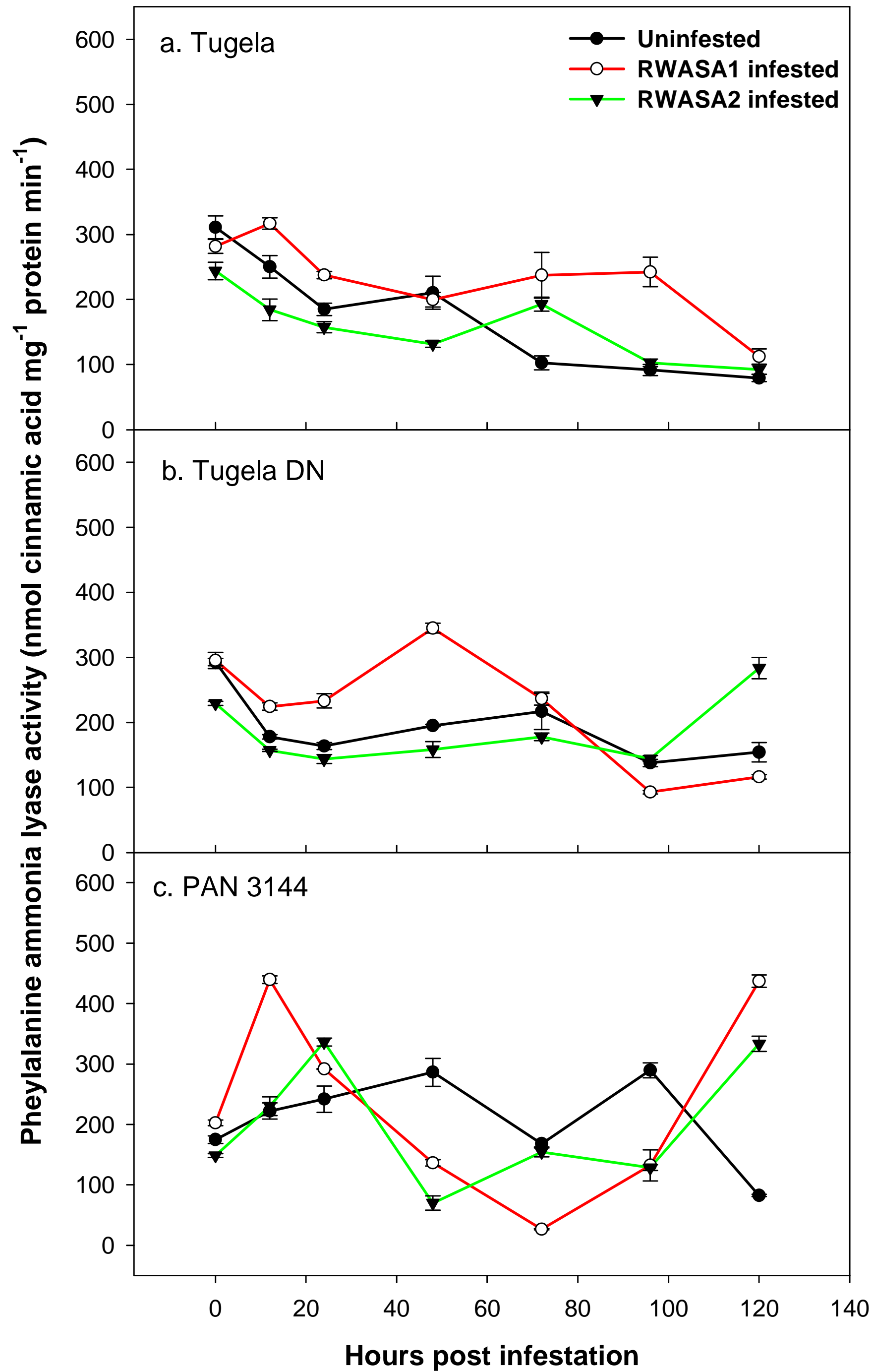

Figure 7.7 Effect of RWASA1 and RWASA2 infestation on the PAL activity of susceptible (Tugela) (a), and resistant wheat cultivars Tugela DN (b) and PAN 3144 (c). Values are means ± SD (n=3).

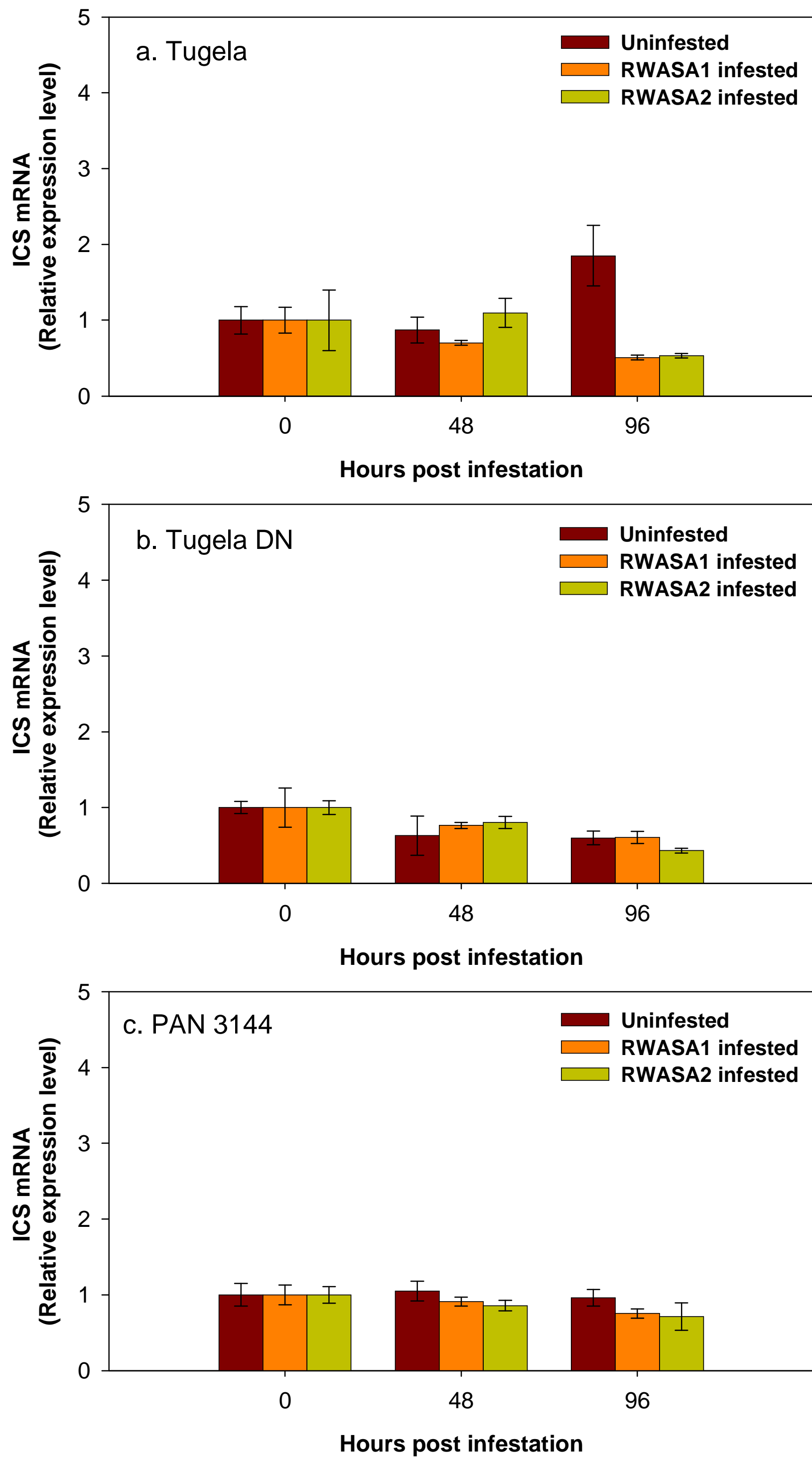

Figure 7.8 Real-time expression analysis of the *ICS* gene following RWASA1 and RWASA2 infestation. Tugela (a), Tugela DN (b) and PAN 3144 (c). Values are means ± 3 SD (n=3).

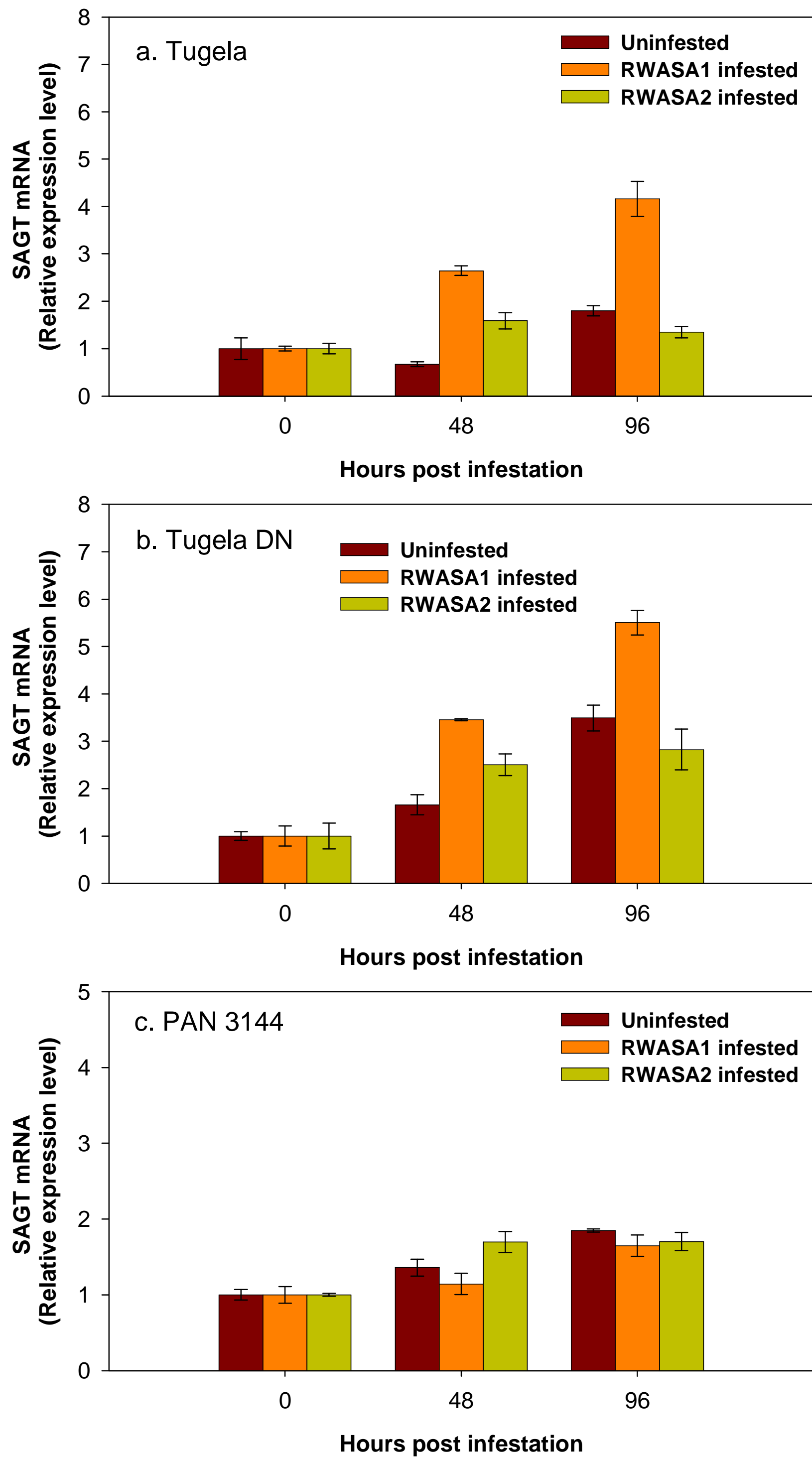

Figure 7.9 Real-time expression analysis of the *SAGT* gene following RWASA1 and RWASA2 infestation. Tugela (a), Tugela DN (b) and PAN 3144 (c). Values are means ± 3 SD (n=3).

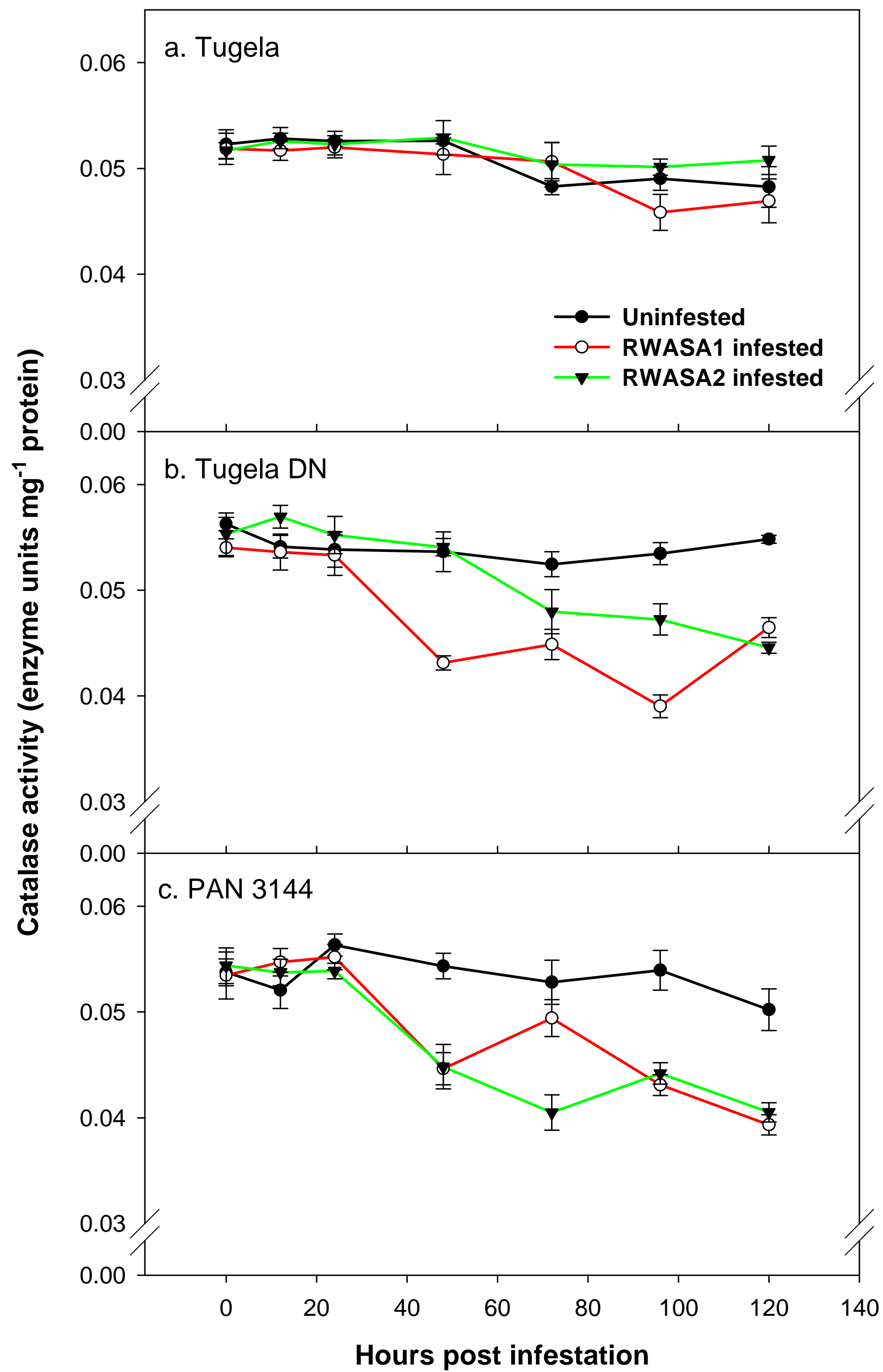

Figure 7.10 Effect of RWASA1 and RWASA2 infestation on the CAT activity of susceptible (Tugela) (a), and resistant wheat cultivars Tugela DN (b) and PAN 3144 (c). Values are means ± SD (n=3).

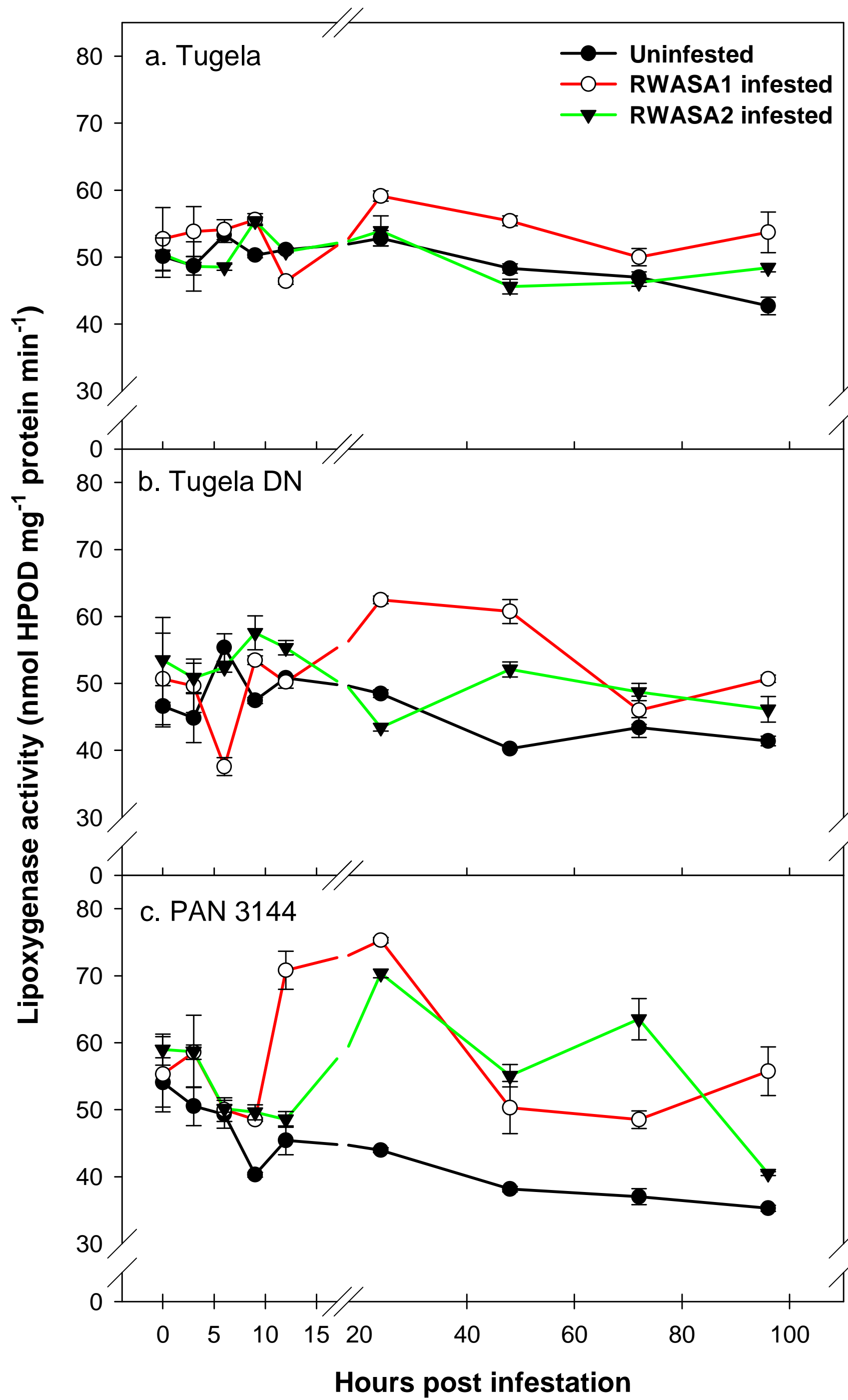

Figure 7.11 Effect of RWASA1 and RWASA2 infestation on the LOX activity of susceptible (Tugela) (a), and resistant wheat cultivars Tugela DN (b) and PAN 3144 (c). Values are means ± SD (n=3).

APPENDIX 2

- **Supporting RT-qPCR data not shown in results (Figs 4.9 and 4.10)**

Before starting RT-qPCR analyses, it is important to obtain good quality total RNA from wheat plants. In Figure 8.1, clear bands are indicated for 28S and 18S rRNA fragments indicating the good RNA quality with no RNA breakdown. This indicates that the RNA was of good quality and no RNA breakdown occurred.

All RT-qPCR analyses were done following the guidelines of quantitative real-time PCR experiments (Bustin *et al.*, 2009). According to these guidelines, real-time results are more reliable, the technique increases the veracity of scientific literature and the consistency between different laboratories.

To ensure the amplification was specific with no primer dimmers for target gene primers, a gradient RT-qPCR reaction was done for *ICS* and *SAGT* primer pairs. Afterwards, the specificity of the primers was confirmed by separating the amplified fragments on an agarose gel (Fig 8.2). According to the results, the optimum temperature of 60 ^{o}C for *ICS* and *SAGT* was chosen. *GAPDH* was provided by the molecular laboratory of our department, the optimum temperature was 60 ^{o}C.

Each experiment included a standard curve consisting of a 5 fold dilution series of total RNA in a concentration range from 100 to 0.0064 ng. A no RNA template control was also added. For each gene, the standard curves were plotted to establish the efficiency of the

RT-qPCR reaction for each run. In Fig 8.3 and 8.5, the standard curves of the target gene *ICS* and *SAGT* and the reference gene *GAPDH* are shown. For a reliable RT-qPCR reaction, the *E* values should be between 90 and 110 %, while R^2-values should be near to one. The melting curves of all three genes indicated no primer dimmer or secondary product formation. The melting temperatures for *ICS*, *SAGT* and *GAPDH* were 80.5 ^{o}C, 76.5 ^{o}C and 83.5 ^{o}C, respectively (Fig 8.4 and 8.6).

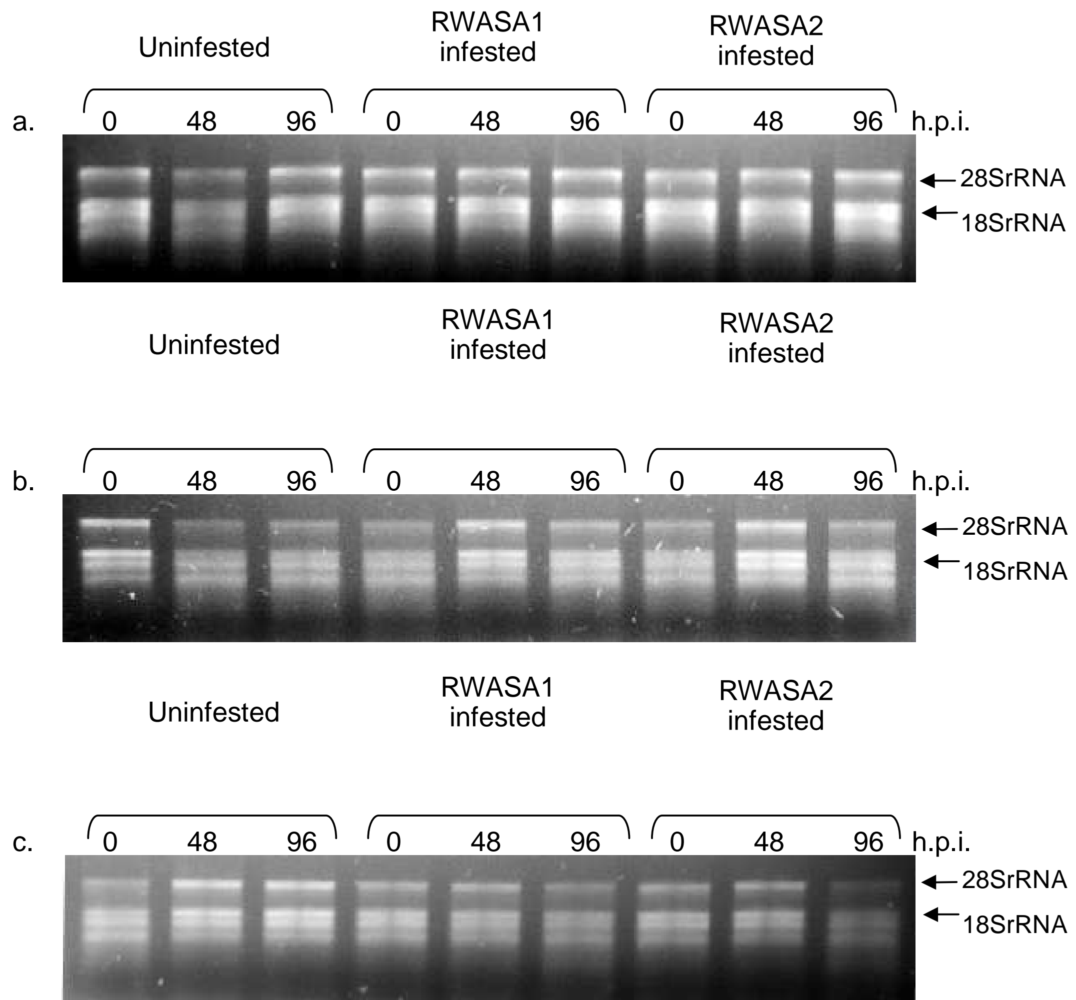

Figure 8.1 Total RNA extracted from three wheat cultivars infested with RWASA1 and RWASA2, respectively. (a) Tugela, (b) Tugela DN and (c) PAN 3144.

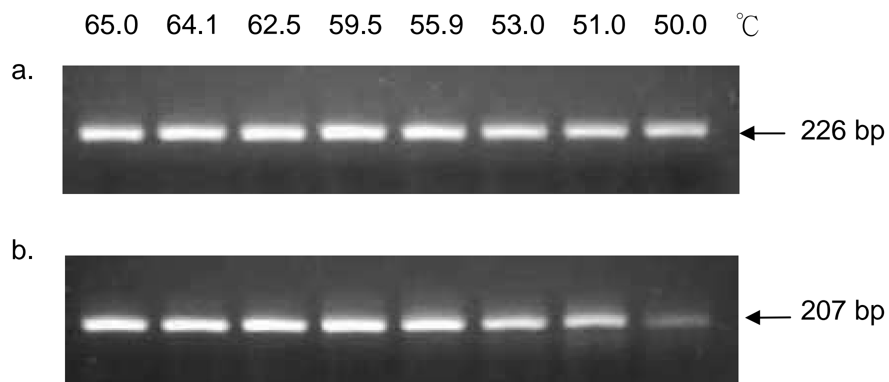

Figure 8.2 Gradient RT-qPCR analysis of *ICS* and *SAGT* gene primers. Amplification of (a) *SAGT* and (b) *ICS*. Fragment sizes and temperature intervals are as indicated.

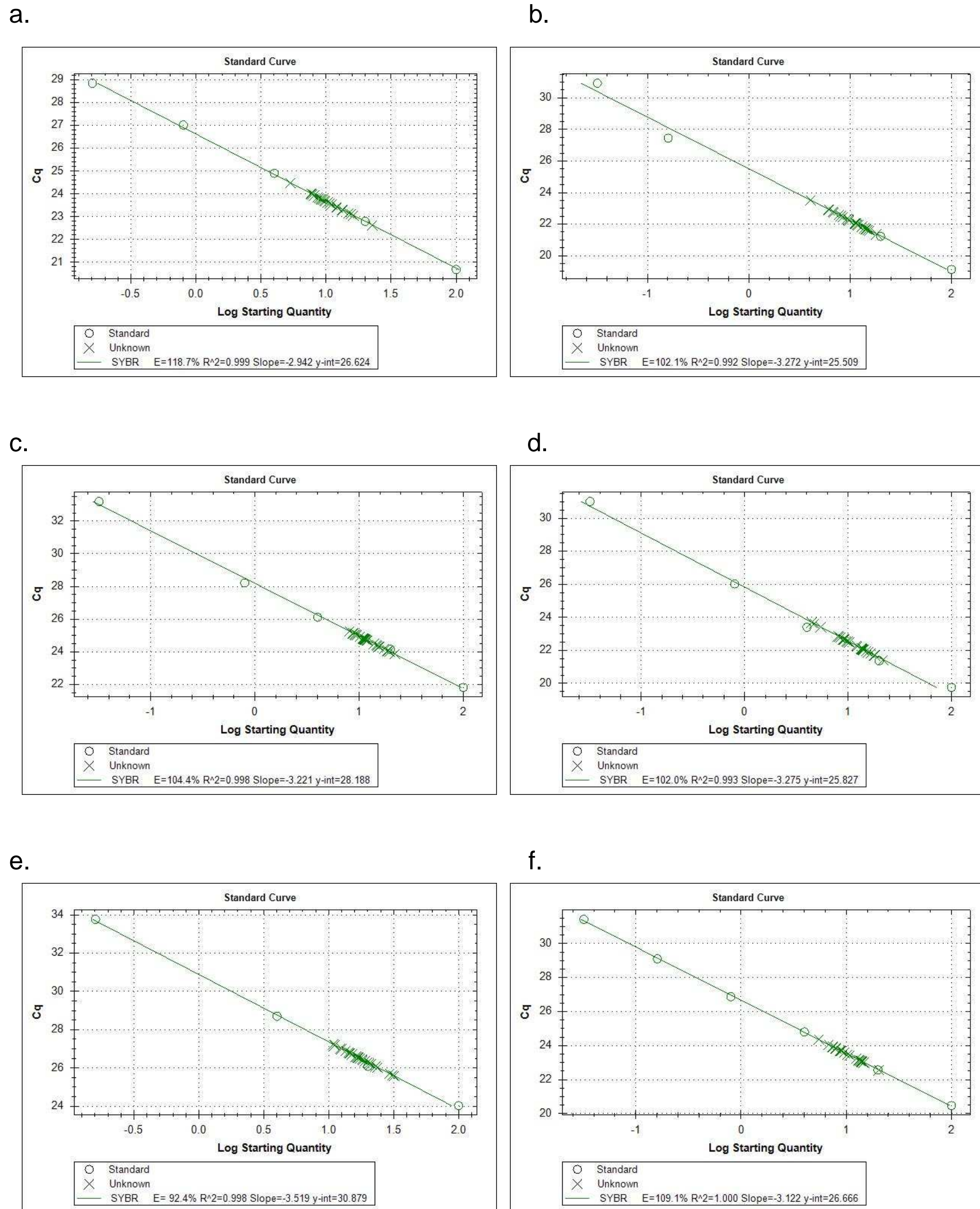

Figure 8.3 Standard curve analyses for the *ICS* gene and the reference gene *GAPDH*. The standard curves of *ICS* (a) and *GAPDH* (b) in Tugela, (c) and (d) in Tugela DN and (e) and (f) in PAN 3144.

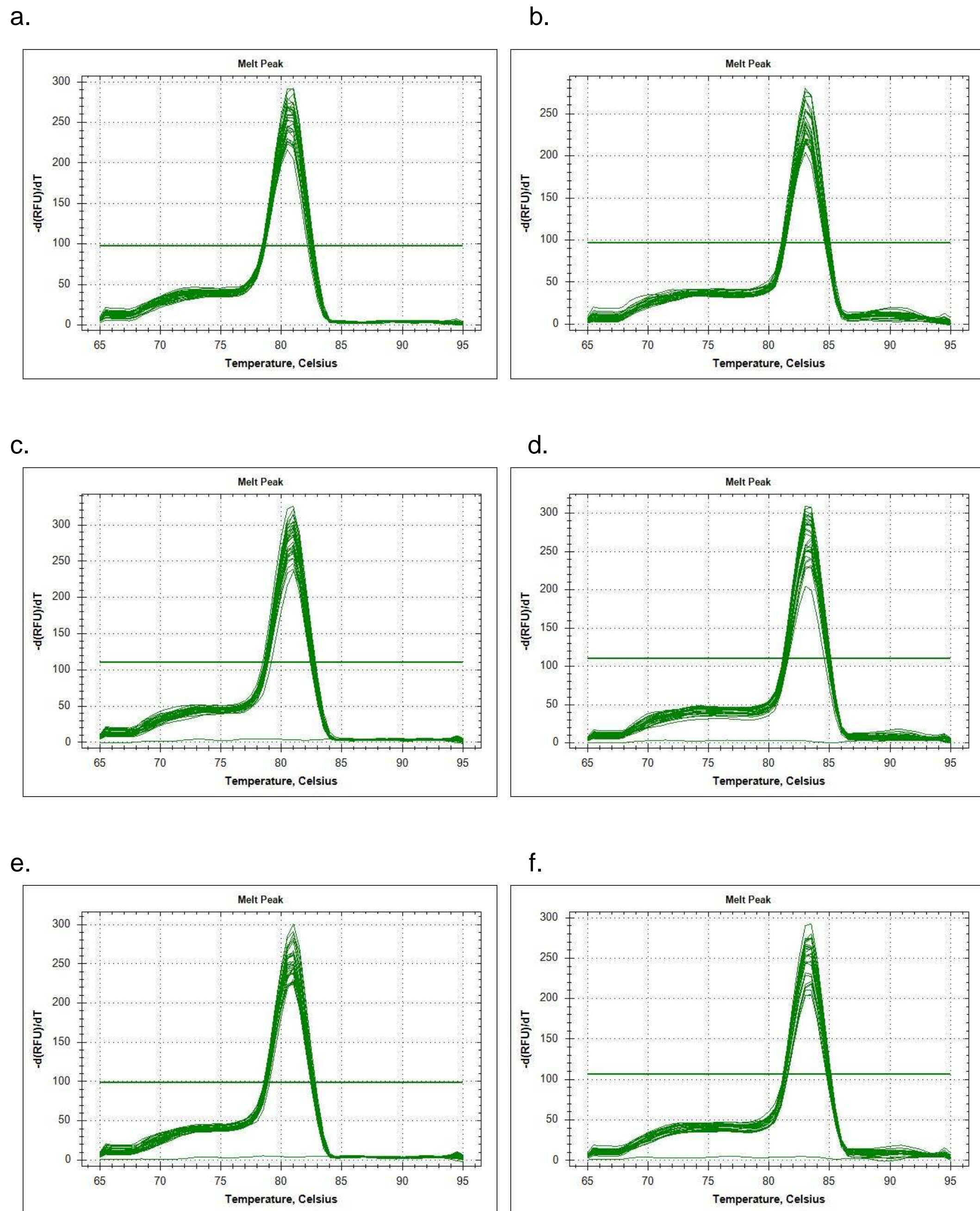

Figure 8.4 Melting curve analyses for the *ICS* gene and the reference gene *GAPDH*. The melting peaks of *ICS* (a) and *GAPDH* (b) in Tugela, (c) and (d) in Tugela DN and (e) and (f) in PAN 3144.

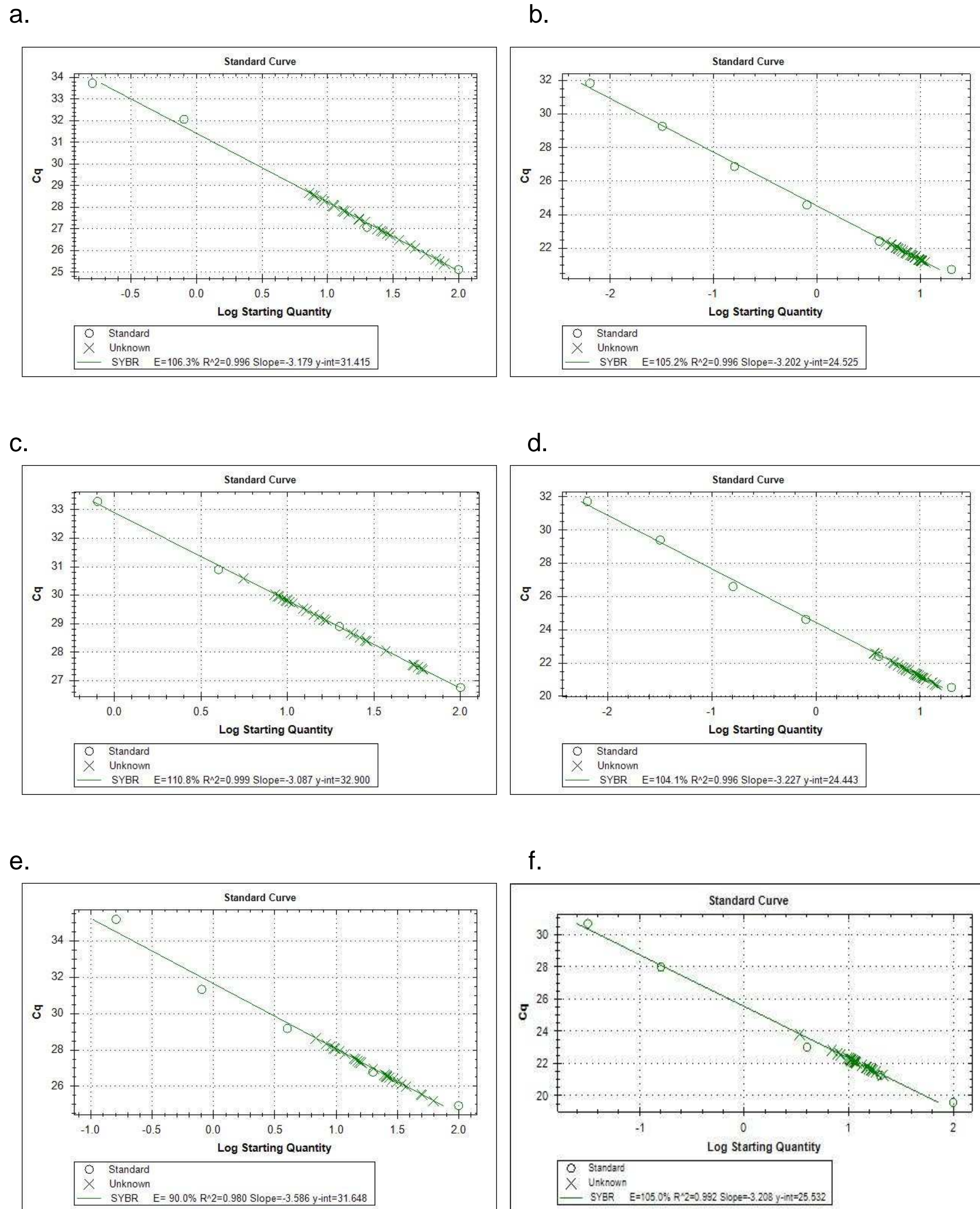

Figure 8.5 Standard curve analyses for the *SAGT* gene and the reference gene *GAPDH*. The standard curves of *SAGT* (a) and *GAPDH* (b) in Tugela, (c) and (d) in Tugela DN and (e) and (f) in PAN 3144.

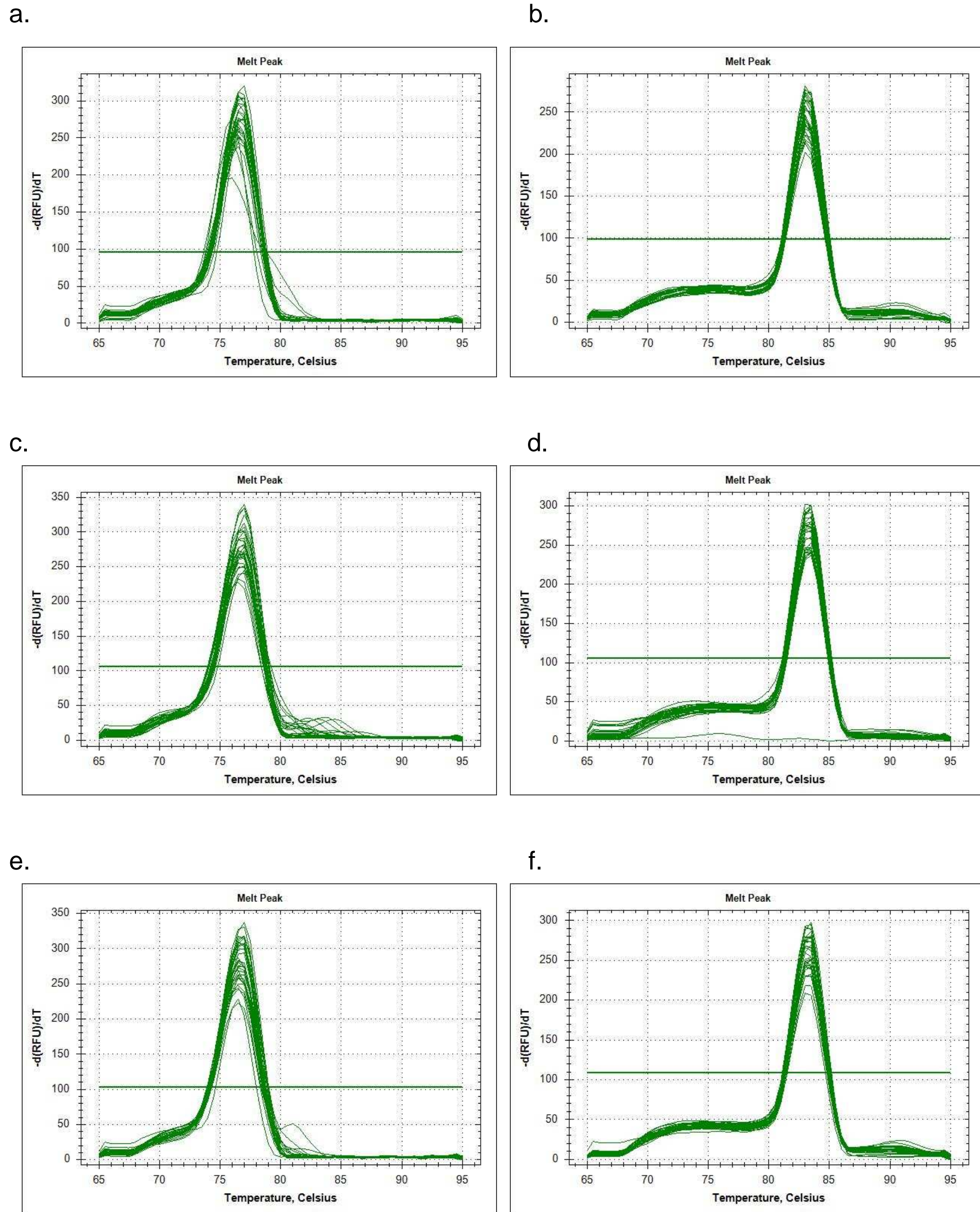

Figure 8.6 Melting curve analyses for the *SAGT* gene and the reference gene *GAPDH*. The melting peaks of *SAGT* (a) and *GAPDH* (b) in Tugela, (c) and (d) in Tugela DN and (e) and (f) in PAN 3144.

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